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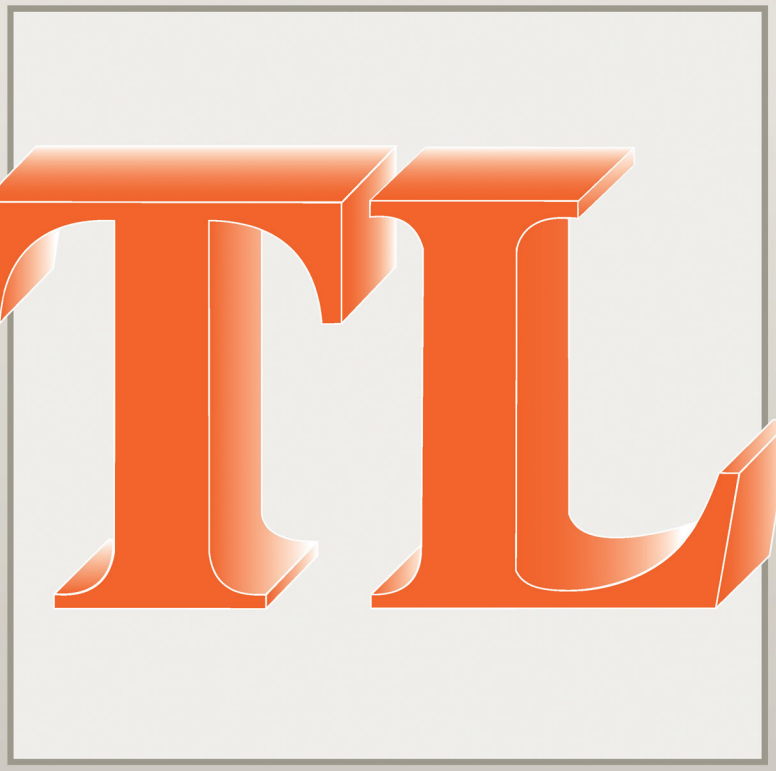

Abstracts of the 58th congress of the European Societies of Toxicology (EUROTOX 2024)
TOXICOLOGY–A QUEST FOR SAFER CHEMICALS AND MEDICINES
Copenhagen, Denmark, 8–11 September 2024

Aims and Scope
An international journal for the rapid publication of novel reports on a range of aspects of toxicology, especially mechanisms of toxicity.
Toxicology Letters serves as a multidisciplinary forum for research in toxicology. The prime aim is the rapid publication of research studies that are both novel and advance our understanding of a particular area. In addition to hypothesis-driven studies on mechanisms of mammalian toxicity, Toxicology Letters welcomes seminal work in the following areas:

- In silico toxicology
- Toxicokinetics
- Physiologically-based pharmacokinetic (PBPK) modeling
- Systems toxicology
- Predictive toxicology
- 3R research in toxicology
- New approach methodology (NAMs)
- Adverse outcome pathways (AOPs)
- Integrated testing strategies

Systematic and narrative reviews and mini-reviews in various areas of toxicology will be published. Clinical, occupational and safety evaluation, hazard and risk assessment, regulatory toxicology, impact on man, animal and environment studies of suffi cient novelty to warrant rapid publication will be considered. Toxicology Letters also publishes editorials, commentaries and contemporary issues in toxicology.

The following types of work are not within the scopes of Toxicology Letters:

- Ecotoxicology studies
- Case studies
- Chemoprevention studies
- Pharmacological investigations

Authors are advised to follow the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments; https://arriveguidelines.org/) and the OECD guidance document on Good In Vitro Method Practices (GIVIMP; https://www.oecd.org/env/guidance-document-on-good-in-vitro-method-practices-givimp-9789264304796-en.htm). In vitro or in vivo investigations conducted at concentrations or doses of no relevance to human or animal exposure will not be considered. Routes of exposure other than those relevant to human or animal exposure need to be justifi ed. Assessment of dose-response should be an integral component of any toxicological research report. Unless adequately justifi ed, studies conducted at a single dose level may not be considered. Test materials must be chemically defined and characterized. Investigations of chemically undefi ned plant extracts or uncharacterized nanoparticles will not be considered.

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Note:

The names of the main authors are bold and underlined. When a main author is not also presenting author, the name of the presenting author is marked with an asterix *.

Abstracts that have been withdrawn for presentation at EUROTOX 2024 Congress are not included in this publication.

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Preface

Dear Friends and Colleagues

It is with immense pleasure that we welcome each one of you, as we convene for the 58th Congress of the European Toxicologists and European Societies of Toxicology, in the captivating city of Copenhagen, Denmark, from September 8-11, 2024, under the resolute banner of "Toxicology – A Quest for safe Chemicals and Medicines."

Allow us to express our profound gratitude for the exceptional quality of the scientific submissions received for this congress, and for the diligent efforts of our distinguished scientific programme committee (SPC). The outstanding commitment of session chairs, speakers and SPC has crafted a vibrant and thought-provoking programme that delves into areas of paramount importance and contemporary relevance, including the groundbreaking applications of artificial intelligence and data sciences in toxicology, the validation and implementation of new approach methodologies, and emerging technologies in preclinical safety testing to name just a few. Our discussions will also encompass crucial topics such as PFAS, risk assessment, next-generation carcinogenicity assessments, quantitative systems toxicology, and the compelling aspects of developmental neurotoxicity. Furthermore, we shall delve into the intricacies surrounding the toxicity of chemical mixtures and the exposome, and dive into the latest developments in micro- and nanoplastics research and toxicological aspects of fire-fighting.

The Congress boasts a meticulously curated agenda, comprising 5 Continuing Education Courses, 28 Scientific Sessions (including 2 sessions for Early Career Toxicologists), and 2 Keynote Lectures. The programme further highlights a diverse array of prize and award lectures, including the EUROTOX Lecture Award, the SOT Merit Award lecture, the HESI Lecture, and the riveting EUROTOX/SOT Debate, a testament to our unwavering pursuit of knowledge and excellence. Moreover, we eagerly anticipate 4 dynamic short oral communication sessions, and the continuous display of over 640 enlightening posters that shall immerse us in cutting-edge research. Furthermore, our gathering shall be enriched by 10 thought-provoking industry hosted events, complemented by a compelling trade exhibition, fostering an environment conducive to forging valuable collaborations and nurturing fruitful interactions.

We would like to express our appreciation and admiration for all those who have tirelessly worked towards the success of EUROTOX, and specifically of the EUROTOX 2024 Congress. Special thanks are due to the immense efforts of the Local Organizing Committee team, as well as the dedication, expertise, and passion demonstrated by each member of our committees (Executive, Nomination, Education, Communication, Registration, Corporate), our Specialty Sections (Carcinogenesis, Immunotoxicology, ERASS, In2TOX, and Molecular Toxicology), our PCO – K.I.T. Group, our experienced, dedicated and efficient Secretariat Office, and last but not least, our individual members and European Societies of Toxicology.

As we convene at this distinguished congress, we wholeheartedly believe that the interactions and dialogues that ensue will serve as a wellspring of enlightenment and inspiration, leading to remarkable advancements in the realm of toxicology and contributing significantly to foster the science and education of toxicology, and influence regulatory and policy frameworks to promote the safety of humans, animals and the environment, and protect global health.

We humbly extend our sincere wishes for a truly exceptional and enriching experience throughout this congress.

With utmost respect and warm regards,

Thomas Weiser
EUROTOX 2024
and EUROTOX 2024 Chair of the
Scientific Programme Committee (SPC)

Prof. Eva Cecilie Bonefeld-Jørgensen
EUROTOX President-Elect
Congress President

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Keynote Lectures

KL01 | Keynote Lecture

Overview of PFAS, toxicology, historical levels in EU vs. health effects and EU risk assessments

T. Kold Jensen

University of Southern Denmark, Odense, Denmark

Tina Kold Jensen is professor in environmental epidemiology and has studied adverse effects of exposure to endocrine disrupting chemicals and impact on both fetuses, children and adults. She the research leader in the Odense Child Cohort, in which more than 100 articles in peer reviewed journals have been published and more than 50 mill DK from external grants have been obtained. She is a member of expert committees in The Danish National Board of Health and The Danish Environmental Agency addressing the adverse health effects of PFAS exposure and knowledge gaps in PFAS and in WHO expert group to evaluate PFAS. In addition, she holds an ERC advanced grant addressing adverse health effects of PFAS exposure.

Per- and polyfluoroalkyl substances (PFAS) are a class of >8000 chemicals often used to make products resist water, stains and heat and they are therefore used in consumer products and are detectable in the environment worldwide making exposure to humans inevitable. Humans are primarily exposed through contaminated drinking water and food. PFAS cross the placenta and are excreted in breastmilk leading to exposure of the fetus and young child, who are especially vulnerable due to rapid growth and development. PFAS are absorbed from the gut and bind to serum proteins and accumulate mainly in the liver, kidney and blood. PFAS are measurable in serum from all humans and have a half-life of up to 5 years. PFAS exposure has been linked to a variety of diseases including cancer, liver and kidney problems, thyroid issues, birth defects, decreased immunity and other serious health problems. EFSA identified reduced response towards routine childhood vaccinations as a critical effect of PFAS exposure. The use of some PFAS have therefore been restricted by the Stockholm Convention, however, they have been replaced by others. A recent Danish review concluded that serum PFOS and PFOA increased from 1988 until the late 1990s followed by a decrease until 2021. A less clear time-trend were observed for the other PFAS.

An increasing number of hot-spot areas in which humans are exposed through contaminated water, soil or food have been reported which has raised public concern. No treatment has been available for high exposed individuals, however, a new study suggests that PFAS elimination can be enhanced in high exposed individual by administration of a bile acid sequestrant, which may be promising especially for women of reproductive age potentially limiting the exposure of the vulnerable fetus and next generation.

https://doi.org/10.1016/j.toxlet.2024.07.008

KL02 | EUROTOX Lecture Award

Endocrine disruptors and microplastics: facing complexity with connection

J. Legler

Utrecht University, Utrecht, Netherlands

In this presentation I will reflect on my experience in research in the field of endocrine disruption (ED), and how lessons learned in the ED field apply to the emerging field of micro- and nanoplastic (MNP) toxicology. I will highlight the challenges of studying endocrine disrupting chemicals and MNPs, and describe how developing innovative solutions to these challenges involves embracing complexity and forging connections between disciplines and stakeholders.

https://doi.org/10.1016/j.toxlet.2024.07.009

KL03 | EUROTOX – SOT Debate

Can the microbiome mediate the toxicity of environmental chemicals?

K. Beekmann[1], **T. Tal**[2]

[1] *Wageningen Food Safety Research, Wageningen, Netherlands*
[2] *Helmholtz Centre for Environmental Health Research, Munich, Germany*

Each year, the EUROTOX congress includes a debate in which leading toxicologists advocate opposing sides of an issue that has significant toxicological importance. The debate continues a tradition that originated in the early 1990s.

This year, the debaters will address the proposition, “Can the microbiome mediate the toxicity of environmental chemicals?” The debaters will provide an introduction to the intestinal microbiome and discuss

how the community of microorganisms in our bodies could potentially influence the harmful effects of chemicals. Debaters will address questions such as

1. "Are microbiome-induced changes in chemical concentrations toxicologically meaningful?";
2. "Are the effects of chemicals on the microbiome more important than the effects of the microbiome on chemicals?";
3. "Does person-to-person variability make it impossible for us to understand the impact of the microbiome on chemicals?"; and
4. "Do model systems and organisms effectively reflect human microbiome-chemical interactions?"

In addition to inclusion as a Keynote Lecture at this meeting, this debate took place already (with the debaters having taken the reverse positions) in Salt Lake City, US, during the 2024 SOT Annual Meeting, March 10–14.

https://doi.org/10.1016/j.toxlet.2024.07.010

KL04 | SOT Merit Award Lecture

Are humans REALLY just big rats and mice? Using the mode of action of aflatoxins, nitrosamines and dioxin-like chemicals for more relevant human-based risk assessments

D. L. Eaton

University of Washington, Washington, USA

My 40+ year research career has focused primarily on the role of biotransformation and disposition as a determinant of chemical carcinogenicity, although my graduate work in the late 1970s was focused on membrane transporters – there were only three known hepatic xenobiotic transporters then: organic anion, organic cation, and "neutral" (ouabain) transporters. A search of "liver transporter" in the Human Protein Atlas now lists 349 different genes!

I also was fascinated by the role of the then recently discovered cytochrome P450 enzymes – there were only two known at the time: cytochrome P450 and cytochrome P448. We know now of 58 different human CYP genes. But much of my early career research focused on the glutathione S-transferase (GST) family of genes. I was fascinated by the discovery that the potent rat liver toxin and carcinogen, aflatoxin B1 (AFB), was not carcinogenic at all in adult mice but was one of the most potent known rat carcinogens.

After several years of work in my new lab, we cloned a GST gene in the mouse that conferred nearly complete resistance to the genotoxic effects of AFB (mGstA3). We subsequently showed that there was an orthologous gene in the rat, rGSTA5, that had high activity toward the genotoxic AFB,8,9-oxide (AFBO) but was not constitutively expressed in the rat liver. But we could turn the rGSTA5 gene on with sulforaphane – present in broccoli and other cruciferous vegetables. We then showed that feeding rats broccoli protected them from AFB genotoxicity/carcinogenicity. We also found that none of the human alpha-class GSTs had any measurable activity toward AFBO, suggesting that humans – relative to rodents – might be highly susceptible to AFB hepatocarcinogenesis, but we found that the human Mu class GSTM1, which is highly polymorphic in the human population, had small but measurable activity toward AFBO. We then showed that human-isolated hepatocytes that were GSTM1-null had about three times more AFB-DNA adducts compared to human hepatocytes that were GSTM1-positive.

Although several studies at the time found that human CYP3A4 had the highest activity of any P450 in human liver toward activating AFB to AFBO, we showed that human CYP1A2 was actually more important – it had a much lower Vmax than CYP3A4 but had a much higher affinity (lower K_M). Thus, at the low concentrations encountered in the human diet, virtually all activation of AFB to AFBO occurred by CYP1A2, not CYP3A4.

More recently, I've been interested in how adverse outcome pathway approaches to risk assessment can do a much better job of estimating human health risks from potential carcinogens and provide a more valid approach to incorporating known species differences between rats, mice, and humans in estimating potential health risks. I will explore two such examples – the importance of using human-based toxic equivalence factors for estimating risk of dioxins and dioxin-like compounds and the importance of species differences in DNA repair in estimating human health risks of the potent rat liver carcinogen, N-nitrosodimethylamine (NDMA), recently found as a contaminant in numerous pharmaceuticals.

https://doi.org/10.1016/j.toxlet.2024.07.011

KL05 | HESI CITE Lecture

No abstract has been submitted.

KL06 | Keynote Lecture

The toxicology of inhaled nanomaterials and examples of risk assessment and regulation

U. B. Vogel

Technical University of Denmark, Denmark

Nanotechnology was the big innovation potential at the turn of the century. Now, more than two decades later, there is scientific consensus that nanosized particles are more hazardous to inhale than larger particles with the same chemical composition. This is especially true for insoluble particles. Inhalation of nanomaterials has been linked to increased risk of cancer, fibrosis and cardiovascular disease, also in AOPs. In addition, some high-aspect-ratio nanomaterials, such as some carbon nanotubes, have asbestos-like properties and are potent carcinogens.

In the presentation, examples of health-based occupational exposure limits will be presented for the process-generated nanoparticle diesel engine exhaust, for insoluble nanoparticles such as carbon black and titanium, for the asbestos-like carbon nanotubes and for ZnO, a soluble nanoparticle.

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Continuing Education Courses (CECs)

CEC01 | Adverse outcome pathways: systematic methods for AOP development & utilization of new technologies and tools

CEC01-01
Introduction into the AOP framework: Integrating Theory and Practice in AOP Development

A. Schaffert[1], S. Murugadoss[2]

[1] *Medial University Innsbruck, Institue of Medical Biochemistry, Innsbruck, Austria*
[2] *Sciensano, Scientific Direction of Chemical and Physical Health Risks, Brussels, Belgium*

In the rapidly evolving field of toxicology, the concept of Adverse Outcome Pathways (AOPs) has emerged as a cornerstone in understanding the mechanistic links between molecular-level perturbations and adverse health outcomes. Recognizing the importance of both theoretical knowledge and practical skills in AOP development, the "Adverse Outcome Pathways: Systematic methods for AOP development & utilization of new technologies and tools" workshop is designed to offer a holistic educational experience for participants at the EUROTOX conference.

The workshop begins with an introductory segment on the AOP framework, setting the stage for deeper dives into AOP development and the practical applications in regulatory contexts. Following the foundational overview, attendees engage in a hands-on practical exercise aimed at consolidating their understanding of the AOP development process. This exercise is conducted in groups, where participants:

1. Create an AOP (network) based on a selected case study, facilitating the transition from theoretical concepts to applied knowledge.
2. Evaluate the weight of evidence, enhancing their ability to critically assess and interpret scientific data for AOP integration.
3. Explore the AOP wiki, allowing the familiarization with the AOP knowledgebase and fostering a deeper comprehension of the AOP landscape.
4. Present their findings, encouraging collaborative learning and the exchange of ideas among peers.

This interactive segment is designed to empower participants with the skills and confidence needed to navigate the AOP wiki and ultimately contribute to the AOP community. Moreover, our workshop provides an exclusive opportunity for participants to learn about the latest developments in AOP methodology directly from experts in the field. The aim is to provide participants with a comprehensive understanding of how to effectively apply the AOP framework in their research or regulatory activities to bridge the gap between theoretical knowledge and practical application in the field of toxicology.

https://doi.org/10.1016/j.toxlet.2024.07.014

CEC01-02
AOP development, the AOP wiki & the way to OECD endorsement

S. Murgadoss

Climate and Environmental Research Institute (NILU), Environmental Chemistry and Health Effects, Kjeller, Norway

No abstract has been submitted.

CEC01-03
The path to a more systematic approach in the OECD AOP development program: integration of systematic evidence integration and assessment

B. Meek

University of Ottawa, School of Epidemiology and Public Health, Ottawa, Canada

Early guidance in the OECD AOP Development Program focused principally on the nature and consistency of AOP component descriptions, as well as the assessment of confidence in described AOPs based on pre-defined considerations. This presentation focuses on recent developments evolving from a program initiative to consider the potential benefits of the incorporation of systematic review methods in AOP development. Resulting revisions to the guidance include addition of a section entitled "the AOP Development Strategy" to the AOP page and more detailed information on the search and assimilation strategies for individual KERs on the KER pages. Application to balance efficiency and transparency in development based on these revisions will be presented and illustrated through examples.

References

[1] OECD (2023) Developers Handbook Supplement to the Guidance Document for Developing And Assessing AOPs. https://aopwiki.org/handbooks/4

https://doi.org/10.1016/j.toxlet.2024.07.016

CEC01-04
Example of a stepwise approach to systematic development of AOPs including available tools/Flash talks on the regulatory use of AOPs

B. Viviani

Università degli Studi di Milano, Dept. of Pharmacological and Biomolecular Sciences "Rodolfo Paoletti", Milan, Italy

The confidence and precision with which AOPs facilitate the extrapolation of data measured at lower levels of biological organisation to predicted outcomes at higher levels, and how measurements of biological effects are linked to their specific causes, is a key requirement for AOPs to support regulatory application. Evidence-based practice helps to achieve this goal. Evidence-based practice assumes that decisions are made by critically evaluating the best available evidence from multiple sources, taking into account all information and data that supports or contradicts a hypothesis. Such an approach helps to avoid biased judgements and increases the transparency, impartiality and rigour of the process.

An evidence-based (systematic) approach to AOP development is articulated in several stages (1): 1. a priori definition of the problem and the method to be used (protocol) to avoid data-driven judgments; 2. scanning and mapping of the evidence to identify relevant MIEs, KEs that may be related to the AO; 3. design of a putative AOP network based on predefined inclusion and exclusion criteria; 4. Systematic literature search (2) and critical appraisal of the prioritised evidence, taking into account human, *in vivo* and *in vitro* studies; 5. Integration of the appraised evidence using the AOP conceptual network according to OECD guidelines (3), synthesising the evidence with consideration of uncertainty. Each step of this approach is supported by the use of specific guidelines, tools and platforms, such as unsupervised machine learning and the AOP wiki to support the systematic mapping of information, systematic reviews and assessment tools to collect and organise the evidence, and to assess the reliability and relevance of toxicity data.

Different case studies are discussed to provide a practical example of a transparent, structured and reproducible approach that supports the development of an evidence-based AOP. The advantages and disadvantages of this approach are discussed, together with a recommendation for the adoption of a fit-for-purpose process for AOPs.

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CEC01-05
Utilizing omics for AOPs: from de novo development and AOP refinement to biomarkers of effect

K. Groh

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Molecular screening and toxicogenomics data, such as transcriptomics, proteomics or metabolomics profiles, have long been proposed for inclusion into chemical risk assessment frameworks and procedures. In prospective risk assessment, omics data can complement and enhance the information obtained in toxicity testing studies, including not only the traditional whole-animal based tests but also the alternative models such as permanent cell lines or zebrafish embryos. In retrospective risk assessment, omics analyses used in the frame of biomonitoring campaigns can provide insights into organismal responses to exposure, generated by analyzing molecular alterations in biosamples collected from humans or from organisms in the environment. However, to enable these types of uses, the linkages between molecular changes and changes occuring at higher levels of biological organization, i.e., cells, tissues, organs and organisms, need to be well understood. This is where the the Adverse Outcome Pathways (AOPs) come into play, as one of the main goals of this conceptual framework is specifically to help organize and evaluate (eco)toxicological knowledge on the successive progression of toxicity from lower to higher levels of biological organization, in order to establish the linkages from molecular to organism-level changes and beyond. This talk will focus on the use of omics data within the AOP framework and discuss how the integration of omics data into AOPs can facilitate its broader application in regulatory decision-making on chemical hazards, in the context of both the prospective and retrospective risk assessment. Discussion related to prospective risk assessment will cover the approaches for using omics data along with data from traditional and alternative toxicity testing endpoints to perform either a de novo development of AOPs, or a refinement of earlier-postulated AOPs. The use of omics and AOPs in retrospective risk assessment will be explored on the example of environmental biomonitoring applications. The talk will conclude by discussing other essential components and identifying further research needs to enable successful integration of omics data and AOPs into regulatory workflows. These include, e.g., development of (i) guidelines for generation and handling of omics samples prior to analysis, which aim to enable collection of comparable omics samples; (ii) guidelines and templates for standardized recording and sharing of omics data, which are meant to enhance the regulators' and other stakeholders' ability to find and retrieve such data, as well as to build a general understanding of its quality; and (iii) guidelines for interpretation and communication of omics results in a manner that is both regulatory relevant and understandable to a regulator not having an in-depth expertise in toxicogenomics. Examples from recent case studies and research initiatives will be used throughout to illustrate the points made and outline ways forward.

https://doi.org/10.1016/j.toxlet.2024.07.018

CEC01-06
Flash talk: use of adverse outcome pathways as an organizing framework for integrated approaches to testing and assessment of developmental neurotoxicity of pesticides

M. Paparella[1], A. Hernandez-Jerez[2]

[1] *Medical University Innsbruck, Medical Biochemistry, 6020, Austria*
[2] *University of Granada, Legal Medicine and Toxicology, 18008, Spain*

Adverse Outcome Pathways (AOPs) can be used as a basis for an Integrated Approach to Testing and Assessment (IATA) of chemicals. Here we demonstrate how European Food Safety Authority (EFSA) -working groups and projects- have used this framework for the assessment of potential developmental neurotoxicity (DNT) of selected pesticides, i.e. deltamethrin, flufenacet [1], and acetamiprid (EFSA 2024, in progress) and 4 more case studies which are in development within the EFSA tendered project DNT-RAP4. In short, the following step-wise approach was used:

1. Define the regulatory problem formulation to be addressed;
2. Search systematically for literature on pesticide specific toxicity and mode of action, and critically appraise the selected evidence for risk of bias;
3. Organize the resulting low risk of bias data from in vitro, animal and human studies into separate lines of evidence for possibly relevant Molecular Initiating Events (MIEs), Key Events (KEs) or Adverse Outcomes (AOs) and assess the consistency of data within each of these lines of evidence;
4. Map the consistency of the chemical-specific lines of evidence with relevant, available AOPs. In case of poor fit, utilize new chemical-specific data to amend the available AOPs or postulate new AOPs;
5. Characterize the uncertainty within the AOP organized data in terms of biological plausibility and empirical evidence for the relationships between the lines of evidence, i.e. the key event relationships (KERs). Expert elicitation tools may be used for this purpose to accommodate diverse expert views;
6. Identify uncertainties that could be reduced with further data and reduce these uncertainties as far as possible;
7. Conclude on the specific regulatory problem formulation and systematically report the AOP-informed IATA including its uncertainties.

Importantly, the uncertainties within and between all lines of evidence, i.e. from *in vitro* to *in vivo* rodent and human, need to be equal-

ly considered for an AOP-based regulatory decision making [2]. Ultimately, such mechanistic AOP-informed IATAs may be used as authoritative, case specific references towards the broader and more standardised regulatory use of non-animal-methods (NAMs) [3] within complex fields regulatory toxicology like DNT.

Here, the approach and its utility to support regulatory decisions are outlined for the specific case studies indicated above.

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CEC02 | Developmental and reproductive toxicology (DART)

CEC02-01
Introduction to developmental and reproductive toxicity (DART) testing

S. Van Cruchten

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The field of developmental and reproductive toxicology (DART) is crucial for assessing the potential risks posed by various chemicals, pharmaceuticals, and environmental factors on human development and reproduction. In this introduction, the main differences between DART studies and general toxicity studies will be discussed first. This will be followed by a general overview on which endpoints of the reproductive cycle need to be addressed. Finally, the general outline of the different studies for pharmaceuticals and chemicals, including some new approaches, will be discussed.

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CEC02-02
Epigenetic changes as biomarkers for developmental neurotoxicity

J. Ruegg

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Developmental neurotoxicity (DNT) is, in the regulatory context, currently only tested using rodent-based test guideline studies, which are long and require many animals and resources. Hence, only a limited number of chemicals have been tested for their DNT properties. Additionally, it has been recognized that the endpoints addressed are not very well predictive for human neurodevelopment. This has raised concerns that chemical risk assessment is currently not protective enough for the human developing brain. Indeed, epidemiological studies have accumulated evidence that chemical exposure is associated with negative neurodevelopmental impacts such as cognitive impairments and changed behaviours. This calls for novel assessment methods that can be used in the regulatory context.

In recent years, efforts have been made to develop an *in vitro* battery (IVB) addressing cellular key events essential for neurodevelopment. A guideline document for this DNT-IVB has recently been approved by the OECD. Concomitantly, several projects at EU level are aiming to upgrade this initial IVB by adding more complexity to the *in vitro* models and addressing molecular events such as epigenetic or endocrine endpoints. This is, among others, part of the Partnership for the Assessment of Risks from Chemicals (PARC). In my talk, I will give an introduction to current DNT testing and the OECD-approved DNT-IVB, and will then continue with results from PARC and other projects that illustrate the usefulness of investigating epigenetic- and endocrine-mediated DNT effects for developing more sensitive test methods and biomarkers for DNT.

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CEC02-03
Current regulatory DART testing

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Safety assessment is required for all compounds to be released on the market, and due to lessons learned in history, specific guidelines have been developed to test the developmental and reproductive toxicity (DART) of these compounds. Differences between pharmaceuticals and chemicals that should be taken into account when developing a testing strategy will be presented. Furthermore, an overview of the DART tests that are currently required for regulatory safety assessment, their design and the main endpoints of these studies will be provided.

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CEC02-04
The virtual human approach to animal-free chemical hazard and risk assessment in developmental toxicology

A. Piersma

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New Approach Methodologies (NAM) with human relevance are increasingly applied for mechanistic understanding of chemical toxicity. Their use in hazard and risk assessment generates research as well as debate. Crucial questions relate to how findings from reductionistic *in vitro* and in silico models should be integrated and translated into the prediction of toxicity in the intact individual. Individual *in vitro* assays cannot fully predict *in vivo* adverse outcome. Also, simply adding up results of batteries of test systems lacks detail as to prediction of adverse effects in the broader context of physiological homeostasis, as to nature, magnitude as well as severity. Clearly, integration within the context of the integral biology of the system targeted by an exposure that might be toxic is necessary for a reliable risk assessment. Human physiology can be described as an ontology, with e.g. genes, enzymes, hormones, receptors, cells, tissues and organs as terms at different levels of complexity, connected by their physiological interrelationships, together forming a homeostatic complex. Many models use to the ontology principle, in toxicology for instance kinetic models connecting organs, and quantitative adverse outcome pathways (AOP) connecting elements of physiology that lead from molecular initiating events to adverse outcomes. The ontological map of human physiology serves as a basis for extracting the AOP network, defined as the physiological network of pathways that, when triggered beyond homeostasis, leads to toxic outcome. Dedicated *in vitro* assays with defined biological domains provide the basic information for testing effects on elements of this AOP network. Emerging computational ontology mod-

els allow data integration to predict toxicity. Various international initiatives such as the OBO Foundry (obofoundry.org) and the Virtual Physiological Human (vph-institute.org) are building integrated ontologies. Proofs of principle have been shown in clinical practice as well as in toxicological effect prediction. Thus, the virtual human approach helps innovating toxicological risk assessment, stepping away from single or simple test battery predictions to physiological integration of *in vitro* and in silico data to human relevant predictions of adverse health effects. Such an integrated approach is necessary to ensure sufficient coverage of toxicological mechanisms and their health consequences. This is essential for reliable application of NAMs in regulatory toxicology as the basis for human chemical safety assessment.

https://doi.org/10.1016/j.toxlet.2024.07.023

CEC03 | Therapeutic Vaccines: how to assess their safety from a toxicology perspective, including species selection

CEC03-01
Introduction to vaccines

S. Gould

Charles River Labs, Evreux, France

Outline of program: introduction to vaccines, in particular therapeutic vaccines, the regulatory space and food for thought

https://doi.org/10.1016/j.toxlet.2024.07.024

CEC03-02
Pharmacology/Immunology

P. Londono Hayes

Simbios Innovation, Ennis, Ireland

This presentation will focus on the pharmacological activity of therapeutic vaccines by exploring the immune system and its response to foreign and self-antigens, within the context of diseases such as cancer, allergy, autoimmunity or chronic infection. We will highlight key aspects of the immunobiology of these human diseases and of the evolutionary diversity of the immune system that shall be considered in determining whether they might be adequately mimicked in animal models. Consideration will also be given to aspects like the mode of action of various types of vaccines (i.e., subunit, mRNA, viral vectors), the choice of immunisation route and the use of adjuvants, in determining the relevance of a species for toxicology evaluation. To conclude, we will explore innovative *in vitro* models that are leading the field towards a future richer in alternative tools to aid the preclinical evaluation of novel therapeutic vaccines.

https://doi.org/10.1016/j.toxlet.2024.07.025

CEC03-03
Clinical Safety issues

M. Pallardy

Université Paris-Saclay, INSERM UMR 996, Faculty Pharmacy, Orsay, France

Will look at the safety of therapeutic vaccines in the clinic, presenting clinical case studies and how we can correlate with toxicology evaluations.

https://doi.org/10.1016/j.toxlet.2024.07.026

CEC03-04
Toxicology

P. Desert

Sanofi Vaccines R&D, MARCY L'ETOILE, France

This presentation will focus on the toxicology of vaccines, including species selection and study designs, in consideration of the principles of prophylactic and with specific considerations of therapeutic vaccines.

https://doi.org/10.1016/j.toxlet.2024.07.027

CEC04 | Toxicology – environmental pollutants

CEC04-01
Introduction to environmental pollution

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The number of chemicals present in the Chemical Abstract Services (CAS) Registry 60 years ago was approximately 200,000. In March 2023, the total had reached 204 million chemicals, with 10−20 million new registrations per year [1]. Some of these, e.g., pesticides, industrial chemicals, and pharmaceuticals, can enter the environment and food chain, potentially causing unwanted effects and disease. The global burden of disease and premature death related to environmental pollution is already three times higher than that for AIDS, tuberculosis and malaria combined, with 7 million premature deaths estimated to occur annually as a result of air pollution [2]. The same study also warns about the likelihood of silent epidemics due to the presence of thousands of commercially available chemicals that are not safety-tested e.g., for their immunotoxicity, reproductive toxicity or developmental neurotoxicity. Besides being harmful to humans, global ecosystems are also adversely affected by the cumulative load of chemical pollution. Environmental contaminants disrupt hormone and immune functions of top predators [3], contribute to the decline of pollinators and the stress on coral reefs, and may as 'planetary boundary threats' irreversibly alter earth systems, on which life depends [4,5].

Of the 350,000 chemicals already on the global market, 95% have not been monitored [6], and the chemical industry is anticipated to double by 2030. Persistent Organic Pollutants (POPs) are chemicals of global concern due to their potential for long-range transport, persistence in the environment, ability to bio-magnify and bio-accumulate in ecosystems, that consequently end up in human food and exert significant negative effects on human health and the environment. However, POPs are not the only contaminants on the agenda of exposure research. Concern is growing about the increasing numbers of chemicals used in both industrial and household applications, leading to a further rise of human exposure and, after emission into the environment, of wildlife and crops [6,7]. Major knowledge gaps remain regarding the spatiotemporal nature and effects of complex exposures, preventing the implementation of data-driven policy at national and international level for risk mitigation and transformational societal change. As human health is interlinked with environmental health, new tools and interdisciplinary approaches are needed to form a holistic knowledge base. Today, environmental and health data are collected in massive amounts attributable to rapid improvements in equipment and storage capacities. These data are often inconsistent due to unharmonized sampling and analytical designs and techniques. Consequently, our abilities to link these data with health effects and in-

crease our understanding about their role with respect to e.g., health outcomes and climate change, are considerably impaired. In response, new holistic approaches are needed, presented during this lecture.

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CEC04-02
Comparison of outdoor and indoor air pollution on cardio-pulmonary health

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Air pollution is an underestimated global issue having considerable consequences on the environment, economy and human health. Though air pollution is a global problem, however the low and lower-middle income countries (LIC and LMICs) bear the maximum burden of air pollution related adverse health (respiratory, cardiovascular) effects. About 9 million people are adversely affected annually due to air pollution related causes worldwide. 4 million of this population is from LIC/LMICs and further most of these people suffer from diseases resulting from exposure to both ambient (outdoor) as well indoor or household exposure to smoke (cook stoves and fuels). Hence both outdoor (urban) and indoor (rural; biomass burning) environment contribute almost equally to air pollution related health risk in LMICs. The United States- Environmental Protection Agency defines the most common air pollutants as "Criteria Air Pollutants" (particulate matter -PM, sulphur dioxides- SO_2, nitrogen dioxides- NO_2, carbon monoxide-CO, ozone- O_3 and Lead- Pb). They can be classified into two main groups: Primary pollutants emitted directly in the atmosphere and secondary pollutants derived from precursor pollutant transformed in the atmosphere through a chemical reaction. On the other hand, the World Health Organization defines air pollution as a critical global problem for human health. Though ambient air pollution affects everyone in the society irrespective of poor and rich, men and women, boys, and girls. However, there are significant socioeconomic inequalities in exposure to and negative health outcomes arising from adverse environmental conditions. The adverse health effects can be due to acute or chronic exposure to air pollution and PMs as well as to several characteristics such as water solubility, size, oxidation property and from the person's susceptibility leading to premature mortality or morbidity. The main route of exposure for humans is through inhalation having the respiratory system as the main target organ. Depending on the size, PMs can reach different part of the lung: extra thoracic and upper respiratory tract (PM10) or deeper lung part – alveolar (fine and ultrafine particles- PM2.5, PM0.1) triggering inflammation that can result into chronic lung disease such asthma, chronic obstructive pulmonary disease (COPD) and chronic bronchitis (CB). Fine and ultrafine particles are of most concern as once they reach the alveolar part, they can be translocated to the bloodstream reaching extrapulmonary organs causing inflammation, oxidative stress and tissue damaging and increased the risk of cardiovascular diseases. Therefore, the lecture will encompass the strategy of translational research integrating epidemiological, clinical, and experimental approaches towards dissecting chronic lung diseases as well as to evaluate the chronic exposure (indoor/outdoor pollution) associated risk of cardiovascular diseases.

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CEC04-03
Exposure and risk assessment of plant protection products

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The Farmer's Toolbox of the 21st century includes several solutions to provide sustainable crop practice. Plant protection products (PPPs) are a more traditional part of this toolbox and are defined as the end-use products intended to be used to protect seeds, crops and non-crops areas from pest pressure. Rigorous human and environmental risk assessment is required, diligently conducted and submitted to regulatory agencies for the registration of these products before they are put on the market, and at regular interval afterwards.

The purpose of this presentation is to provide background information on regulatory as well as technical aspects of human health risk assessment for plant protection products, from both hazard and exposure perspective. Hazard characterisation of PPPs of chemical and biological origin will be reviewed in detail in its principle and some applicative aspects, related to farming and non-farming conditions. Current state of integration of NAMs (New Approach Methodologies) in hazard characterisation will be considered. Exposure assessment methodologies of different tiers will also be reviewed and provide the participants with a snapshot of current and future exposure assessment methodologies and possible risk management measures; this will cover several non-dietary and dietary exposure scenarios, including exposure from environmental sources. Global and regional aspects of risk management decision making. Exposure improvement via precision and digital agriculture improvements will be described.

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CEC04-04
Pollutants and female reproduction

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Fertility rates in Europe have remained below the population replacement level for nearly half a century. One in six individuals experiences unwanted childlessness, and around 5% of babies are born following medically assisted reproduction. While factors such as education, family planning, and advanced maternal age play undeniable roles in this fertility decline, the impact of environmental pollution warrants attention. Epidemiological studies have linked chemical exposure to various fertility outcomes, including time-to-pregnancy and live birth rates, all indicative of a couple's reproductive health. Female-specific fertility markers have received less scrutiny although women are biologically the bottleneck of human reproduction. Fertility in women is limited by the oocytes, that are all formed prenatally. When the reserve of oocytes runs out, fertility is lost and menopause commences.

Our research has examined relationships between chemical exposure and ovarian biology through various cohorts and *in vitro* models. The cohort studies, focusing on over 70 different chemical contaminants and metabolites, have revealed associations between exposure and reduced ovarian reserve, altered follicle sensitivity to hormones, and diminished quality of ovulated oocytes. Moreover, in line with literature, we have identified correlations between chemical exposures and increased risk of infertility.

Chemicals of concern identified in our studies include persistent organic pollutants (e.g., PFOS, DDT/DDE, and PCBs) and semi-persistent compounds (e.g., phthalates). Lifestyle analysis suggests that higher levels of PFAS and phthalates in ovaries correlate with increased

consumption of fish and eggs, and the use of personal care products, respectively. To elucidate epidemiological associations, experimental studies are imperative. Such studies can clarify causality and uncover mechanisms of action. Consequently, we have investigated the chemicals of concern in various *in vitro* models, ranging from cell lines to primary cells and ovarian tissue cultures. Transcriptomic profiling has uncovered multiple novel potential targets of chemical toxicity in ovaries, ranging from lipid metabolism and glycolysis to ATP production.

In conclusion, environmental chemicals, including classic endocrine-disrupting compounds like DEHP, associate to reduced fertility in women and affect ovarian cellular processes in ways that extend beyond traditional endocrine pathways. Our findings, alongside literature, indicate associations between chemical contaminants and reduced female fertility, suggesting that current health risk assessments fail to adequately safeguard female reproductive health. Present guidelines for reproductive toxicity testing, which rely on rats and cover only high-production-volume chemicals, are threrfore inadequate. Developing novel methodologies to identify chemicals with reproductive toxic properties should be prioritized.

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CEC04-05
Group assignment about PFAS

A. Vieira Silva

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For the group assignment, the participants do not need to make any special preparations. The participants will be allocated into groups and discuss the adverse effects on human health, following environmental contamination with PFAS.Each group will have a theme: 1) sources and PFAS exposure routes 2) exposure assessment and data sources 3) development of risk assessment strategies and exposure limit derivation 4) risk management and mitigation strategies 5) strategies for communication and information dissemination. After the discussion part, the chairman of the group will share the group's conclusions with the general assembly.

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CEC05 | The open science movement: challenges and opportunities for toxicology

CEC05-01
What is "Open Science" and why does it matter?

P. Whaley[1]

[1] *Johns Hopkins Bloomberg School of Public Health, Evidence-Based Toxicology Collaboration, Baltimore, USA*
[2] *Lancaster University, Lancaster Environment Centre, Lancaster, UK*

This introductory talk will set up the themes of the CE session, giving participants a broad introduction to the core concepts of open science and its motivations. This will focus on one of the biggest challenges faced by the scientific community today: research has never been produced at such a high rate, but reusing data from studies is so difficult that only a tiny fraction of the results of researchers' efforts can successfully be acted upon. Inscrutable methods, wrong results, unrepeatable studies, and unextractable data all obstruct the reuse of scientific data.

Open Science is about offering a comprehensive solution to this problem, rethinking the incentives, infrastructure, and outputs of research such that science is fairer, more efficient, more reliable, and more reusable. It does this through a range of techniques and technologies including: protocol publication; preprints; open data standards; use of data repositories; data dictionaries, ontologies, and other ways of ensuring smooth data sharing and reuse. This presentation will introduce the major concepts of open science to the audience, to prepare them for the more detailed talks and activities to follow.

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CEC05-02
Scientific data can be messy, unreliable, and hard to find and use: how open science helps with this

N. Larigaldie

Aarhus University, Denmark

No abstract has been submitted.

CEC05-03
Practical steps for making your research FAIR

K. Hair

University of Edinburgh, Edinburgh, UK

This talk explores practical steps for implementing the FAIR (Findable, Accessible, Interoperable, Reusable) principles in your research. Attendees will learn about tools, technologies, and practices which facilitate open science across the research lifecycle. The presenter aims to empower researchers to work in a transparent and reproducible way from the initial planning stages of research through to the reporting phase of a study, highlighting practical tips along the way.

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CEC05-04
What funders want

M. Shatz

NIEHS, Office of Data Science, Morrisville, USA

Every year the US National Institutes of Health (NIH) receive 40–50 billion dollars of taxpayers money to achieve its mission to "seek fundamental knowledge about the nature and behaviour of living systems and the application of that knowledge to enhance health, lengthen life, and reduce illness and disability". To maximise return on investment, the data produced by NIH-funded studies should be FAIR. To attain this goal, in January 2023 the NIH issued their updated Data Management and Sharing Policy (DMSP). This has profound implications for recipients of NIH grants and mirrors similar initiatives in Europe and across the world. In this presentation, the Scientific Program Manager of the Office of Data Science at the US National Institute of Environmental Health Sciences will introduce the DMSP and provide guidance to authors on how they can plan for compliance with the DMSP and similar policies in Europe.

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CEC05-05
How to work with journals to achieve your open science goals

P. Whaley[1]

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[2] *Lancaster University, Lancaster Environment Centre, Lancaster, UK*

The Editor-in-Chief of *Evidence-Based Toxicology*, a new open-science journal for toxicology and environmental health, will present some examples of open science workflows and manuscript options that are increasingly being introduced by publishers. These include preprints, protocol publication, "Registered Reports", the publish-review-curate model of publishing, open peer-review, and other concepts. The presenter will explain what each policy is, why it is thought to be helpful, and how researchers can engage with the policy to improve the accessibility and reusability of their data. The presenter will also offer tips and advice on how to get the best out of journals, in order to achieve your open science goals.

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CEC05-06
Accessing FAIR chemistry and toxicity data from US-EPA Dashboards

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Public domain and open databases serving the domains of chemistry and toxicity data continue to expand in number, scope and diversity. The online websites, and increasingly their associated application programming interfaces (APIs) can provide access to an enormous breadth of data that can be queried and harvested to bring together data for various purposes including simple data gathering, assessments, database development and derivative software tools. While the harvesting of data is not difficult, the aggregation and utilization of *quality* data can be a challenge. This presentation will provide an overview of available resources that toxicologists may find of value and highlight the importance of data integrity in such resources.

Over the past two decades the US Environmental Protection Agency has developed a curated database of chemicals, DSSTox, which provides the foundational database of curated chemistry on which multiple public software applications have been built. These data, when integrated with *in vivo* hazard data, *in vitro* bioactivity data (i.e.; ToxCast/Tox21), physicochemical property data, can be invaluable to toxicologists as they develop an understanding of chemicals of interest. This presentation will provide an overview of the importance of data curation, urge caution with the sourcing and harvesting of data from public sources, and demonstrate public tools which have been developed on the aggregation and curation of public data sources. This will include an introduction to the US EPA CompTox Chemicals Dashboard (https://comptox.epa.gov/dashboard) and the cheminformatics modules (https://www.epa.gov/comptox-tools/cheminformatics).

This abstract does not necessarily represent the views or policies of the U.S. Environmental Protection Agency.

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Sessions

S01 | Complex chemical mixtures: assessment of chemical mixture drivers and adverse human health and environmental effects

S01-01
Risk assessment of complex chemical mixtures – an overview of approaches and recent developments

A.M. Vinggaard[1], B. I. Escher[2], M. Scholze[3], M. J. Valente[4], M. Lamoree[5], T. Hamers[5], S. Schmeisser[6], M. Herzler[6]

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2 *UFZ, Helmholz Center for Environmental Research, Leipzig, Germany*
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4 *Technical University of Denmark, Kgs.Lyngby, Denmark*
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6 *German Federal Institute for Risk Assessment, Berlin, Germany*

This presentation aims to provide an overview of key findings and insights derived from years of research in mixture toxicology. An overview of various experimental and theoretical approaches for assessing mixture effects of chemicals and for risk assessment of chemical mixtures will be given, while evaluating pros and cons associated with each approach. Outcomes of experimental *component-based mixture studies* have had significant implications for risk assessment of chemical mixtures as results affirm the efficacy of the concentration-addition principle in facilitating confident predictions of mixture effects. Thus, with reliable hazard and exposure information on single compounds, mixture effects can be predicted with relatively good confidence. In many cases substantial mixture effects have been detected and predicted when chemicals targeting shared biological pathways have been combined at their No Adverse Effect Levels. While synergy occasionally manifests in experimental studies at higher doses, its prevalence at low exposure levels of environmental chemicals, typical of human scenarios, is hypothesized to be rare, a phenomenon predicted earlier by the funnel hypothesis. This stated that as the number of chemicals in a mixture increase, the range of deviations from additivity decreases, and that biological endpoints requiring high doses will deviate more from additivity than endpoints requiring low doses (Warne and Hawker, 1995).

Risk assessments of *complex real-life chemical mixtures* containing a multitude of very different substances are more challenging, as comprehensive data on both exposure (individual versus aggregated data, biomonitoring levels or predicted levels) and hazard (*in vitro* or *in vivo* data) are required. Thus, critical bottlenecks persist due to the scarcity of high-quality hazard and exposure data occurring simultaneously for many compounds as well as the lack of mechanistic information required to establish an assessment group. Experimentally, complex chemical mixtures can be addressed in *whole mixture studies* especially in *in vitro* bioassays, in which the integrated effect of the mixture is assessed. When combined with effect-directed analysis, chemical mixture drivers can be identified. This approach requires that the selected bioassays measure a molecular initiating or key event linked to a relevant adverse outcome and in such cases, it may in future be possible to derive effect-based trigger values for specific adverse effects in human tissues. The ongoing PANORAMIX project under the GreenDeal plan tackles these challenges, aiming to advance our understanding of evaluating and managing the risks associated with chemical mixtures.

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S01-02
Whole mixture assessments of water, food and human blood

B. Escher[1], J.-P. Antignac[3], M. Audebert[6], P. Cenjin[2], T. Hamers[2], M. João Portugal Couto Valente[7], L. Khoury[6], M. König[1], M. Lamoree[2], J. Lee[1], Y. Ma[7], M. Margalef Jornet[2], S. Motteau[3], K. Renko[5], M. Scholze[4], A.M. Vinggaard[7]

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6 *PrediTox, Toulouse, France*
7 *Technical University of Denmark, National Food Institute, Lyngy, Denmark*

In vitro bioassays have a long tradition for hazard assessment of chemicals as well as for water quality monitoring. Here we apply these tools to mixtures extracted from pooled samples covering the continuum of environment to human, including water, fish, milk and human serum. We developed a test battery of 22 *in vitro* bioassays that broadly covered developmental neurotoxicity, thyroid hormone system disruption, reproductive toxicity, genotoxicity and adaptive stress responses. Putative adverse outcome pathways synthesized from diverse literature sources served as a starting point for the selection of the bioassays. Most assays responded to wastewater with effecs and cytotoxicity decreasing from wastewater over surface water to drinking water. Serum samples pooled from diverse population groups in Australia and Europe were extracted with solid-phase extraction yielding broad-spectrum extracts of chemicals of a medium hydrophobicity range including

neutral and charged organic chemicals. 50–60% of the bioassays responded to the dosed serum extracts. All groups of mode of action were affected with the exception of genotoxicity. High specificity was observed for disruption on thyroid hormone system and neurotoxicity. Through a global profiling approach (suspect screening), 24 endogenous chemicals and environmental pollutants were identified with high confidence and were quantified, among many more qualitatively confirmed. Mixture modelling using detected concentrations and bioassay data for single chemicals demonstrated that little of the effect could be explained by the quantified chemicals. Endogenous compounds also contributed to mixture effects and future work will need to establish a baseline of effects caused by endogenous compounds and differentiate those from the effects triggered by environmental pollutants. Bioassays are a promising tool to identify bioactive chemical mixtures in environmental and human biomonitoring.

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S01-03
Effect-directed analysis for determination of chemical mixture drivers in environmental and human samples

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In our daily lives, we are exposed to a multitude of chemicals via our food and drink and our living environment. These chemicals originate from many sources, many of which are known but there are sources and chemicals that we are not yet fully aware of. It is therefore very difficult to obtain a comprehensive assessment of exposure, because we don't always know what chemicals to aim for.

To obtain insight in the environmental and human exposome, we have developed an Effect-Directed Analysis (EDA) strategy that combines fractionation using liquid chromatography (LC) followed by high resolution mass spectrometry (HRMS) and parallel *in vitro* toxicology to determine the biological response, to ultimately identify the chemicals that are responsive in the bioassays used.

In the last decade, we have worked towards high resolution fractionation using an in-house developed instrument, the FractioMate™, in combination with workflows to achieve an easier, semi-automated evaluation of the HRMS data ultimately resulting in a higher identification success rate.

The type of chemicals we can identify are dictated by the choice of *in vitro* bioassay, logically, when using an assay to assess thyroid hormone displacement, the chemicals identified will cause exactly that effect. EDA has matured from its application in a series of water quality oriented projects, using *in vitro* bioassays (in multi-well plate format) focusing on endocrine disruption (estrogenic, androgenic, glucocorticoid, progestagenic, thyroid), but also mutagenicity and antibiotic activity.

Currently, development of several aspects of EDA are important for its further implementation to identify chemical mixture risk drivers: i) expansion of the application of EDA to other matrices, e.g. food and human-related, ii) expansion of the battery of bioassays used, for wider coverage of chemical classes and endpoints, iii) continued advancements with regards to HRMS data acquisition and evaluation.

In the EU-funded Panoramix project, entitled 'Providing risk assessments of complex real-life mixtures for the protection of Europe's citizens and the environment', we address these developments through collaboration with key European partners. We aim to identify mixture risk drivers in samples from the environment-food-human continuum, using a wide variety of bioassays and advanced HRMS data acquisition, processing and evaluation methods. In this lecture, examples will be given of the application of EDA to samples from the aquatic environment, for which a high throughput data evaluation workflow was developed. Furthermore, first results will be shown of EDA on human blood samples, including cord blood, focusing on thyroid hormone system disruption using the *in vitro* FITC-T4 transthyretin binding assay.

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S01-04
Advancing mixture risk assessment from a regulatory point of view

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The EU Chemicals Strategy for Sustainability aims to better protect human health and environment from the effects of simultaneous exposure to multiple chemicals, also referred to as mixture risk assessment or cumulative risk assessment. Current regulatory frameworks for chemicals mainly rely on the risk assessment of individual substances. In practice, however, humans and environment are continuously exposed to a multitude of chemicals from different sources and there is a growing scientific consensus that such effects need to be better integrated into chemical risk assessment processes.

Within this remit, EFSA issued a guidance document on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals as well as a follow-up guidance on scientific criteria for grouping chemicals.[1,2] Furthermore, with regards to pesticides, several important milestones have been achieved towards the regulatory implementation of dietary

cumulative risk assessment (CRA). These include the establishment of cumulative assessment groups for several effects and the development of a systematic approach for the prioritisation of substances and organ systems that require refined CRA.[3] EFSA also established several partnerships aiming at building capacity with the risk assessment community to carry out such CRAs, and developing standardised tools that will ensure efficient and harmonised implementation.[4,5]

However, further efforts are needed to overcome regulatory silos and to develop innovative approaches for mixture risk assessment across regulatory domains. In this context, EFSA commissioned a first roadmap for action on the Risk Assessment of Combined Exposure to Multiple Chemicals (RACEMiC).[6] Building on the experience gained in the pesticide area, this roadmap mainly focuses on extending mixture risk assessment to other chemicals such as contaminants, food additives and food flavourings. To also address integration of non-dietary sources of exposure into EFSA's risk assessment processes, a second roadmap for action on Advancing Aggregate Exposure to Chemicals is currently under development.[7] Pending the finalisation of this roadmap, it is already clear that the use of new data streams, such as human biomonitoring data, must be explored and a research project investigating new opportunities on monitoring and surveillance data for chemicals was initiated. In addition, such comprehensive approaches will require EFSA to strengthen cooperation with other EU agencies and the EU research community at large. EFSA is therefore also engaged as an associated partner in the Partnership for the Assessment of Risks from Chemicals (PARC).

This talk will give an overview of ongoing initiatives of EFSA in this area and provide insights on how EFSA promotes the development and implementation of mixture risk assessment within its regulatory framework.

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S02 | Data science in drug discovery and development

S02-01
Data Science in derisking drug target and chemistry

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Early derisking of drug targets and chemistry is essential to provide drug projects with the best chance of success. Target safety assessments (TSAs) use target biology, gene and protein expression data, genetic information from humans and animals and competitor compound intelligence to understand the potential safety risks associated with modulating a drug target. However, there is a vast amount of information, updated on a daily basis that must be considered for each TSA.

We have developed a data science-based approach that allows acquisition of relevant evidence for an optimal TSA [1]. This is built on expert-led conventional and artificial intelligence-based mining of literature and other bioinformatics databases. Potential safety risks are identified according to an evidence framework, adjusted to the degree of target novelty. Expert knowledge is necessary to interpret the evidence and to take account of the nuances of drug safety, the modality and the intended patient population for each TSA within each project [1].

Alongside understanding the potential risks associated with inhibiting or activating a drug target, it is key to evaluate the different lead candidates emerging from discovery chemistry to understand their potential for toxicity. We have developed a deep generative adversarial network (GAN)-based framework capable of deriving new animal results from existing animal studies without additional experiments [2]. Using pre-existing rat liver toxicogenomic (TGx) data, we generated Tox-GAN transcriptomic profiles with high similarity to the corresponding real gene expression profiles,Tox-GAN holds great promise for generating high-quality toxicogenomic profiles without animal experimentation.

Over the past 20 years, screening for activity at cardiac ion channels such as hERG has considerably reduced attrition due to cardiovascular toxicity. Recently, we proposed that a similar approach could be taken to reduce seizure liability [3]. We developed an *in vitro* seizure panel based on microelectrode array (MEA) of human induced pluripotent stem cell (hiPSC) neurones and a panel of 15 ion channels with strong links to seizure. The panel is able to detect seizurogenic compounds and has great utility in compound selection and in problem solving.

Overall, ion channel screening, Tox-GAN and TSAs take full advantage of the most recent developments in data science and can be used within drug projects to identify and mitigate risks, helping with informed decision making and resource management. These approaches should be used in the earliest stages of a drug project to guide decisions such as target selection, discovery chemistry options, *in vitro* assay choice and end points for investigative *in vivo* studies.

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S02-02
Molecular representation learning for drug discovery

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Molecular representation learning has advanced drug discovery applications by facilitating efficient predictions of bioactivity and toxicology profiles. In this presentation, we will demonstrate how these techniques enable researchers to effectively identify promising drug candidates and evaluate their safety profiles, surpassing traditional methodologies in both accuracy and efficiency.

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S02-03
FDALabel: enabling full text searching of drug labeling

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Prescription Drug Labeling, commonly referred to as the package insert or prescribing information, contains critical science-based information about FDA-approved medications. Drug labeling contains 17 main sections, including those widely used to study drug safety and efficacy, drug-drug interactions, and pharmacovigilance research such as Boxed Warning, Indication and Usage, Dosage and Administration, Warnings and Precautions, and Drug Interactions. The information is collected from preclinical and clinical trials during drug discovery and development, as well as through post-market surveillance. The large volume and complexity of labeling documents presents challenges in efficiently accessing comprehensive drug information. To address this, we have developed FDA software called FDALabel (https://nctr-crs.fda.gov/fdalabel/ui/search) which hosts over 140,000 drug labeling documents with a user-friendly interface. The database includes prescription drugs, biological products, and over-the-counter (OTC) medications. Accessible through Amazon Web Services (AWS), FDALabel facilitates easy retrieval of information via customizable searches by exploring entire documents or specific drug labeling sections and subsections. Furthermore, FDALabel integrates with other FDA drug-centric databases such as Drugs@FDA, Orange Book, Pharmacological Classes, and GSRS (Global Substance Registration System), providing unique ingredient identifiers (UNII) for active and inactive drug substances. Case studies demonstrate the application of FDALabel, including utilizing the MedDRA standard dictionary for extracting adverse reactions and conducting biomarker searches for personalized medicine. With over 1,000 daily users currently, FDALabel serves as a rich resource for researchers, regulators, drug developers, and the public. It plays an essential role in enhancing the transparency, advancement, and safety of drug information to promote public health.

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S02-04
Computational Toxicology in Drug Safety

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Computational toxicology uses *in silico* tools to support integrative approaches for toxicological research and chemical safety assessments via predictive modeling. Traditional approaches are frequently based on quantitative structure-activity relationship studies, which use representations of chemical structures as molecular descriptors which exploit the structural similarity of molecules. The difficulties arise from the limited amount and heterogeneity of data, as well as the complexity of predicting of *in vivo* endpoints based on *in silico* and *in vitro* data.

Modern Machine Learning based on deep neural networks (DNNs), which form the basis of Artificial Intelligence (AI), is gaining popularity in the field of computational toxicology. These methods can digest large amounts of information and provide reliable predictions for new molecules. They can also incorporate non-traditional information, such as images produced by high content screening, to better utilize results of *in vitro* measurements. Emerging solutions include the use of meta- and transfer-learning approaches, which enable the pre-training of models on a large corpus of chemical data of different modalities, and then efficiently applies the models to small datasets using few-shot and zero-shot learning approaches, providing potential alternatives to read-across methods. The estimation of the accuracy of such predictions, which allows for the discrimination between reliable vs non-reliable predictions, is another important direction of the development of these methods. Being considered for a long time as black boxes, DNNs can become interpretable via eXplainable AI (XAI) methods. In combination with Large Language Models, which can explain the reasoning of the methods using natural language, these approaches open new perspectives for the use of interpretable and trustworthy AI in toxicology.

I will recapitulate the recent developments in AI methods, and discuss their impact on computational toxicology and drug safety as showcased in the studies performed within the Advanced Machine Learning for Innovative Drug Discovery (AIDD) https://ai-dd.eu project, as well as studies published in a special issue of ChemResTox [1].

This study was partially funded by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie Actions grant agreement "Advanced machine learning for Innovative Drug Discovery (AIDD)" No. 956832 and Doctoral Networks grant agreement "Explainable AI for Molecules – AiChemist" No. 101120466.

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S03 | PFAS compounds and Immunotoxicity

S03-01
Using NAMs to address PFAS immunotoxicity: *in vitro* and *in silico* approach

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Per- and polyfluoroalkyl substances (PFAS) are a class of synthetic chemicals widely used in industry, to which people and the environment are exposed. Human studies have shown that PFAS exposure is linked to immunosuppression, increased risk of infections, and a diminished response to vaccinations, yet the precise mechanisms of action remain largely unexplored. Therefore, this presentation aims to elucidate the immunotoxic effects of PFAS and uncover the mechanisms behind them by leveraging New Approach Methodologies (NAMs)-based studies. An integrated testing strategy (ITS) consisting of *in vitro* and *in silico* predictions was developed to bridge this knowledge gap. Based on the *in vivo* evidence of reduced antibody produc-

tion, suitable *in vitro* models were identified that allow the evaluation of parameters relevant for the immunotoxicity *in vivo*. The effects of PFAS on the most important immune cells were studied using only human models, and the results obtained with the selected NAMs support the *in vivo* evidence, demonstrating the value of the *in vitro* human immune system model selected also for the identification of gender different. Furthermore, mathematical fate and distribution models were employed to identify nominal concentration of PFAS in the *in vitro*, while a Physiologically Based Kinetics (PBK) model facilitated quantitative extrapolation from *in vitro* results to potential *in vivo* outcomes. Building on the PBK model's data, the 'Universal Immune System Simulator' was utilized to extend our ITS, enabling us to assess immunosuppressive effects also on potentially vulnerable populations. In accordance with data present in the literature, also the *in silico* results obtained from this study evidenced an immunosuppressive effect in the models used, with differences between the selected PFAS. In summary, the results corroborate the immunosuppressive potential of PFAS observed *in vivo*, highlighting the effectiveness of the ITS in modeling PFAS dynamics and kinetics through alternative, human-relevant methods.

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S03-02
PFAS exposure, vaccine antibody concentrations, and risk of infection among West African infants

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Globally, humans are exposed to PFAS from various routes including contaminated food and drinking water, and PFAS are transferred across the placenta and into breast milk thereby causing peak exposures in infancy. PFAS exposure has been associated with reduced antibody response after childhood vaccination, but the majority of studies have been performed in high income countries. With this study, we aimed to examine the impact of PFAS exposure in two West African countries with high infant morbidity.

We measured serum-PFAS concentrations among 437 infants (median age: 168 days) from Guinea-Bissau and Burkina Faso and 200 mothers from Burkina Faso. Children were followed to age 15 months in Burkina Faso and to age 24 months in Guinea-Bissau. Measles antibody concentrations were measured in serum obtained before (age 4 or 9 months) and after measles vaccination (age 9 or 15/24 months). Information about health facility contacts for infections was obtained from maternal report and records kept at health facilities.

We found six types of PFAS in the serum of Guinea-Bissau infants and Burkina Faso infants and mothers and calculated the sum of the six PFAS types. The median serum-sumPFAS concentrations were markedly lower in this study population compared to infants and mothers in high-income countries.

In Guinea-Bissau, increased infant serum-PFAS concentrations were associated with reduced pre- and postvaccination measles antibody concentrations, but these findings could not be reproduced among Burkina-Faso infants. However, increased infant serum-PFAS concentrations were associated with higher rates of infections among infants in both Burkina Faso and Guinea-Bissau, and in Burkina Faso, increased maternal serum-PFAS concentrations were likewise associated with higher infant infection rates.

Although immunotoxicity is considered the most sensitive endpoint for PFAS exposure, the clinical relevance of the potential immune system changes has been debated. This study indicates that the harms of PFAS exposure have a clear clinical relevance – even in a population with low PFAS exposure.

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S03-03
Effects of PFAS on T cell membrane fluidity and its immunotoxic consequences

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Perfluorinated alkyl substances (PFAS) pose a possible danger to the health of exposed people due to their almost ubiquitous distribution and, depending on the chain length, their high persistence in the environment and living organisms. PFAS are a large group of substances, characterized by their amphotericity. Epidemiological evidence correlates PFAS in serum with immunosuppression as most sensitive effect and liver toxicity as one important of several other effects. Indeed, the connection with reduced antibody production after vaccinations currently represents the basis for the risk assessment for 4 substances (PFOA, PFOA, PFNA and PFHxS). However, the molecular basis for impaired immune responses as well as for liver toxicity is unclear. One possibility could be an increase in cell membrane fluidity due to membrane-integrated PFAS. The formation of solid membrane structures, the so-called lipid rafts, controls signal transmission between immune cells and is influenced by cell membrane fluidity. Signaling via cell-surface receptors is crucial for T and B cells in adaptive immunity. We exposed a human liver cell line (HepG2) and T cell line (Jurkat) to PFOA (perfluorooctanoic acid) or PFNA (perfluorononanoic acid). Toxicity correlated with chain length and exposure time. Liver cells exhibited greater membrane fluidity by PFOA, as assessed by a fluorescent excimer assay, which correlated with an increase of membrane stabilizing cholesterol content in the membrane after PFOA and PFNA treatment. On the contrary, in Jurkat T cells PFOA seemed to lower membrane fluidity and preliminary results suggest that, when T cells were stimulated via their antigen receptor with anti-CD3/anti-CD28 antibodies, the presence of PFOA could induce higher IL-2 secretion, contrary to expectations. These results provide a basis for further studies on a connection between changed cell membrane fluidity and T cell as well as B cell activation. Furthermore, the involvement of a changed cholesterol distribution, possibly as a compensatory reaction, might pose a further aspect to consider in the assessment of PFAS-induced membrane effects. Ultimately, such molecular knowledge might provide a basis for subsequent studies to derive potency factors for various PFAS.

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S03-04
Effects of PFAS exposure on B cell functions

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Exposure to per- and polyfluoroalkyl substances (PFAS) produces marked reduction in antigen-specific antibody responses in experimental rodent models. These toxicological data are supportive of findings of reduced antibody responses to vaccines in people who have been

exposed to PFAS. The reductions in antigen-specific antibody responses in experimental rodent models occur when exposed animals are immunized with either T cell-dependent or T cell-independent antigens, suggesting that the deficiency in antibody production lies at the level of the B cell. However, earlier studies reporting antibody reduction did not report reductions in the overall number of B cells in the spleen. Thus, experiments were conducted to dive more deeply into B cell subsets in mice exposed to the legacy PFAS, perfluorooctanoic acid (PFOA) at a dose and duration known to suppress the T cell-dependent antibody response (TDAR). Male and female C57BL/6 mice were orally dosed with 0 or 7.5 mg/kg of PFOA for 15 days; one subset was immunized with a T cell-dependent antigen five days before dosing ended and another subset received no immunization. One day after dosing ended, animals were humanely euthanized, spleens were removed, and prepared into single cell suspensions. The subset of spleen cells from immunized animals were stained with markers to identify the following B cell subsets: naive, marginal zone, follicular, plasmablasts, and memory. From the subset of spleen cells that were not immunized, naive B cells were isolated, activated ex vivo with anti-CD40+ and IL-4. After a 24 hour incubation, activation was verified with flow cytometry and mitochondrial markers were evalauted with a mitochondrial stress test kit on a Seahorse XFe96. Alterations were found in numbers of follicular cells and plasmablasts in male animals and shifts in mitochondrial energy use were detected in B cells collected from both male and female animals. These data suggest that exosure to PFAS may inhibit the ability of B cells to differentiate and/or proliferate due to altered bioenergetics. Future work will continue to explore this potential mechanism of suppression of the antigen-specific antibody response across different PFAS structures.

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S04 | New developments in micro- and nanoplastics research

S04-01
Inflammation-related key events stimulated by micro- and nanoplastics

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The presence of micro- and nanoplastic particles (MNP) in our environment has raised increasing public and political concern. Increasing evidence suggests that humans are exposed to these MNP, mostly via inhalation or ingestion. The EC-Horizon project POLYRISK (https://polyrisk.science) aims to establish an IATA/AOP-based tiered approach to assess human health risks of MNP. In our research, we focus on assessing immunotoxicological effects of MNP.

We have tested a series of primary, secondary, environmentally aged (incubated in River Rine water) and chemically altered MNP for their potential to affect dendritic cells or macrophages. MNP used in this study include primary and weathered PS, secondary PVC, PA, PP (with or without talc) and PE. The secondary PE and PP (w/o talc) particles appeared to have oxidized groups on their surface.

Data shows that MNP can be engulfed by macrophages (PMA-stimulated human THP1 cells) and human blood-derived dendritic cells (DCs). The effects of virgin as well as secondary MNP (0, 10 or 100 µg/ml) on THP1 macrophages were limited to decrease of mitochondrial activity (Alamar blue), increase of cellular leakage (LDH), and stimulation of lysosomal activity. None of these MNP stimulated gene expression of NFκB or release of pro-inflammatory cytokines (IL-6, TNFα, IL-1β). On the other hand, secondary PP and PE with oxidized surface groups did increase NFκB gene expression and release of cytokines by THP1 macrophages. Virgin and weathered PS particles were tested using DCs, and only weathered PS did stimulate DC activity (increased costimulatory molecules CD83, CD86) and as consequence allogeneic T cells. DC activation appeared to result from environmental contaminants.

In conclusion, of all MNPs tested only those that contained active surface groups or environmental components appeared to be immunostimulatory, whereas primary and secondary MNP rather reduced macrophage activity and viability (at relatively high concentrations of 100 µg/ml). Further research is needed to reveal molecular mechanisms and to translate *in vitro* findings to real-world exposure scenarios.

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S04-02
Redox-mediated toxicity of micro- and nanoplastics in intestinal models

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Micro- and nanoplastics (MNPs) have been detected in various human tissues, including blood, lung, colon, placenta, liver, kidney and spleen, yet there is limited knowledge about their health effects. A primary route of MNP exposure for humans is through ingestion, with the intestine acting as the first line of defence. Similar to other (nano)particles, alterations in the cellular redox state represent a fundamental step in the toxicological pathways associated with MNPs.

In this talk, I will elaborate on the link between MNPs and redox responses within *in vitro* intestinal models (Caco-2, Caco-2/HT29-MTX-E12), and how physicochemical properties of MNPs are of importance. Because of the diversity in sizes, polymer types, and shapes of MNPs, we use a range of both commercially available and environmentally relevant particles to tackle the complexity of MNPs (e.g. polystyrene beads and fibres, polyvinyl chloride fragments, low density poly ethylene fragments). I will focus on the interplay between mitochondria and redox state by looking into the expression of redox-related genes, H_2O_2 levels, mitochondrial DNA content, footprint and mitochondrial network morphology.

Overall, our findings show that smaller MNPs (<1µm) are more easily taken up, and translocated across the intestinal barrier, than larger MNPs. None of the MNPs induce a cytotoxic response, but distinct redox responses (such as higher *HMOX1* gene expression) and mitochondrial stress responses (including higher mitochondrial DNA content) are observed depending on the size and shape of the MNPs. Weathering by artificial stomach acid changes the behaviour of the MNPs, and thus their underlying redox responses.

These results stress the importance to further explore the relationship between the physicochemical properties of MNPs, their uptake kinetics and redox responses to fully comprehend the hazard that MNPs may pose to the intestinal barrier.

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S04-03
Towards a risk assessment framework for micro- and nanoplastic particles for human health

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Background: Human exposure to micro- and nanoplastic particles (MNP) is inevitable as they have been detected in all environmental compartments as well as in numerous foods and beverages. However, human health risk assessment is challenging for several reasons. MNP represent a highly heterogenous class of particles that can be made from different polymers, which often contain plenty of additives. MNP show broad size distributions, often with irregular shapes/morphologies. Moreover, they can be contaminated with plenty of environmental pollutants and can change in several properties due to weathering. Conceptual approaches how to characterise human health risks are rare. Overall, (validated) methods are largely missing. Data is scarce and, if at all existing, sometimes questionable.

Methods: We reviewed state-of-the-art approaches and concepts for risk assessment covering chemicals and in particular polymers as well as particles conserving fibres and nanomaterials to identify suitable elements that be used or adapted for risk assessment of MNPs. Among others, we identified the following useful concepts, i) polymers of low concern (PLC), ii) poorly soluble low toxicity particles (PSLT) and iii) fibre pathogenicity paradigm (FPP). Furthermore, we broadly screened relevant guidance documents, standards, scientific publications and publicly available reports including deliverables or published standard operating procedures (SOPs) to identify promising methods, which may serve as a reasonable starting point in the absence of methods being specifically established/ validated for MNPs.

Results and Conclusion: Here we propose a practical and modular risk assessment framework for MNPs, focusing primarily on inhalation as a key exposure route for humans. It should be easily amendable, for instance to also cover other exposure routes. It combines several integrated approaches to testing and assessment (IATAs). We also provide guidance and (to the extent possible) suggest suitable methods that cover the characterization of physicochemical properties, exposure and hazard assessment. We have put a particular emphasis on New Approach Methodologies (NAMs) and also considered grouping, where suitable. The framework has been improved in iterative cycles taking into account expert feedback and it is currently being tested in several case studies. Overall, our framework is an important step forward to tackle human risk assessment of MNP.

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S04-04
Acute and subacute toxicity of inhaled micro- and nanoplastics using advanced In Vitro models

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Recent studies have shown that the 3D printing process release not only volatile organic compounds (VOCs) but also micro- and nanoplastics (MNPs), posing potential health risks to users. Once inhaled, these particles may be deposited in various parts of the respiratory system, potentially causing acute, subacute, and chronic adverse effects. Traditionally, regulatory authorities require data from animal models to assess inhalation toxicity. However, animal models, such as rats, do not always provide relevant human toxicity data due to the greater sensitivity of rats compared to humans, largely because of the complexity of the rat nasal turbinates. Therefore, human-based non-animal methods are highly recommended. Among these, advanced *in vitro* models made of primary human bronchial epithelial cells represent promising models as they closely mimic the human *in vivo* lung microenvironment, reproducing the biophysiology of human airway epithelia, including a functional mucocilliary system and mucus secretion. Moreover, they can be maintained for up to 6 months without losing their biological characteristics, making them suitable for acute and subacute hazard assessments. Here, we show the application of an advanced *in vitro* pulmonary model to evaluate the acute and subacute effects of MNPs released from 3D printing. We tested the acute and subacute toxicity of polycarbonate (PC) MNPs (<5 μm) with and without single-wall carbon nanotubes (SWCNT), and polypropylene (PP) MNPs (<5 μm) with and without silver nanoparticles (Ag). Repeated short-term exposures (4 hours per day) were performed over a period of up to 28 days and several endpoints were evaluated: cytotoxicity, cell barrier integrity, inflammation, genotoxicity and oxidative damage. Results suggest that repeated short-term exposures to MNPs induce acute but mainly subacute effects (7 to 28 days exposure). Our findings suggest that long-term exposure to MNPs with and without nanomaterials induce cytotoxicity, inflammation and genotoxicity in human bronchial cells. Despite exhibiting relatively high uncertainty, the advanced *in vitro* pulmonary model proved to be a useful tool for detecting subacute effects of 3D printing MNPs. It was capable of discerning differences in particle toxicity with and without nanomaterials, thereby aiding in the inhalation risk assessment of particulate materials.

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S05 | Young scientist session: emerging researchers in the field of *in vitro* and *in silico* toxicology

S05-01
Functional mapping of neurodevelopmental disorders to ontology-based DNT NAMs for Next Generation Risk Assessment of chemicals

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In the pursuit of advancing human risk assessment of chemicals without animal testing, the European H2020 ONTOX project introduces an innovative solution [1]. This presentation delves into developing a novel approach utilizing ontology-based artificial intelligence-driven New Approach Methodologies (NAMs) in line with the next-generation risk assessment. Our focus lies in predicting the developmental neurotoxic (DNT) effects of chemicals, particularly those resulting in decreased cognition in children following prenatal exposure to chemicals.

Our methodology begins with a comprehensive compilation of existing information on neurodevelopmental disorders, emphasizing the functional impairments within key neurodevelopmental processes. Subsequently, we assembled and curated a DNT ontology encompassing a physiological map describing the neurodevelopmental processes in the developing human brain and an adverse outcome pathway (AOP) network delineating chemical-induced cognitive impairments in children. Finally, we complemented this approach with a tailored and characterized human *in vitro* test battery to assess the chemicals' DNT effects.

The resulting physiological map of the developing brain serves as a multi-layered knowledgebase, detailing neurodevelopmental processes, major brain cell types, and developmental timeline in the first layer of the map, a cell-cell interaction map in the 2nd layer, and 4 submaps focusing on the development of 4 major brain cell types in the final layer. Notably, this map incorporates impaired cellular and molecular functions observed in various neurodevelopmental disorders, facilitated by a plugin integrated into the map visualization platform. Additionally, the nodes of the maps are overlayed with transcriptomic data from developing healthy and afflicted brain tissues, enriching the understanding of disorder-specific molecular signatures. Simultaneously, we curated an AOP network comprising 16 AOPs available in the AOP-Wiki and scientific literature. We then identified neurodevelopmental disorders-related gaps in the existing key events of the available AOPs using the ICD-11 International Classification of Diseases system. Finally, we characterized the biological applicability domain of the selected *in vitro* assays using specific inhibitors and activators of selected signaling pathways. This allowed us to establish connections between the signaling pathways governing these processes and neurodevelopmental disorders characterized by dysfunction within these pathways.

By linking disturbed signaling pathways, proteins, and AOP key events associated with neurodevelopmental disorders to corresponding neurodevelopmental processes, our approach provides a robust foundation for predicting chemical impacts on the developing human brain. The final AI-driven ontology-based strategy, coupled with tailored *in vitro* assays and exposure assessment, marks a significant advancement in next-generation risk assessment of chemicals.

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S05-02
In silico toxicology from a deep tech's perspective

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Chemical safety assessment is increasingly including in silico methodologies such as QSAR (Quantitative Structure-Activity Relationship) models. These computational approaches use AI-based and other algorithms to correlate chemical structures with biological or chemical properties, offering a high-throughput, cost-effective, and ethically favorable alternative to traditional experimental methods.

Recognizing the need for accessible QSAR applications, we have developed ProtoPRED, a user-friendly web platform designed to enhance the accessibility of QSAR models for in silico evaluation of a complete panel of endpoints, including the possibility to perform an exhaustive toxicological analysis.

We have developed specific QSAR models by using experimental data from public databases, following OECD guidelines and a rigorous data curation process that ensures the reliability and homogeneity of the datasets. ProtoPRED's models are built using 4500+ molecular descriptors, including 2D and 3D descriptors, selected through a combination of automated and manual processes. Several machine learning algorithms were explored in order to find the most appropriate ones for building each model. All models were tested on independent validation datasets and their quality was statistically assessed.

Models are included in the ProtoPRED platform through a suite of prediction modules covering a broad spectrum of endpoints relevant to toxicology, ecotoxicology, physicochemical properties, and ADME (Absorption, Distribution, Metabolism, and Excretion) profiling. Two additional modules specifically cater to the regulatory contexts of REACH and ICH M7 norms, facilitating compliance with international chemical safety standards and providing automatically the QMRF and QPRF dossiers required for regulatory purposes. There is also a module which deals with QSAR models for nanomaterials and one for the prediction of genotoxicity using the integrated testing strategy proposed by REACH. The platform's design emphasizes ease of use, enabling both experts and non-specialists to conduct sophisticated chemical safety assessments with minimal training.In summary, ProtoPRED represents a significant stride towards integrating advanced QSAR models into the regulatory and research workflows of chemical safety assessment. It exemplifies how modern in silico techniques can complement traditional testing methods, offering a more ethical, economical, and efficient pathway to understanding chemical toxicity and protecting public health and the environment, contributing to the green toxicology approach. Some examples of the use of QSAR models included in ProtoPRED will be discussed.

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S05-03
The development of a human liver-based *In Vitro* test battery to detect liver steatotic potential of chemicals from various applicability domains

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The fields of toxicology and chemical risk assessment are currently witnessing a paradigm shift moving away from animal testing towards the use of non-animal and human-based New Approach Methodologies (NAMs) [1]. In this respect, the European Horizon 2020 project ONTOX, "Ontology-driven and artificial intelligence-based repeated dose toxicity testing of chemicals for next-generation risk assessment", is set to generate NAMs for the prediction of chemical-induced hepatotoxicity, among others [2,3]. In this regard, the current work aims to establish an *in vitro* test battery in a human hepatoma HepaRG cell model encompassing a range of functional assays. Each of the assays targets a key event within the recently optimized adverse outcome pathway network for chemical-induced liver steatosis [4]. To evaluate the applicability domain of the *in vitro* test battery, 6 data-rich steatogenic chemicals from various sectors were selected, including 3 pharmaceuticals (sodium valproate, tetracycline HCl and amiodarone HCl), 2 plasticizers (trimethyl phenyl phosphate and perfluorohexanesulfonic acid), and 1 pesticide (cyproconazole). The HepaRG cell model was exposed to a concentration range of CC_{10} (*i.e.* the concentration inducing 10% cell death) and two ten-fold serial dilutions of the 6 (non)-pharmaceutical chemicals for 72 hours of daily exposure. The developed *in vitro* test battery successfully detected intrahepatic lipid accumulation induced by each steatotic chemical in a concentration-dependent manner. Moreover, alterations in fatty acid uptake, lipid efflux and mitochondrial fatty acid beta-oxidation were differently modulated by the chemicals. Overall, these results suggest that the established *in vitro* test battery exhibits capability in assessing the liver steatotic potential of chemicals from various applicability domains.

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S05-04
Simulating kidney tubular crystallopathy *in vitro*

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Kidney crystallopathy is characterized by the presence of crystals within renal tubules, causing various harmful effects. The interplay of crystal-induced tubular obstruction, cytotoxic effects, and inflammatory responses is central to the pathogenesis of crystallopathy, making them a distinct category of kidney disorders with specific histological features and associated challenges in diagnosis and treatment. The objective of this research is to investigate the mechanisms underlying crystal-induced kidney inflammation and to identify the process involved in inflammasome activation within proximal tubules. Our study focuses on simulating kidney crystallopathy and its impact on inflammasome components, caspase activity, cytokine production, and the NF-κβ pathway using *in vitro* models.

The study utilized conditionally immortalized proximal tubule epithelial cells (ciPTECs) to mimic kidney tissue and investigate the effects of uric acid, a known crystal-forming metabolite, and the influence of acidification on crystal formation under different pH conditions. A series of *in vitro* assays were explored to evaluate cytotoxicity, oxidative stress, mitochondrial function, inflammation, and the expression of NLRP3 inflammasome-related markers at both gene and protein levels.

Exposure of ciPTECs to uric acid under acidic conditions significantly reduces cell viability and triggers oxidative stress and inflammation compared to normal physiological pH levels. Moreover, crystal formations were shown to occur at a concentration of 800 µg/ml of uric acid. The study reveals that uric acid induces the expression of inflammasome-related markers such as caspase-1, ASC, and TNFα at the mRNA level, along with increased levels of IL-1β protein. These effects are more pronounced at higher uric acid concentrations and lower pH levels.

The results of the study will be utilised as a conceptual basis toward establishing and expanding upon a test battery of *in vitro* assays that could further characterise crystal-forming and potentially nephrotoxic chemicals by measuring individual key events toward the generation and evaluation of adverse outcome pathways for crystallopathy-related kidney failure.

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S06 | Consideration of dose-response in the assessment of genotoxic carcinogens

S06-01
Mechanisms driving non-linear dose-response relationships in genotoxicity

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Risk assessment of chemical carcinogens is an important task in toxicology. Although exposure has been effectively reduced in recent decades, low levels of carcinogens are still present in food and in the workplace and are often unavoidable. An important and widely accepted distinction concerns genotoxic and non-genotoxic carcinogens; for the latter group the existence of no-effect concentrations (thresholds) is assumed. In contrast, genotoxic carcinogens, their metabolic precursors and DNA-reactive metabolites are considered risk factors at any concentration, since even one or a few DNA lesions can in principle lead to mutations and thus increase the risk of tumors. However, in recent years, updated risk assessments for genotoxic carcinogens have been proposed (e.g., [1]). They consider mechanistic knowledge of the substance (group) under investigation, including the cellular response to DNA damage, but also endogenous exposure due to physiological metabolic processes. Together with significant improvements in analytical techniques for quantifying even background levels of DNA lesions and mutations, as well as the increasing application of "omics" approaches, refined dose-response assessments appear appropriate in case of well-investigated compounds. Specific examples of genotoxic carcinogens to which humans are exposed both exogenously and endogenously are formaldehyde and acetaldehyde. In other cases, such as benzo[*a*]

pyrene diolexpoxide, the assumption of linear dose-response relationship appears to be more appropriate. Finally, special attention is given to some carcinogenic metal compounds that are considered indirect genotoxins, accelerating mutagenicity through interactions with the cellular response to DNA damage, but at particularly low exposure conditions. Thus, a refined strategy for assessing the carcinogenic risk associated with exposure to genotoxic compounds is proposed.

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S06-02
Consideration of dose-response in the assessment of genotoxic carcinogens

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The Health and Environmental Science Institute (HESI) Genetic Toxicology Technical Committee (GTTC) and other expert groups including the International Workshop on Genetic Toxicology (IWGT), have assessed the use of genetic toxicity data for risk assessment purposes. Other toxicological endpoints are often used to calculate health-based guidance values (HBGV), and genetic toxicity data should also be used in a quantitative manner for risk assessment, either as an adverse outcome, or as a key event towards the cancer adverse outcome. Key concepts around this topic, are which statistical method should be used to define which metric for use as a point of departure (PoD), with the benchmark dose (BMD) approach in PROAST at a critical effect size (CES) of 50% being supported. To calculate a HBGV, the rodent derived BMD lower confidence interval (BMDL50) is converted to a human value by multiplication to human body weight, and division by a series of uncertainty factors (UF). Current data indicate that the default UF are reasonable i.e. 10 for interindividual and 10 for study duration with current effect severity UF at a recommendation for 2 to 10. There are some examples of environmental chemicals and pharmaceutical impurities where relative potency has been shown between *in vivo* genotoxicity and cancer bioassay data, which show that the human population is at no increased risk when using genetic toxicity data in risk assessment.

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S06-03
Next-generation testing strategies for quantitative assessment of genomic damage

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In human health risk assessment of chemical substances, genotoxicity hazard for regulatory purposes is considered one of the more important endpoints to consider because of its possible impact on human health. Usually, genotoxicity testing starts with a standard battery of *in vitro* toxicity tests, which is needed to cover all genotoxicity endpoints: gene mutation, clastogenicity and aneugenicity. The interpretation of test results from this *in vitro* battery typically involves a dichotomous (yes/no) evaluation. However, over the past decades our knowledge on mechanisms underlying a wider range of genomic damage has advanced. In addition, a paradigm shift in applied genetic toxicology is moving the field towards a more quantitative dose-response analysis and point of departure determination with a focus on risks to exposed humans. Therefore, the Genetic Toxicology Technical Committee of the Health and Environmental Sciences Institute has developed a framework for assessing the risk of genomic damage via exposure to chemical substances [1]. The framework entails a systematic approach with the aim to quantify risk levels for substances that induce genomic damage contributing to human adverse health outcomes. In this lecture, the utility of the next-generation framework to quantitatively model human risk on the basis of genetic damage will be demonstrated through a few case studies. Key observations based on the case studies will be presented, together with the needs to further improve the framework as well as its applicability for regulatory purposes.

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S07 | Emerging technologies in pharmaceutical preclinical testing

S07-01
The current landscape of non-animal preclinical assessment for biopharmaceuticals

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The development of biopharmaceuticals is a complex process, traditionally involving extensive use of animal models to assess safety and efficacy. Advances in scientific methodologies and ethical considerations are now driving a shift towards non-animal preclinical assessment techniques. This shift is guided by the 3R principle: Replacement (substituting animal tests with alternative methods), Reduction (minimizing the number of animals used), and Refinement (enhancing techniques to reduce animal suffering).

Key non-animal methods include in vitro techniques and computational models. Cell-based assays and organ-on-a-chip systems allow for detailed assessment of drug interactions and mimic human organ functions. Computational models help predict biological responses and potential toxicity.

Regulatory acceptance is crucial for adopting these methodologies. Engaging with regulatory agencies through scientific advice meetings ensures alignment with expectations and supports the rationale for non-animal methods.

This overview will provide insights into the latest scientific advancements, regulatory frameworks, and practical considerations for implementing non-animal methodologies, ultimately aiming to improve clinical outcomes.

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S07-02
CRO perspective of emerging technology use for preclinical decision-making

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No abstract has been submitted.

S07-03
Non-animal preclinical safety testing strategy

J. Brown

PETA Science Consortium International e.V., Germany

No abstract has been submitted.

S08 | Innovative *in vitro* screening tools for assessing the risk of immunotoxic chemicals

S08-01
Endocrine disrupting chemicals (EDCs)-Receptor for activated C kinase 1 (RACK1) liaison: from bridging the immune and the endocrine systems to EDC screening tool

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Background: RACK1 (receptor for activated C kinase 1) has a central role in the immune system due to the strong correlation between its expression and PKC-mediated immune cells activation. This results in pro-inflammatory cytokines TNF-α and IL-8 modulation *in vitro*, *in vivo* and *ex vivo* [1]. A hormone-related regulatory element for androgens and glucocorticoids (ARE/GRE) in RACK1 gene promoter mediates its transcriptional regulation, suggesting that hormone-active substances can alter RACK1 modulation, affecting the immune response [2]. EDCs (endocrine disrupting chemicals) can induce immune alterations by exerting inflammation-enhancing and immunosuppressive actions and a role for EDCs in the increased incidence of cancers, autoimmune diseases, and allergies in most industrialized countries has been hypothesized [3]. Hence, we aimed to assess how EDCs interfere with the immune response by modulating RACK1 expression and to elucidate the mechanisms behind their immunological implications.

Methods: To investigate EDCs ability to modulate RACK1 expression, THP-1 cells were exposed to different concentrations of anti-androgens p,p'DDT, p,p'DDE, vinclozolin (VCZ), atrazine (ATZ) and cypermethrin (CYP), estrogen-active compounds diethylstilbestrol (DES), zearalenone (ZEA) and ethynyl-estradiol (EE) and, finally, perfluorooctanesulfonic acid (PFOS), diethyl-phthalate (DEP), bisphenols A, AF and S (BPA, BPAF, BPS). Luciferase reporter assay, qPCR, Western blot analysis, specific sandwich ELISA and flow cytometric analysis were performed.

Results: p,p'DDT, p,p'DDE, VCZ, ATZ, CYP (all AR antagonists), PFOS and DEP (GR agonists) induced a significant decrease in RACK1 transcriptional activity, RACK1 expression, LPS-induced IL-8 and TNF-α production and CD86 expression. On the other hand, DES, ZEA and EE (through GPER activation) increased RACK1 transcriptional activity and its expression, which paralleled an increase in LPS-induced IL-8, TNF-α production, and CD86 expression all dependent on RACK1/PKCβII activation. Flutamide completely prevented DES-induced RACK1 transcriptional activity and protein expression, confirming a role for AR in RACK1 transcription regulation. Finally, while BPS displayed upregulating effects on RACK1 production and consequent cytokine release, BPA and BPAF initially downregulated RACK1 but specific inhibitor pre-treatments unmasked upregulating effects and shed light on their mechanism of action [4–10].

Conclusions: The complex effect resulting from the activity as antagonist or agonist of hormone-active substances shows how RACK1 modulation and its PKC-mediated downstream effects in the immune context are of important interest. Therefore, RACK1 represents a bridge between the immune and the endocrine systems, indicating its relevance as target of steroid-active substances and EDCs. This offers the possibility to exploit RACK1 as a tool to screen EDCs for their immunotoxic potential.

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S08-02
The importance and recent development of screening tools in the immunotoxicological context

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Immunotoxicity testing has long been considered impossible without the use of animals. The reasoning for this was that the immune system is too complex and dynamic to be mimicked *in vitro*. Indeed, the complete adverse outcome pathway (AOP) of allergic or autoimmune(-like) diseases cannot be reproduced in a single or even a combination of multiple test methods. On the other hand, because these immune system-based outcomes are extremely personalized they are also not reproducible in standard animal toxicity tests. In addition, onset of the diseases of interest are co-influenced by chemicals and microorganisms, thus further hampering hazard prediction using *in vitro* or animal tests.

Chemical-induced inflammatory diseases, e.g. allergies (i.e. contact and drug allergy) and autoimmune diseases are known to involve both the innate and adaptive arm of the immune system. Innate-immune components (including a range of innate leukocytes and humoral fac-

tors, but also epithelial barrier cells) are important in shaping, instructing and helping the adaptive immune cells, i.e. T and B cells. In this sense the innate immune system helps the adaptive immune system to be tailored for an effective response. Indeed, many studies in the last decades also pointed out that the innate immune system is crucial in initiating chemical-induced inflammatory diseases. Often inflammation is used in these cases as a single crucial key event, but in fact inflammation can be dissected into multiple key events. Recently, three of these KE have been identified as overarching KE that fit to many adverse outcome pathways and can also be addressed using straightforward *in vitro* screening tools. These three KE include: 1. Tissue resident cell activation; 2. Increased proinflammatory signaling; and 3. Leukocyte recruitment and activation.Now, these inflammatory hub-KEs can be plotted on different immunotoxicity related cases, such as contact allergy, food allergy, and drug induced liver injury.

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S08-03
Exploring applicability domain and predictive value across *in vitro* platforms to detect potential dermal sensitizers

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Current test guidelines for the assessment of skin sensitization utilize *in vitro* and *in chemico* approaches which map to multiple key events (KE) in the adverse outcome pathway (AOP) for skin sensitization. None of these methods can currently be used as a stand-alone assay to determine skin sensitization potential. *In vitro* and *in chemico* tests have been incorporated into defined approaches (DAs), which allow these new approach methods (NAMs) to be used in combination to inform skin sensitization potential. To assess the ability of commonly used NAMs to evaluate complex chemicals and formulations for their potential to induce skin sensitization and explore their applicability domain, we evaluated 181 chemicals with existing local lymph node assay (LLNA) reference data nominated by partner agencies in the U.S. National Toxicology Program. These chemicals were tested in the direct peptide reactivity assay (DPRA; KE 1), KeratinoSens™ assay (KE 2), and Human cell line activation test (h-CLAT; KE 3). Results for the chemicals tested in the DPRA, KeratinoSens™ and h-CLAT, were evaluated for prediction of skin sensitization hazard and potency classification according to GHS categories. Three different DAs: 2 out of 3 (2o3), Integrated Testing Strategy (ITSv2) and Key Event 3/1 Sequential Testing Strategy (KE 3/1 STS) were used. LLNA results were used as historical reference data. The skin sensitization hazard and/or potency classification results for each NAM and DA were compared with *in vivo* outcomes. A subset (31) of these chemicals was tested in the GARDskin assay. Concordance and performance of the GARDskin results were compared to "classic" DAs and individual test methods, including LLNA reference data. For hazard classification, concordance between assays was higher among NAMs than for the LLNA. The highest hazard concordance was noted for comparisons against ITSv2 whereas the lowest hazard concordance for all methods was seen for comparisons against the LLNA. Hazard classification concordance with the LLNA was higher for DAs that incorporated GARDskin than those that did not. Concordance for potency classification was highest between the KE 3/1 STS DA and the ITSv2 DA. For potency classification, the highest concordance was observed between the two ITSv2 options (classic vs. GARDskin). Overall concordance for any of the DAs with LLNA was low, with ITSv2 with the GARDskin method being the highest at 69%. Concordance was highest among the DAs when GARDskin was used as the KE3 endpoint. DAs did not always have the highest concordance with the LLNA, but they engender confidence because they cover multiple key events in the AOP. Overall, NAM concordance with human data was similar to and sometimes better than LLNA concordance with human data. Results from NAMs varied by chemical group but showed that testing and application of DAs may be useful alternatives to animal testing for complex substances.

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S09 | Advances in class-based and grouping approaches to chemical assessment

S09-01
Introduction: the role of methods harmonization in grouping chemicals for hazard and risk assessment

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Environmentally sound management of hazardous chemicals is more likely to be achieved in a sustainable way if there are coordinated international, regional and national efforts to assess the health effects of chemicals. It is recognized that resources to assess and manage chemicals are insufficient globally, and the use of the limited resources available needs to be optimized. If risk assessments of chemicals can be performed using internationally accepted methods, then assessments can be shared and thus avoid duplication of effort. Harmonized methods can also incorporate advances in scientific knowledge and promote sound science as a basis for risk management decisions, as well as promoting transparency. The use of class-based approaches, instead of more resource-intensive chemical-by-chemical approaches, can play a role in making optimal use of resources. Classes can be defined in different ways depending on the decision context. These different definitions need to be understood from different organizations and perspectives, so that harmonized approaches can be considered.

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S09-02
Informatics approaches and evidence mapping to establish the foundation for class-based research and risk assessments

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Health assessors and regulatory agencies generally assess hazard or risk from environmental exposures on a chemical-by-chemical basis that is time and resource-intensive. However, many commercial chemicals are structurally related compounds that may have similar toxicological effects. As such, there is a growing need for organizations to implement class-based assessment methods and consider chemicals together, grouped by physiochemical and toxicological properties. The European Organization for Economic Co-operation and Development (OECD) has guidance on grouping of chemicals for assessing hazards collectively. In the United States, class-based approaches have been applied to some subsets of major chemical groups such as the per- and polyfluroalkyl substances (PFAS) and organohalogenated flame retardants (OFRs). In each case, methods for assessing groups of chemicals require not only the time and effort normally required for performing a risk assessment, but also the additional work to assess multiple chemicals simultaneously. Systematic reviews are the gold standard methodology to summarize and critically assess existing evidence on potential health risks associated with an environmental exposure. These methods provide a rigorous framework designed to minimize bias and maximize transparency. However, the rigor and comprehensiveness of the systematic review approach is time-consuming, resource intensive and designed to address narrowly focused questions. Systematic evidence maps (SEM) utilize the rigor of systematic review methodology to identify, categorize, and present health effects evidence in an interactive format. Because SEMs enable users to better understand and manipulate broad evidence bases, they are ideal methods to support decision making necessary for class-based approaches. The SEM format is also is also well suited for informatics and automation approaches because the format relies on objective data capture of study features such as exposure, outcomes, and study details rather than more subjective judgements typical of a systematic review. This presentation will use examples from current SEMs on health effects associated with exposure to OFRs and to chemicals in personal care products to illustrate the utility of SEMs for class-based hazard and risk assessment as well as the growing possibilities for implementing evidence informatic approaches.

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S09-03
Risk governance of per- and polyfluroalkyl substances (PFAS) as a chemical class

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No abstract has been submitted.

S09-04
Evidence-based approaches to support the development of endocrine-mediated adverse outcome pathways: Challenges and Opportunities

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In the context of a paradigm shift in toxicology and ecotoxicology towards New Approach Methods (NAM) and Integrated Approaches to Testing and Assessment (IATA), Adverse Outcome Pathways (AOP) represent a valuable systematic approach and a practical framework for the organization and understanding of toxicological knowledge. Within the past decade, AOPs have captured the attention of regulators and researchers alike, and became an integral part of research activities like the Horizon 2020 EURION cluster of projects focused on developing endocrine disrupters (EDs) test methods. Like for other fields, the use of evidence-based methods (EBM) could increase the quality and efficiency of AOP development and application processes. This presentation, will update a Perspective article recently published in Frontiers in Toxicology – Regulatory Toxicology, and draw on the EURION cluster's collective experience to overview the challenges and opportunities of EBMs application to endocrine-mediated (EM) AOP development. We illustrate that; (1) systematic evidence mapping may support problem formulation in complementing canonical knowledge and identifying key event relationships (KER) for which systematic review (SR) is appropriate, (2) some selected machine learning tools (MLT) are identified as suitable to support the earlier stages of SR adapted to endocrine-mediated AOP development such as problem formulation or the design of search strategies, (3) their implementation for information retrieval ought to be validated and compared with manual methods, (4) whilst desired and promising, EBM's feasibility and their application to the appraisal of evidence or the evaluation strength of the overall body of evidence is not yet demonstrated.

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S10 | Practical Application of New Approach Methodologies (NAMs) for human health risk assessment

S10-01
Making safety decisions for a sunscreen active ingredient using next-generation risk assessment: benzophenone-4 case study

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Next Generation Risk Assessment (NGRA) is centred on exposure-led and hypothesis-driven methodologies, aiming to protect human health. This presentation will delve into ongoing efforts to advance *ab initio* NGRA for systemic toxicity where points of departure (POD from diverse *in vitro* bioactivity methods) are compared with internal exposure estimates from physiologically-based kinetic (PBK) models. To illustrate this approach, we evaluated the safety of benzophenone-4 (BP-4), a UV filter present at 5% in a body lotion. This chemical was chosen due to regulatory concerns related to potential endocrine disrupting properties. To characterise bioactivity, *in vitro* pharmacological profiling assays, CALUX assays (covering the estrogen, androgen steroidogenesis, and thyroid modalities), high-throughput transcriptomics in HepG2, MCF-7, and HepaRG cell models, and a cellular stress panel in HepG2 cells were conducted. For each assay a POD was calculated.

The tiered approach to estimating key kinetics parameters showed that BP-4 was not metabolised by the liver and does not cross biological membranes easily. Clearance predictions within PBK models generally rely on the assumption that chemicals partition into or through cells. In this case, with limited membrane permeability observed, additional *in vitro* NAMs were explored to better understand the transport

of BP-4 across the cells and delineate its route of elimination (i.e. renal). The results showed that BP-4 is a substrate of OAT1 (SLC), OAT2 (SLC), OAT3 (SLC) and a substrate of the efflux transporters, BCRP (ABC) transporter and MRP4 (ABC) transporter. The impact of transport kinetics on the estimates of plasma C_{max} was evaluated resulting in the selection of the most conservative value for the risk assessment. Due to the limited membrane permeability, a human isolated kidney proximal tubule cell model (aProximate) which expresses the relevant transporters was used to further investigate the potential bioactivity of BP-4. Comparisons between the resultant Cmax and *in vitro* PoDs were made, and bioactivity:exposure ratios (BERs) were calculated. To contextualize these BERs for informed safety decision-making, the outcome of a larger evaluation with 48 chemicals and 94 exposure scenarios will be presented. Briefly, we found that for the majority of these (>90%), safety decisions based on BERs from the NAM-based workflow are protective of human health. This presentation will end with a discussion on the areas of the BP-4 risk assessment that would require further refinement to enable a confident low risk conclusion.

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S10-02
New Approach Methodologies (NAMs): a quantitative *in vitro* to *in vivo* extrapolation case study with perfluorinated compounds

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With the increased call to enforce the 3R (Reduce, Replace, and Refine) principles for chemical safety, major developments have been achieved with New Approach Methodologies (NAMs) and towards the Next Generation Risk Assessment (NGRA). Relying on cell culture models as primary tools for predicting toxicity, in the place of animal experiments, presupposes their Quantitative *In vitro* to *In vivo* Extrapolation (QIVIVE) with the integration of toxicokinetics.

Physiologically based kinetic (PBK) models are computational approaches based on a mathematical representation of absorption, distribution, metabolism and elimination providing a predictive framework for QIVIVE. A QIVIVE case study is presented here for per and poly-fluoroalkyl chemicals (PFAS), a large group of synthetic chemicals that have been linked with several adverse health effects. Amongst them blood lipid perturbations, as a risk factor for cardiovascular disease, hepatotoxicity, and suppression of the immune system. Selected *in vitro* readouts were considered as surrogate biomarkers for the respective PFAS-induced *in vivo* effects and were used as a basis for the extrapolations. Concentration-response data obtained from the *in vitro* studies were converted into corresponding external human dose-response relationships, using PBK model-facilitated reverse dosimetry. Next to this, PFAS biokinetics were studied in the cell system either experimentally or with the application of an *in vitro* distribution model.The calculated oral equivalent effect doses generated through this approach overlap with the current European dietary PFAS exposure, indicating that the latter may lead to interference with lipid metabolism, as well as, with immune system function. These findings demonstrate an impactful application of the QIVIVE methodology, which can be further expanded to rationalize the future selection of PFAS that should be prioritized for further testing and hazard characterization.

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S10-03
Use of NAMs within an IATA for potential mitotoxicants

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Various efforts have been made to propose strategies on how to innovate risk assessment of chemical substances. New concepts suggest the integration of exposure and hazard assessment models and the implementation of **New Approach Methodologies (NAMs) into Next-Generation Risk Assessment (NGRA).** While some case studies that demonstrate the applicability of NGRA approaches have been published, full operationalization of NGRA concepts in regulatory risk assessment of chemicals is limited. The Horizon 2020-funded ASPIS cluster develops a workflow for NGRA entitled **ASPA (Alternative Safety Profiling Algorithm)** ASPA's objectives are to provide visual and flexible guidance for collecting data and to connect new and available knowledge for data interpretation, enhancing the traceability of the decision-making process. The NAMASTOX virtual dashboard will act as an NGRA web tool for the practical implementation of non-animal chemical risk assessment. Here, some aspects of ASPA will be illustrated, using mitochondrial toxicants as an example. In particular, the pesticide **tebufenpyrad** will be investigated, concerning its potential neurotoxicity, and the fungicide **picoxystrobin** will be used as example of a potential developmental toxicant.

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S10-04
Pragmatic one health frameworks based on New Approach Methodologies to assess risks of human disease following exposure to chemicals and pathogens

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No abstract has been submitted.

S11 | New era of cardiotoxicity risk assessment – where we are, challenges and opportunities

S11-01
Cardiotoxicity risk assessment: an example of how the weight of Evidence approach can be used for promoting NAMs and 3Rs

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Specific classification criteria should be used in a weight-of-evidence approach for the assessment of cardiotoxicity of chemicals and, thus, reduce cardiovascular adverse effects in the general population after exposure to chemicals. Classification should be based on the following scientific evidence (findings), in a way that would reduce uncertainties:

- Anatomical and histopathological data;
- Echocardiographic data on contractility (e.g., LVEF, LVFS), documentation of cardiac frequency and/or implementation of other cardiac imaging modalities (e.g., MRI);
- Biochemical data, of generic nature (e.g., circulating oxidative stress markers), of more specific nature (e.g., oxidative stress markers of the cardiac tissue) and heart specific biomarkers (e.g., cardiac enzymes);

- Identification of pathways and mode of actions, which modulate the changes observed in different parameters after exposure to chemicals;
- In silico data, such as adverse outcome pathways (AOPs), omics, in vitro, organs on a chip, physiologically based pharmacokinetic models (PBPK), etc.

The current presentation will describe a methodological approach (weight of evidence) to the collection of in silico, *in vitro* and *in vivo* data by regulators and scientists in order to identify cardiotoxic chemicals from a regulatory perspective and, consequently, endorse legislation measures to protect human health from relevant exposure.

In this context, respective preliminary results obtained will be discussed (published and unpublished data) on evaluation of two important echocardiographic indices, namely ejection fraction and fractional shortening, specific indices and biochemical markers (antioxidant stress and iinflammation) and histopathological data from rats. The results show that they should be further investigated in order to set regulatory criteria and highlight the need for targeted research to this end.

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S11-02
Frameworks for cardiotoxicity assessment: integrating key characteristics, AOPs and failure modes

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Our reliance on animal studies in drug safety assessment stems from our confidence that their broad and complex biology is a reasonable model of human biology and toxicological response. Applying less complex NAMs with a narrower but potentially more human-relevant biology requires that we understand the questions we're asking, the biology we're modeling, their context of use, the endpoints we should measure, and how to make decisions from the data we derive from those systems. A number of biological frameworks have recently been defined including "key characteristics of cardiovascular toxicants", adverse outcomes pathways, and a cardiovascular failure mode framework. These attempts to define and structure what we know about cardiotoxicants and how the cardiovascular system responds to toxic insult can provide useful guides to building, validating, and applying relevant NAM-based modeling systems. This presentation will provide an overview of these frameworks, their strengths and limitations, and their application to the development and application of cardiac-relevant NAMs.

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S11-03
New Approach Methodologies for cardiac toxicity assessment: the ALTERNATIVE perspective

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Humans are continuously exposed to a vast amount and variety of potentially toxic chemicals present in the environment. Different chemical classes (such as fertilizers, pesticides, heavy metals, and plasticizers) are continuously in contact with human bodies and may persist in the environment [1]. Additionally, residues of drugs (i.e., antibiotics, parasiticides, antimycotics, and anti-cancer) and their metabolites have been detected in environmental compartments, including surface water, groundwater, soil, air, and biota. The recent EU pharmacovigilance legislation recognizes that pollution produced by pharmaceutical residues is an emerging environmental issue [2]. Moreover, the combined effects of chemicals and pharmaceuticals are still debated, but studies show how these interactions could negatively affect the prognosis of the diseased population [3], especially of patients with cardiovascular diseases (CVD). CVD deaths have constantly dominated the chart in the last 30 years [4], and it has been hypothesized that the high incidence may be related to environmental exposure to exogenous toxic chemicals [4]. Despite the high CVD incidence in the population, cardiotoxicity is not addressed as a separate endpoint in current toxicology studies.

In this context, the ALTERNATIVE project applies a multidisciplinary approach by: A) producing an easy-to-handle, reliable technological solution representing the 3D bioengineered cardiac tissue and its micro-physiological environment through bioreactors, microfluidics and electrical stimulation printed sensors; B) evaluating the molecular pathways associated with chemical mixtures by multi-omics approaches; C) implementing *in silico* ML models (Toxicokinetic/Toxicodynamic, Quantitative-Structure Activity Relationships, Physiologically Based Kinetic) models for cardiotoxicity prediction of chemical mixtures; D) identifying and developing an Adverse Outcome Pathway (AOP)-integrating toxicological and epidemiological data to guide the design of an Integrated Approach to Testing and Assessment (IATA) and facilitate regulatory uptake of the *in vitro* system.

By integrating the different technologies and competencies, ALTERNATIVE aims to (i) support the evaluation of chemical mixture effects in terms of cardiotoxicity, (ii) update existing rules and/or define new ones for regulatory aspects associated with the assessment of chemical compounds, (iii), put the ALTERNATIVE approach forward as an example of a new way for the integrated system of cardiotoxicity evaluation of the future.

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S11-04
Derisking cardiovascular safety throughout the pharmaceutical product lifecycle

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Since the turn of the century, both functional and structural (i.e., histopathology) cardiovascular (CV) toxicity issues have remained a major reason for adverse drug reactions, safety related attrition and product withdrawal during late-stage clinical development and even post approval. The presentation will offer a perspective on the evolution of CV safety assessment of pharmaceuticals considering the scientific and technological advancements in drug safety science, the paradigm shifts of the drug discovery and development process and the continuously evolving regulatory landscape. Gaps in CV safety sciences resulted from the inability to identify safety hazard across the CV system and interconnected organs and physiological functions; to predict, risk assess, manage, and mitigate against drug CV safety liabilities; and to apply principles of governance on the generation, integration, and use of experimental data. Challenging aspects of CV safety have included an understanding of the molecular mechanisms of CV toxicities to develop, validate and deploy human-based or human-relevant in silico, *in vitro* and *in vivo* test systems to address human specific or selective forms of toxicities. Examples of strategies that are being deployed and will be presented include the assessment of drug effects on the electrocardiogram (ECG, in particular the QT interval), cardiac contractility, blood pressure, clinical pathology, and histopathological readouts. The increasing diversity of drug modalities presents further challenges for nonclinical and clinical development requiring further research to develop suitable CV test systems using relevant technologies. Optimally managing and mitigating CV safety risk has come from the greater refinement of safety margin estimates, the provision and use of human-relevant safety biomarkers, and understanding of the translation from in silico, *in vitro*, and *in vivo* studies to human. Opportunities do exist to evolve our testing paradigm in order to select and progress optimized drugs with increased confidence in success; to refine and adapt the clinical monitoring at all stages of clinical development resulting in an optimized benefit/risk assessment; to increase likelihood of regulatory acceptance in a way compatible with an expedited and streamlined drug discovery and development process to benefit patients; and to avoid the unnecessary use of animals studies and encourage alternative approaches.

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S12 | Skills for early career toxicologists

S12-01
Skills for early career toxicologists

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No abstract has been submitted.

S12-02
Career development: industry

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Careers in industry have their own challenges but can be highly rewarding. In this talk I will share my experiences of moving from academia to industry and back again, including setting up a successful Pharma business. I will highlight key challenges, opportunities and setbacks, including the learning from mistakes made along the way. I will also draw some conclusions around similarities and differences between industry and academia. Overall, the session aims to raise awareness and provide some tips and suggestions to improve your skills or to provide information to help you plan your future.

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S12-03
Career development academia

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No abstract has been submitted.

S12-04
Career development CRO

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No abstract has been submitted.

S12-05
Career development regulatory

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EU regulatory agencies such as EFSA, EMA and ECHA, but also the national ones, such as ANSES, BfR, FSAI, provide many employment opportunities for toxicologists. As an example, EFSA currently has approximately 550 members of staff, of which 80 have a background in toxicology. Of these 80, around 50 are formally employed as toxicologists and the other 30 are engaged in more managerial activities. Among the 50 active toxicologists, 30 deal with human or animal health and 20 are specialised in environmental toxicology. In those functions, they cover most of EFSA's sectors, from chemical contaminants to regulated products such as food and feed additives and plant protection products to novel foods and nutrient sources. The majority of the toxicologists at EFSA have a PhD in a toxicology-related field or a veterinary degree, although those who have benefitted from a formal training in toxicology at undergraduate or post-graduate level are a minority. The employment opportunities range from junior level posts to senior officers. The nature of the tasks performed as a toxicologist working in EFSA is assessing dossiers and secretariat support to Scientific Panels and Working Groups, and unlike some of the national regulatory agencies such as the BfR or ANSES, EFSA does not have any laboratories or testing facilities. A key feature of the work as a toxicologist at EFSA is that it focuses on regulatory toxicology, and as such very much addressing the prevailing sectoral legislations and linked

data requirements, and chemical risk assessment methodologies. However, equally important are the skills needed to support the EFSA in its science communication work and to engage with stakeholders, applicants, EU Member States and the European Commission, among others.

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S12-06
Finding European research funding and writing a competitive research proposal

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Contemporary toxicologists are expected to be widely visible and active at the international level as well as to master a plethora of skills, including project proposal writing. The latter mainly applies to academic settings, where most scientists usually depend on external funding to perform their research. Throughout the years, project proposal writing has become an art, and to some extent even a true business, which is highly competitive. This has resulted in overall low success rates, especially holding for European project proposals. Although initially discouraging for researchers, rejection of project proposals is an integral part of the learning curve and will eventually improve writing skills. This will be addressed in this presentation. Focus will be put on the different stages of project proposal writing, including the prospective work, the preparative work and the actual writing exercise. An overview of sources of European funding will be provided along with a discussion of the typical organization of a European project proposal. Several recommendations will be shared by the speaker originating from own experience built up with writing successful and unsuccessful project proposals submitted to various European funding programs over the past decade.

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S13 | Integrating exposome and risk assessment approaches: a joint strategy for chemical mixture assessment

S13-01
Strategy for real-life mixture risk assessment using HBM data

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Human populations are exposed on a daily basis to numerous chemicals through the environment and diet. The growing concern regarding exposure to chemical mixtures has gained attention in numerous European Member States and by several European Institutions. However, the traditional approach for risk assessment is usually conducted on a substance-by-substance basis, given the complexity of assessing risks from exposure to chemical mixtures. The Partnership for the Assessment of Risks from Chemicals (PARC) Real-life Mixtures project [1] aims to consolidate on international guidelines from EFSA and OECD. Complementary to and in synergy with current activities at the European Agencies, this project is developing practical tools, methods, and a general strategy to enable an effective mixture risk assessment based on human biomonitoring (HBM) data to address policy questions. This presentation will outline the proposed strategy to perform mixture risk assessment in the regulatory context and its practical implementation. The strategy is based on applying HBM data, knowledge developed for mixtures in risk assessment and in the field of toxicology, exposome and epidemiology. This strategy is organised in three steps: 1) Prioritisation of mixtures; 2) Collection and organisation of HBM and hazard data (including toxicokinetic information); and 3) Mixture risk assessment for prioritised mixtures and effects. Its application was carried out primarily on prioritised chemical families and their respective common effect: pesticides and neurotoxicity, metals and developmental neurotoxicity or nephrotoxicity, and PFAS and immunotoxicity. HBM data from several European studies were harmonised and uploaded in the Monte Carlo Risk Assessment (MCRA) software, which is part of the PARC model network. Additionally, hazard data related to internal and external toxicological thresholds were organised around common effects. Statistical analysis, kinetic modelling, analysis of the link with biomarkers of effect, and risk assessment were implemented in the MCRA software, and applied in a harmonised way to the different HBM datasets. This strategy will be extended to mixtures of substances from several chemical families identified from the combination of exposure and hazard data. In addition to producing relevant knowledge for chemical mixture risk assessment, the PARC Real-Life Mixtures project will propose a coherent implementation of the data and models for evaluating the risk of mixtures for end-users including EFSA panels/ECHA working groups, national experts responsible for risk assessment of mixtures and other stakeholders.

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S13-02
Update on EFSA's activities in the area of cumulative risk assessment of pesticides

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The EU Regulation requires EFSA (European Food Safety Authority) to take into consideration the cumulative and synergistic effects in the risk assessment of pesticides. In the last 15 years, EFSA invested time in the development of methodologies to conduct dietary cumulative risk assessment (CRA) which resulted in the publication of the assessments for effects on the thyroid [1], nervous system [2,3] and craniofacial alterations [4]. This exercise has proved to be more complex than initially expected, therefore EFSA and the EU Commission developed an action plan that set out priorities for the ongoing work on method development and the subsequent implementation of the methodology [5].

Among the action points are (i) the development of a prioritisation method to allow the identification of pesticides and organ systems that may present the highest risk in terms of dietary exposure, (ii) the es-

tablishment of new cumulative assessment groups for the organs identified in the prioritisation exercise, (iii) development and implementation of prospective CRA to assess the impact of new pesticide authorisations in the cumulative risks and (iv) the integration of non-dietary exposure in CRA.

EFSA has recently published a scientific report [6] describing the outcome of the prioritisation method on around 350 chemicals and 16 target organ systems. As a first step, pesticides expected to have a marginal contribution to the cumulative risk are identified by applying a risk-based approach: individual hazard quotients are calculated and only substances exceeding the set cut-off values are retained for further assessment. In the second step, prioritised substances are grouped based on their ability to cause toxicological effects on common target organ systems. Combined exposure calculations are performed allowing the identification of high priority organ or biological entities to be addressed in future cumulative risk assessments. Overall, the method allowed the reduction of the initial number of substances and organs by about 80% and 70%, respectively. The following organs and biological entities were identified as high priority for further assessment: reproductive function, development, liver, kidney, male reproductive system and haematological system. Uncertainties in the modelling procedure and methodological assumptions are considered, concluding that risk estimates, as performed, are more likely to be overestimated than underestimated.

Other recent EFSA's activities related to retrospective and prospective CRAs will be also presented.

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S13-03
Case studies showing the applicability of the mixture risk assessment strategy using HBM data

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Human populations are continuously exposed to various chemicals through the environment and diet. The Partnership for the Assessment of Risks from Chemicals (PARC) Real-life Mixtures project has proposed a strategy based on human biomonitoring (HBM) data, knowledge developed for mixtures in regulatory risk assessment and in the fields of toxicology, exposome and epidemiology to perform mixture risk assessment. This presentation will show the application of this strategy to case studies. Chemical with their specific effects of concern were selected based upon priorities expressed by EU Agencies and the European Commission. Overall, five case studies are being addressed in the project: 1) Pesticides – nervous system effect, motor division 2) Pesticides – nervous system effect, brain and/or AChE inhibition 3) PFAS – immune effect 4) Metals – kidney effect 5) Metals – developmental neurotoxicity. HBM data from several European countries such as France, Belgium, the Netherlands, Spain, Greece, Czech Republic, Slovenia, Norway, Denmark, Germany, Finland, Luxembourg and Croatia were harmonised and uploaded in the Monte Carlo Risk Assessment (MCRA) software. One challenge is to identify appropriate toxicological values associated to the selected common effect of the mixture components. As the use of HBM data to perform risk assessments is recent, the establishment of internal toxicological values such as a Human Biomonitoring-Guidance Value (HBM-GV) is still in its early stages. Moreover, HBM-GVs are usually established for the critical effect, which may differ from the selected common effect. Thus, approaches such as the use of an internal reference point or conversion of an external toxicological value into an internal dose were proposed and applied to compensate for the absence of HBM-GVs. This was the case for some metals for which internal threshold were derived from internal reference points in including uncertainty. For the pesticides, reverse dosimetry using the urinary concentration data and the urinary excretion fraction was applied to convert internal exposure to external exposure. The estimated external exposure levels were then compared to the no observed adverse effect levels established by EFSA and total margin of exposure were calculated. For the PFAS, as an HBM-GV was available for PFOA, and internal relative potency factors (RPFs) were available for others, risk assessment was performed using the RPF approach. The selected case studies present the diversity of available information, the proposed solutions in the first-tier mixture risk assessment, and highlight the uncertainties that must be addressed in the second tier of the mixture risk assessment. Furthermore, the strategy will be extended to mixtures crossing regulatory silos identified from co-exposures. These first results will inform risk managers in implementing appropriate risk mitigation measures by identifying substances that have the highest contribution to the risks.

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S13-04
Towards interoperable modelling tools for integrative risk assessment – the PARC model network approach

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Integrating models and data from various disciplines such as exposure science, epidemiology, and toxicology is vital for understanding health effects linked to chemical exposure and for improving decision making. This is for example illustrated by the growing scientific and regulatory interest towards assessments involving aggregate and cumulative exposure, possibly integrating human biomonitoring (HBM) data. However, models and data are often created by separate parties using different concepts, data standards, and programming languages. This diversity hampers efficient and practical implementation of workflows linking models and data for integrative assessments. Clearly there is a need to make data and models more FAIR (findable, accessible, interoperable, reusable).

The Partnership for the Assessment of Risks from Chemicals (PARC) aims to develop solutions to increase FAIRness, leading to a network of linked modelling tools and data. Within this framework, workflows are under development, levering these connections. Starting from existing dietary risk assessment workflows, we introduced workflows for standardized assessment of chemical mixture risk based on HBM data. These specific workflows are accessible via the Monte Carlo Risk Assessment (MCRA) platform (https://mcra.rivm.nl), and efficiently integrate HBM data, exposure models for multiple sources, kinetic models, dose-response models and risk assessment models. This was achieved by employing harmonized data standards such as the PARC standard for HBM data (https://hbm.vito.be/tools) and interoperability standards for linking e.g. exposure models and Physiologically Based Kinetic models. Stakeholders have been trained on the use of the workflows for several assessments such as mixture risk assessment of heavy metals on kidney health or IQ loss, PFASs with an impact on the immune system, and pesticides affecting the nervous system. The workflows prove effective in enabling efficient and transparent integrative assessments, resulting in consistent analysis, output and interpretation across various users, studies and datasets. This may stimulate adoption in the context of regulatory risk assessment. The effectiveness of these workflows will be illustrated by practical applications.

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S14 | New insight into mechanisms of food-borne chemical carcinogens

S14-01
Mechanistic insight into the bioactivation of alkylbenzenes from computational studies

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Alkenylbenzenes are aromatic compounds found in several vegetables that can cause genotoxicity when bioactivated to 1′-hydroxy metabolites by specific isoforms of the cytochrome P450 (CYP) family. These hydroxylated intermediates act as proximate carcinogens and can be further converted into reactive 1′-sulfo-oxy metabolites, which are the ultimate carcinogens responsible for their genotoxicity and carcinogenicity. Therefore, their site-specific hydroxylation by CYPs is the upstream molecular event leading to genotoxicity. Although some alkenylbenzenes either have been or are in the process of being banned as a food or feed additives in many countries, they still enter the food and feed chain being abundant in spices, herbs and essential oils, representing a hazard to human and animal health. Knowledge on CYP isoform-specific bioactivation of alkenylbenzenes provides important insights into which alkenylbenzenes can be bioactivated by humans as well as possible human interindividual differences, but just for a few alkenylbenzenes found in food such information is available. Furthermore, the analysis of inter-species differences across alkenylbenzene bioactivation in farm and companion animals is largely overlooked. For the sake of filling related knowledge gaps, a 3D molecular modelling approach was applied to tackle such an analysis of CYP-mediated bioactivation from a mechanistic point of view. Specifically, the study addressed the possible bioactivation of apiol and dillapiole in humans and showed that they might not be efficiently transformed by the main CYPs known for bioactiving other alkenylbenxenes (due to an improper arrangement at the catalytic site), while suggesting CYP1A1 as an additional biotransforming CYP for safrole [1]. With regards to farm and companion animal species, interspecies differences in terms of safrole bioactivation in dog, cat, rabbit, pig, goat, sheep and chicken have been investigated integrating bioinformatic to molecular modelling. In addition, the animal species have been proritized for further dedicated investigation and a high priority has been highlighted for cats [2]. This study shows the promising application of 3D in silico methods to investigate the bioactivation of alkenylbenzenes. It also expands the current toxicological understanding of those compounds relevant to food and feed safety, supporting the weight of evidence with regards to CYP bioactivation mechanisms from a molecular standpoint.

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S14-02
Mechanisms of colorectal carcinogenesis triggered by heme iron from red meat

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Colorectal cancer (CRC) is one of the most common tumor entities worldwide, with an increasing incidence and mortality in younger adults over the last two decades. Colorectal carcinogenesis is a multistep process that involves various etiological factors. These include genetic predisposition, inflammatory bowel disease, lifestyle factors and dietary habits. Particularly, the consumption of red and processed meat was shown to increase the risk for CRC development. This talk will highlight three areas of your research, which focus on the detrimental effects of dietary heme from red meat in human cell culture models, intestinal organoids and mouse models. First, the effects of dietary heme on DNA damage, gut microbiota and intestinal inflammation in colorectal carcinogenesis will be presented. Second, the genotoxic and cytotoxic mode of action of heme iron will be dissected with a focus on heme oxygenase-I. Third, the relevance of oxidative stress for heme iron-triggered toxicity in intestinal epithelial cells and engaged cellular defense pathways will be discussed.

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S14-03
3D chromatin structure at the intersection of toxicology and disease

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Understanding the mechanism by which heavy metals drive diseases is imperative to human health. My lab explores the crossroads of chromatin biology, RNA biology, environmental toxicology, and cancer. Using an inorganic arsenic (iAs) model, we have uncovered foundational insights into epigenetic gene regulation, chromatin architecture, and oncogenesis. This approach goes beyond traditional perspectives, emphasizing the pivotal role of chromatin structure in health and disease. We discovered that iAs exposure at the the one-dimensional chromatin level drives the over-expression of histone H2B variants in iAs-mediated carcinogenesis. We futher show that these variants could act like 'oncohistones', although the mechanisms by which H2B variants dysregulate gene expression are still unknown. A second discovery shows that iAs could modeulate the 3-dimensional strcuture of chromatun by impacting the binding of CTCF, the master weaver of the genome, via targeting CTCF binding to target sites . This "loss of function" alters 3D genome organization and activates distal oncogenes. We suspect a similar mechanism operates in other iAs-induced disease (like neurodegeneration). Finally, we discovered that iAs exposure generates a circular form of SATB2 mRNA, and that this circSATB2 is translated into protein. SATB2 normally tethers chromatin to the nuclear matrix, where this higher-order structure is important for proper cell differentiation. Our data suggest that circSATB2 "protects" SATB2 mRNA from degradation, thus dysregulating SATB2 during cellular differentiation, leading to cancer. If true, it suggests a novel circRNA-microRNA-mRNA regulatory axis in health and disease. Thus, continuing these studies will not only provide new mechanistic insights, biomarkers, and therapeutic targets for iAs-induced disease, but will continue opening exciting new areas in chromatin biology and non-coding RNAs in gene regulation and toxicology.

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S14-04
Role of replication stress in ochratoxin A carcinogenicity

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Ochratoxin A (OTA) is a mycotoxin and widespread food contaminant that presents a health concern due to its nephrotoxicity and renal carcinogenicity. OTA is not DNA-reactive, but has been shown to cause weak genotoxic effects in mammalian cells. The molecular mechanism underlying OTA genotoxicity and the contribution of genetic toxicity to OTA carcinogenicity are still unclear and continue to be a cause of uncertainty in OTA risk assessment. In reviewing the available information on the mode of action of OTA genotoxicity and carcinogenicity, the European Food Safety Authority recently concluded that the specific spectrum of mutations and chromosomal damage induced by OTA, consisting of chromosome hypercondensation, abnormally separated chromatids, multipolar mitotic spindles, endoreduplication, polyploidy and aneuploidy, may arise from unresolved replication stress. Since replication stress is increasingly recognized as a major source of genomic instability and hallmark of cancer, the overall objective of our work was to understand the role of replication stress in OTA genotoxicity. Specifically, our aims were (1) to test if OTA interferes with DNA replication, (2) to establish a causal link between replication stress, mitotic aberrations and genomic damage induced by OTA and (3) to characterize the cellular response to OTA mediated replication stress.

Using the DNA fiber assay to analyze replication fork dynamics at single molecule resolution, we were able to show that OTA significantly delays replication fork progression in human kidney cells, providing direct experimental evidence for perturbation of DNA replication by OTA. Exposure of cells to OTA resulted in a significant concentration-related increase in γH2AX. Co-localization of γH2AX and 5-chloro-2´-deoxyuridine (CldU) incorporated into cells during S phase and visualization of γH2AX in the extended chromatin fiber assay revealed a significant increase in γH2AX along newly replicating DNA, indicating a replication-coupled mechanism of OTA induced DNA damage. Further evidence that OTA may act predominantly in S phase was provided by experiments in synchronized cells, demonstrating an increase in γH2AX in cells exposed to OTA during S phase, but not during mitosis. However, the ATR-Chk1 and ATM-Chk2 DNA damage response pathways did not appear to be efficiently activated in cells exposed to OTA. This may allow cells with under-replicated DNA or unresolved DNA damage to escape checkpoint control and continue into mitosis, leading to mitotic defects and chromosome segregation errors. Overall, our results highlight replication stress as an early key event in OTA genotoxicity and support a mechanistic link between replication stress, genetic damage and mitotic aberrations induced by OTA. Current work is aimed to confirm these effects at the target site of OTA carcinogenicity in rat kidney, with particular emphasis on dose-response characterization as a basis for risk assessment.

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S15 | Ontology-driven and artificial intelligence-based repeated dose toxicity testing of chemicals for Next Generation Risk Assessment

S15-01
Overview and hazard identification strategy

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The 3Rs concept, calling for replacement, reduction and refinement of animal experimentation, is receiving increasing attention around the world, and has found its way to legislation, in particular in the European Union. This is aligned by continuing high-level efforts of the European Commission to support development and implementation of 3Rs methods. In this respect, the European project called "ONTOX: ontology-driven and artificial intelligence-based repeated dose toxicity testing of chemicals for next generation risk assessment" was initiated in 2021 with the goal to provide a functional and sustainable solution for advancing human risk assessment of chemicals without the use of animals in line with the principles of 21st century toxicity testing and next generation risk assessment. ONTOX will deliver a generic strategy to create New Approach Methodologies (NAMs) in order to predict systemic repeated dose toxicity effects that, upon combination with tailored exposure assessment, will enable human risk assessment. For proof-of-concept purposes, focus is put on NAMs addressing adversities in the liver, kidneys and developing brain induced by a variety of chemicals. The NAMs each consist of a computational system based on artificial intelligence and are fed by biological, toxicological, chemical and kinetic data. Data are consecutively integrated in physiological maps, quantitative adverse outcome pathway networks and ontology frameworks. Supported by artificial intelligence, data gaps are identified and are filled by targeted *in vitro* and in silico testing. This presentation will specifically focus on the use of mode-of-action ontology frameworks for hazard identification as part of the ONTOX NAMs.

Among other applications, these ontologies can serve as the conceptual basis for setting up animal-free and human-relevant batteries for the toxicity testing of chemicals. This will be demonstrated in this presentation. Particular attention will be paid to the liver, which is a frequent target for systemic toxicity because of its unique location and function in the organism. A tiered ontology-driven approach for the prediction of steatotic and cholestatic liver toxicity induced by chemicals and relying on combined in silico/*in vitro* testing as well as on expression and functional analysis will be presented.

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S15-02
Biokinetic frameworks for hazard characterization

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Biokinetics constitutes a pivotal aspect of risk assessment, elucidating systemic availability, organ-specific accumulation, and variations across general and special populations, as well as differences in exposure routes. It becomes even more crucial in next generation risk assessment due to distinct bioavailable dose and exposure scenarios observed in *in vitro* assays and *in vivo* settings. Consequently, different models are used for modelling the biokinetics in *in vitro* and *in vivo*.

Physiologically-based kinetic (PBK) models, being mechanistic in nature, utilize physiological and chemical-specific parameters to predict temporal internal concentrations following external exposures. Similarly, *in vitro* models leverage mechanistic insights from assay compartments and chemical-specific attributes to predict concentrations in the medium or within cells. Together, these models enable quantitative *in vitro* to *in vivo* extrapolation and thus, hazard characterization data extrapolation.

Within the ONTOX project, we are refining in silico biokinetic models to align with various ontologies. The initial step involves characterizing these tools and identifying gaps relative to both biological and chemical domains within the ontologies. This gap analysis guides efforts to extend the tools appropriately.

In this presentation, I will outline the strategies employed in ONTOX for characterizing and analyzing gaps in the models, with a focus on PBK models. For PBK modeling, we focused on the open-source Open Systems Pharmacology software (PK-Sim® AND MoBi®), testing various strategies for inputting parameters based on Quantitative Structure-Activity Relationships (QSARs) and available databases. These high-throughout PBK (HT-PBK) simulations were compared with *in vivo* pharmacokinetic profiles.

Accurately reporting uncertainties within this HT-PBK framework is essential for its utilization in first-tier assessments of ab initio chemicals. The uncertainties can inform the need for refinement of certain parameters for more accurate predictions in a second tier. This will depend on the chemical and the tissue of interest. Hence, we are focused on characterizing the uncertainties related to tissues/compartments and chemical classes related to the ONTOX disease ontologies.

By incorporating uncertainty quantification and adversity-specific considerations, this approach enhances the accuracy and reliability of NAMs hazard characterization, ultimately contributing to more informed and efficient decision-making for chemical safety and regulatory purposes.

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S15-03
Probabilistic exposure assessment from food and cosmetics

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Human exposure to environmental chemicals may originate from multiple sources through different routes, such as oral, dermal and inhalation. The aggregated exposure from these routes can only be estimated as internal exposure since absorption, distribution, metabolism and excretion (ADME)of chemicals are route dependent. Probabilistic external exposure estimates include the variability in the exposure estimate and can be used as input to physiologically based pharmacokinetic (PBPK) models to predict internal aggregated exposure from all routes. Here we describe a probabilistic exposure assessment from food and personal care products (PCPs) with perfluorooctanoic acid (PFOA) as an example, and compare the aggregated internal exposure estimate with measured PFOA in serum. For this purpose, Norwegian data from the human biomonitoring study of the EuroMix project was used [1]. For two 24-hour study periods separated by 2–3 weeks, adult volunteers (44 males and 100 females) kept detailed diaries on their food consumption (i.e. type/brand, weight, time, and packaging material) and PCP use (i.e. type/brand of product, time and number of applications). At the end of each 24h recording period serum samples were collected and used for quantification of environmental chemicals. Probabilistic estimates of individual external exposure to PFOA from diet and PCPs were calculated using a custom-build model in R. A PBPK model on PFOA, first published by Loccisano in 2011 [2] and used by EFSA in 2020 [3], was implemented into R with further improvements in two compartments describing urinary and fecal excretion. The external exposure estimates were used for internal aggregated exposure via the PBPK model. The code for the probabilistic exposure estimations and PBPK model is available on GitHub [4]. Simulated blood concentrations of PFOA were compared with measured concentrations of PFOA in serum. The aggregated internal exposure estimates using the PBPK model shows that for both males and females the major contributor is diet. The estimated lower bound (LB) aggregated internal exposure to PFOA was in the same range but lower than the measured blood concentrations, indicating that the LB estimates are underestimations. Importantly, for seven females in the EuroMix biomonitoring study the internal exposure to PFOA was higher from PCPs than from diet. PCPs and diet contributed in the same range to the internal PFOA exposure for several women participating in EuroMix. Overall, the probabilistic exposure estimates combined with PBPK modelling represents valuable tools in understanding the sources of PFOA exposure, for predicting aggregated internal exposure, and thereby contributing to probabilistic risk assessments.

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S15-04
The use of artificial intelligence in risk characterization through probabilistic risk assessment

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The synergy of data generation and improvement of computers and algorithms has increased the power of AI more than billion-fold since AI was coined in 1956: Data in the world double every 18 months, i.e., 90% of all date were produced in last three years; computer double in capacity every 24 months (Moore's law) and AI algorithms double in capacity every 3 months since 2010. For most human skill tests, AI performs better than 90% of us. For toxicology and safety pharmacology, AI promises support for data retrieval, evidence integration (systematic reviews, risk assessments), predictive toxicology of untested compounds, digital pathology and support in reporting. The prospects of animal replacement with better accuracy in (human) prediction, ethical benefits and cost-effectiveness are enormous. Beyond this, accelerated assessments with automated data analyses, real-time monitoring and complex analyses come into reach with user-friendly prediction tools. These changes also promise to democratize knowledge, encourage open-access databases, algorithms and publications. As a copilot for toxicology, it empowers researchers, regulators, consumers and industry.

Uncertainty and probability are two sides of the same coin. Taking a probabilistic approach to risk assessment, however, was always hampered by the considerable effort associated and often a lack of mathematical literacy. The possible game changer is the advent of artificial intelligence (AI). In fact, probabilistic risk assessment only becomes beautiful by AI. It excels on data retrieval and integration based on pattern recognitions allowing the most probable evidence integration. Not pretending any certainty in suggested classification but explicitly expressing the probability of an accurate result, it can serve as a co-pilot to our risk assessments. The EU ONTOX project aims to design exactly this, a prototype of a predictive tool, which can be benchmarked for further optimization.

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S16 | PFAS and health: state-of-the-art in some areas of controversy

S16-01
Human exposure to PFAS: what did we learn? Does dose matter?

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Poly- and perfluoroalkyl substances (PFAS) are synthetic chemicals that are ubiquitous in food, water, air and soil. Human biomonitoring has demonstrated the presence of these synthetic chemicals in serum samples worldwide. The most abundant compounds in human serum are perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) with a half-life in humans of several years. PFOS and PFOA concentrations in serum samples of 1957 teenagers (12 to 18 years old) recruited from the general population in 9 different sampling sites spread over Europe between 2014 and 2021 varied for PFOS between 0.6–1.7 μg/L(P10) and 2.7–6.2 μg/L(P90) and for PFOA between 0.3–1.0 μg/L(P10) and 0.9–2.7 (P90) μg/L [1]. But in some hot spot sites in Europe, serum concentrations may be tenfold higher. Near the 3M site close to Antwerp, PFOS concentrations were measured in 301 teenagers. P90 went up to 18 μg/L and P95 to 30 μg/L. Next to living in a PFAS hot spot and consumption of locally grown eggs and food, other factors such as more frequent consumption of fish and seafood, consumption of offal, being male, having a lower body mass index, being from a household with a high educational level increase significantly the risk for having higher PFOS serum concentrations [2,3]. In 14% of the European teenagers, the serum concentrations of the sum of PFOS, PFOA, PFNA and PFHxS exceeded the human biomonitoring health-based guidance values of 6.9 μg/L. In the Flemish 3M hot spot area, 71% exceeded the guidance value. This value is the internal dose that corresponds to the external Tolerable Weekly intake established by EFSA (2020) and it is considered as a safe level below which no adverse health effects are to be expected [4]. The European studies carried out in HBM4EU and the Flemish hot spot study had the opportunity to include molecular effect markers measured in the same individual samples as the exposure biomarkers. Changes in serum levels of reproductive hormones were associated with serum PFAS levels, in the hot spot study but also in the studies of the general population which span a much lower PFOS gradient [5]. This shows the complexity of exposure response analysis and emphasizes the need for further reducing exposure even at what we call "background" exposure levels.

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S16-02
PFAS and lipids: Epidemiological evidence of causal associations

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This talk responds in part to the apparent paradox: if the evidence of PFAS increasing cholesterol is so strong, why is there not a clear impact on cardiovascular disease (CVD)? While there are numerous papers linking one or more PFAS to increased cholesterol, principally PFOA or PFOS, measured in human serum [1]. However, there is some concern as to whether to consider this a causal relationship, and it has generally not been used as a basis for setting health-based guidance values for PFOA [2,3]. There are plausible mechanisms whereby these associations could be explained via correlated routes of metabolism and excretion [4,5], and there is little support from toxicological data. Furthermore, while total cholesterol appears to rise with increasing PFOA or PFOS, cardiovascular disease does not [6]. Conversely there is persuasive evidence from triangulation of epidemiological evidence [7] that there are causal associations between PFOA or PFOS and lipids. The evidence includes a range of study designs each potentially vulnerable to different types of bias, and the various designs generally indicating a positive exposure-response: these are the cross-sectional studies already mentioned, ecological studies where clear contrasts of exposure are evident [8], the use of measured or modelled serum PFAS, and longitudinal studies with changing PFAS exposure profiles [9].Analyses of the large database of biomonitoring data in over 40k adults in the C8 study of a PFOA exposed population, show a significant association with total cholesterol and LDL in cross-sectional analyses, but not cardiovascular disease in a cohort follow up study [6]. This may be explained due to PFOA affecting multiple clinical markers: It is associated with increases in both LDL and HDL, and a decrease in CRP. Thus, the increased CVD risk to be expected from increased LDL may be compensated by increases in other markers indicating reduced CVD. Comparison with ecological analyses in the same, suggest that about half of the apparent cross-sectional association may be considered causal and half due to uncontrolled confounding. This population had large contrasts in daily intake between different subgroups, in other places the causal component would be expected to differ depending on the amount of contrast in intake across the population.

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S16-03
Recent advances in the risk assessment methodology for PFAS mixtures: Derivation of relative potency factors (RPFs) for PFAS

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Per- and polyfluoroalkyl substances (PFAS) often occur together as contamination in exposure media such as drinking water or food, and are found to co-occur in human blood. Over the past years, several risk assessment approaches have been developed to assess the health risk from exposure to PFAS mixtures [1,2]. These approaches share that they assume dose-addition of PFAS with regard to their cumulative toxic response [3,4].

One of the approaches for risk assessment of PFAS is the 'Relative Potency Factor' (RPF) approach [5,6,7]. In this approach, the mixture components' toxicity is expressed as a potency factor relative to an index compound [5]. Although there is convincing evidence that PFAS affect the same toxicological endpoints with different potency, there is controversy what underlying toxicological data are suitable to derive RPFs and subsequently, which RPFs are to be applied in risk assessment.

The combination of the intrinsic toxicity and the bioaccumulation potential together determine the potency of a PFAS. Consequently, RPFs based on external intake doses are not the same as internal (systemic) RPFs based on blood concentrations, or *in vitro* RPFs [6]. It is therefore important to consider the dose-metric corresponding to the RPFs, and to apply the correct set of RPFs when assessing for instance intake of PFAS via drinking water (external RPFs) or PFAS blood concentrations (internal RPFs).

Cumulative exposure to PFAS mixtures cannot be dismissed in European populations [8], and therefore is important to take into account in risk assessment. Further research and scientific debate to work toward agreement for RPFs for PFAS is however needed. It is therefore important to harmonize RPFs for PFAS in an international context such as at the WHO level or within another established regulatory structure. This would channel discussions on establishing criteria and setting values for RPFs.

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S17 | Toxicology – a quest for safer firefighting

S17-01
Use of biomarkers of effect in evaluating toxicity in firefighters: Results from the US Fire Fighter Cancer Cohort Study

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Purpose: Firefighters are occupationally exposed to carcinogens in products of combustion and per-and polyfluoroalkyl substances (PFAS) and are at increased risk of cancer and adverse reproductive outcomes. [1–4] Firefighters are also at increased risk for post-traumatic stress disorder (PTSD). [5] This presentation will include data on biomarkers of effect evaluated within the Fire Fighter Cancer Cohort Study (FFCCS).

Methods: The FFCCS is a community-engaged prospective multicenter cohort study evaluating firefighter exposures, effects, and preventive interventions in the United States (US). Firefighters are involved in all stages of the research. Blood and urine samples are collected at study enrollment, after specified exposures, and approximately every two years for the duration of the study. Samples are analyzed for exposures and/or effects of exposures or stored for future analyses. Biomarkers of effect commonly evaluated include epigenetics (peripheral blood DNA methylation and microRNA expression), serum anti-müllerian hormone (AMH) which is a measure of reproductive reserve in women, and urine metabolites.

Results: As of February 2024, >4,800 structural, wildland, volunteer and airport firefighters have enrolled in the FFCCS, including >900 women and >1,300 minority firefighters. 1,200 firefighters have been evaluated for serum PFAS, 1,242 for DNA methylation analysis, 776 for microRNA analysis, 678 for AMH analyses, and 100 for urinary metabolome analyses. Differential DNA methylation including in genes associated with cancer has been found comparing incumbent and recruit firefighters adjusting for age, within individual firefighters followed over a two-year period both associated with and not associated with cumulative fireground exposure, with serum levels of several PFAS chemicals, and with Hispanic ethnicity. [6–9] Differential microRNA expression including markers associated with cancer has also been found comparing incumbent and recruit firefighters adjusting for age, within individual firefighters followed over nine-month and two-year periods [10] and associated with wildland-urban interface (WUI) exposures and serum levels of PFAS chemicals. Serum AMH is reduced in women firefighters with a self-reported clinical diagnosis of PTSD. Urine metabolomic differences have been found comparing pre-and post-exposure and associated with Hispanic ethnicity.Discussion: Epigenetic biomarkers that may be related to increased cancer risks have been associated with PFAS, other exposures and firefighter ethnicity. Serum AMH analyses have identified PTSD as a potentially impactful exposure that may reduce reproductive reserve. Urine metabolomic markers change with exposure and firefighter ethnicity. Future work will continue to evaluate these changes over time across subgroups (women, wildland, minority and volunteer firefighters) and different regions of the US and identify and test strategies to prevent or mitigate these biological changes.

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S17-02
Exposure and effect markers during firefighting activities – Danish intervention studies

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Epidemiological studies based on Danish registries have shown that in addition to a slight increase in risk of cancer, Danish male firefighters have increased risk of cardiovascular disease and infertility compared to Danish employed males [1–3]. Firefighting training is a requisite for being a firefighter, initially and continuously throughout their career and constitutes a semi-controlled exposure situation, allowing the investigation of exposures and associated biological mechanisms-of-action. Firefighting activities include a combination of stressors such as strenuous work, heat, smoke and soot known to be able to affect cardiovascular and reproductive health, while smoke and soot also being risk factors for cancer.

Since 2015, we have studied young subjects enrolled in a Danish educational programme aimed at training firefighters. Our research has involved the characterization of exposure and assessment of effect biomarkers before and after extinguishing fire exercises, using the subjects as their own controls. We have previously reported that inhalation exposure primarily occurs when respiratory protective equipment is removed and that subjects are exposed to polycyclic aromatic hydrocarbons (PAH) during exercises [4]. Furthermore, we observed adverse changes in cardiovascular markers [4], and increased levels of DNA damage associated with PAH on skin and urinary hydroxy-pyrene levels after firefighting [5].

After the dissemination of study results reported in 2017–2018, changes were made to the spatial organization layout and procedures of the firefighting exercises, which could potentially affect exposure and effect levels. In our subsequent repeated measurement studies, we enrolled 83 firefighter trainees, in addition to the initial 53 subjects. We have observed and monitored the changes and furthermore, designed the following studies to assess interventions related to hygiene procedures and different training conditions. These interventions aim to reduce skin exposure and to create gradients of the heat, workload, smoke, and soot.

We assess smoke exposure with stationary and person-borne equipment for particle number concentration, and the content of selected PAH in skin wipes and of hydroxy-PAHs in urine. As biomarkers of effect, we measure functional cardiovascular markers, lung function and DNA damage in peripheral blood mononuclear cells. In our newest study, in addition to assessing the previously mentioned markers, we are also investigating effects on core temperature and testicular thermoregulation, urinary markers of oxidative stress, and serum analyses of troponin, selected microRNA, and specific hormones.

We will discuss the impact of the observed interventions in exposure levels and effect changes in preliminary results on markers of health effects.

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S17-03
Fire toxicity – elephant in the room?

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Firefighters are at an increased risk of exposure to toxic fire effluents and subsequently at an increased risk of suffering adverse health outcomes [1,2]. Chemical and building regulations are designed to ensure that exposure to materials within residential, commercial and industrial buildings are safe. However, there are currently no requirements to consider how the safety of those materials might change in the event of a fire – i.e. there are no requirements to measure and quantify the toxic fire effluents produced by burning materials. Compared with natural materials (wood, wool, cotton, leather, etc.), widely used synthetic polymers burn more quickly, have faster flame spread, generate more heat and produce not only higher numbers of hazardous gases and particulates, but also much higher concentrations of toxic chemicals [3].

Fire effluents can be released in the form of particulates which will include aerosols, dusts, fibres, smoke and fumes or gases and vapours. Some of these fire effluents such as asphyxiants and irritant fire gases have immediate adverse effects on health after only a single or short exposure [3]. Chronic toxicants are predominantly associated with delayed or chronic effects and disorders such as genotoxicity, mutagenicity, carcinogenicity, neurological disorders etc., which generally occur due to repeated exposure to toxins. Regardless of the fuel, carcinogens such as benzene, polycyclic aromatic hydrocarbons and other volatile organic compounds are released in almost all fires. Polychloro- and polybromo dibenzo-p-dioxins and dibenzofurans, polychlorinated biphenyls and polybrominated diphenyl ethers, bio-accumulative and persistent organic pollutants associated with adverse health conditions, have been also identified in fire smoke from burning halogen containing fuel. Concerns over adverse health effects and bioaccumulation have led to the replacement of some of polybrominated diphenyl ethers, gas phase halogenated flame retardants commonly used in consumer products and building materials, with the other group of chemicals such as organophosphorus flame retardants. Similarly, per- and polyfluoroalkyl substances, commonly found in firefighters' gear and firefighting foams, have been identified as persistent organic pollutants with bio-accumulative properties [4].

Firefighters' exposures to various chemicals can occur throughout different phases of fire intervention, including attack, knockdown, and overhaul, as well as within their work environment, such as the fire station. They can inhale, ingest, or absorb a variety of chemicals dermally during or after attending a fire. This presentation will discuss firefighters' occupational exposure to fire toxins and their associated health risks.

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S18 | Advances and applications in quantitative systems toxicology to support chemical safety assessment

S18-01
From qualitative adverse outcome pathways (AOP) to quantitative systems toxicology (QST) models: Modeling approaches – challenges & opportunities

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Various efforts are being made to bridge the gap between qualitative adverse outcome pathways (AOP) and broader quantitative systems toxicology (QST) models. While modelling early key events (KE) in an acute toxicity setting may often be straightforward, the extrapolation to more complex toxicological situations (prolonged exposure, oral dosing, etc.) can rapidly become very tricky. Several time dimensions need to be considered in QST modelling: the time a toxicant is present at the target site at sufficient concentrations to trigger the MIE; the time an upstream KE needs to be activated in order to trigger a downstream key event; the time to accumulate sufficient cellular damage to lead to an adverse outcome on the level of the organism. In this talk, some of the challenges and opportunities of QST modelling will be presented and discussed based on the example of a neurodegeneration pathway via mitochondrial toxicity. Concretely, this presentation will include an introduction to the main tools that we are using in safety assessment modelling and a more in-depth demonstration of a selection of them. Here, we will try to cover different aspects of the hybrid modelling of QST, from point-of-departure (PoD) predictions (*e.g.*, *in vitro* biokinetics, physiologically based toxico-kinetics (PBTK) using the Simcyp® simulator), to dose-response and response-response profiling using a Bayesian approach (*e.g.*, Markov-chain Monte Carlo algorithm, MCMC), and uncertainty characterization and propagation. This project received funding from the European Union's Horizon 2020 Research and Innovation program under Grant Agreement No. 964537 (RISK-HUNT3R), which is part of the ASPIS cluster. The European Commission is not responsible for any use that may be made of the information it contains.

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S18-02
Application of quantitative systems toxicology to support chemical safety assessment in the cosmetics industry

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Ensuring consumer safety for cosmetic products is a key industry priority that has driven the continued development and implementation of new assessment methods over the past 20 years. Driven by consumer ethical concerns, scientific advancements, and legislative changes the safety evaluation of new cosmetic ingredients for consumer requires the use of non-animal approaches. A key approach currently being assessed is called Next Generation Risk Assessment (NGRA), which provides a human-relevant, hypothesis-driven and exposure-led tiered framework. This allows flexibility to combine exposure information, in silico alerts, computational models and points of departure from non-animal *in vitro* assays to generate a transparent WoE. At lower tiers within the framework, where no bioactivity is detected at human-relevant concentrations, a low risk safety decision can be made on the assumption that no bioactivity implies no adversity. This type of safety assessment protects human health without predicting hazards that may happen at higher exposures. However, when bioactivity cannot be excluded at human-relevant concentrations, higher-tier tools are needed to refine the risk assessment and address the remaining uncertainties. This talk will highlight how quantitative AOPs (qAOPs)can be used as a higher tier within the NGRA framework, when specific mechanisms that drive concern have been identified. This is especially important for hazard characterisation when differentiating between adaptive and adverse effects associated with key events in an AOP. In the talk, the benefits, and limitations of qAOPs, and how they could be used within the context of NGRA will be shown by drawing on several examples, spanning inhalation (inflammation and fibrosis) and systemic toxicity (Nrf2 activation and oxidative stress). The qAOP modelling approaches used in these examples will range from systems biology (i.e., bottom up, mechanistic) to Bayesian statistical models. An industry perspective on both short- and long-term future challenges of how these different approaches could be selectively applied within a tiered risk assessment framework will also be discussed.

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S18-03
Regulatory perspective for the opportunities afforded by quantitative systems toxicology in chemical safety assessment

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The European Food Safety Authority (EFSA) carries out risk assessments in relation to food and feed safety, and in the case of chemicals, provides health-based guidance values. The data provided to EFSA are mostly derived from *in vivo* animal toxicity studies, using traditional endpoint measurements and conventional extrapolation approaches to derive health-based guidance values from critical points of departures such as NOAELs or BMDLs. However, EFSA is following closely and investing in the development and regulatory application of new methodologies and tools, the so-called New Approach Methodologies or NAM as part of its scientific strategy. The use of NAMs in regulatory chemical risk assessment has so far been quite limited in EFSA. To overcome these challenges and generate opportunities for NAMs in regulatory chemical risk assessment, EFSA launched a contract to develop a roadmap for action on NAMs to reduce animal testing. The roadmap aims to define EFSA's priorities for the incorporation of NAMs

as well as to inform a multiannual strategy for increasing the use of NAMs in human health risk assessment to minimise the need for animal-based verification studies. As part of its work in the area of NAMs, EFSA has developed a number of online tools to model and predict the ADME properties of chemicals within and across species in order to quantitatively integrate the different NAM data streams together using a weight of evidence approach. Challenges and opportunities from a regulatory perspective will be discussed.

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S19 | Getting scientific confidence for NAM-based regulatory assessments

S19-01
PARC and other current EU scientific initiatives

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Several EU projects have been working on the development of New Approach Methodologies (NAM) resulting in an increasing number of *in vitro* and *in silico* for the testing of potential toxic effects of chemical substances. Despite the variety of methods available regulatory uptake remains a challenge.

This is partially due to limited readiness of methods not sufficiently safeguarding the level of robustness or validity needed for regulatory implementation. On the other hand, at the regulatory level a broad understanding of the chances of NAM with respect to address human relevance, analyse effects of multiple compounds and mixtures by screening approaches or contribute to a better understanding of mechanisms of toxicity needs to be created.

Projects like PARC or the ASPIS cluster projects involving method developers as well as regulators and ensuring a high level of exchange and creation of a common understanding of needs and challenges will help to overcome these difficulties.

This presentation describes PARC and other current European research and regulatory initiatives ensuring the implementation of 21st century toxicology methods in the 21st century.

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S19-02
Global initiatives to gain scientific confidence in New Approach Methodologies (NAMs)

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The International Collaboration on Cosmetics Safety (ICCS) is a global multi-stakeholder not-for-profit organization focused on advancing the adoption of animal-free assessments of cosmetics, and their ingredients, for human health and environmental safety. ICCS has a diverse cross-sectorial membership, including cosmetic product and ingredient manufacturers, cosmetic and chemical trade and research associations and non-governmental organizations (NGOs). ICCS has a three pillar framework of Science, Education & Training, and Regulatory Acceptance, and this presentation will delve into the Regulatory Acceptance pillar providing an overview of strategies and initiatives used by governments and legislators worldwide to increase the implementation and uptake of New Approach Methodologies (NAMs).

Global initiatives to increase scientific confidence in NAMs will be described in countries and regions such as the European Union, the United States of America, Canada, and China. This will include the status of cosmetic testing bans, efforts to promote the uptake of NAMs, and collaborations nationally and internationally. In addition, ICCS work groups created with the aim of fostering further collaboration will be presented, including NAMs 101, Best Practice Guidance, and Progressing Integrated Approaches for Testing and Assessemnt (IATA) for Systemic Toxicity.

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S19-03
Working effectively together to build confidence in Non-Animal based regulatory assessments

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Humane Society International is a leading animal protection NGO that aims to protect all animals around the world by tackling the cruelest practices, including testing on animals for chemical regulatory compliance. HSI is the founder of the Animal Free Safety Assessment (AFSA) Collaboration, which brings together diverse stakeholders including industry, academia, contract research organisations, consultants, and method developers to advance the use of non-animal methods (NAMs) in global regulatory frameworks, and replace redundant animal test with effective NAMs.

Confidence in NAMs is critical to their use in regulatory safety assessments, yet there is a disparity in confidence amongst key stakeholder groups. NAMs have been used for many years in ingredient defence, but less so in regulatory decision-making. Collaborations and educational programmes such as the AFSA Collaboration and AFSA Master Class are efforts designed to address this disparity.

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S20 | Opportunities and challenges for PB(P)K models to uncover the role of maternal transfer in developmental toxicity

S20-01
Principles of quantitative *In Vitro* to in vivo extrapolation (QIVIVE) applying physiologically based kinetic (PBK) modelling for DNT IVB

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The process of quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) involves converting an *in vitro* concentration associated with bioactivity to an external exposure level. This conversion is achieved by utilizing a physiologically based kinetic (PBK) model to determine a plausible exposure level that results in a tissue or plasma concentration equivalent to the *in vitro* concentration. The predicted exposure level can then be compared with actual or estimated human exposures to assess potential health risks. A document on "quantitative *in vitro* to *in vivo* extrapolation" (QIVIVE) within the framework of the OECD Developmental Neurotoxicity *in vitro* battery (DNT IVB) Initial Recommendations, published by OECD in 2023, has been prepared. This document serves as a collection of recommendations and considerations regarding the principles

and steps involved in utilizing QIVIVE to interpret DNT IVB data for downstream applications in hazard characterization and risk assessment. It is not intended to be an exhaustive technical guide for regulatory applications. The QIVIVE conversion process includes selecting an *in vitro* point of departure (POD) and determining its corresponding *in vivo* tissue or plasma/blood concentration. This step also involves linking the tissue or plasma/blood concentration to an external dose using a physiologically based kinetic (PBK) model. Similar to the Tiered testing concept, the selection of a PBK model structure follows a Tiered approach, with the choice of a specific tier being influenced by the available data for model calibration and evaluation. Furthermore, the assumptions and limitations of a model are dependent on the purpose of the analysis and the accepted level of uncertainty. The document will be presented alongside examples to illustrate the potential application of the QIVIVE approach within the context of DNT IVB.

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S20-02
Maternal and maternal-fetal PBPK models for estimating therapeutic efficacy and safety in pregnancy

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Physiologically Based Pharmacokinetic (PBPK) Modeling has evolved as a tool to make predictions of drug and chemical pharmacokinetics. The models can be adjusted for the physiology of a single subject, a certain life-stage, or a population. An additional advantage of PBPK models is, depending on the data available for a defined compartment, they can estimate the concentration of the drug, chemical, or toxicant in the specific compartment, such as the fetal compartment. This ability is particularly important in life-stages, like pregnancy, where effects or concentrations in human fetuses are largely unknown and under-explored for ethical and liability reasons. PBPK models can mimic maternal physiology and the changes that occur. This allows a more accurate estimation of maternal PK and thus a better environment for predicting fetal PK. Although details in fetal and maternal physiology may differ from model to model, PBPK modeling can help establish a starting point for safety and efficacy. Regulatory agencies are moving toward less reliance on animal data, especially when there is not a validated *in vivo* model for a specific disease state or life-stage. Therefore, more regulatory agencies are accepting *in vitro* and in silico methods. This presentation will review maternal and maternal-fetal PBPK models of drugs and discuss the considerations for safety and efficacy for this population as it relates to PBPK modeling.

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S20-03
Maternal-fetal physiologically-based (pharmaco)kinetic (PBK) modeling in the field of clinical pharmacology: insights and future directions

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Over the past decades, physiologically-based (pharmaco)kinetic (PBK) modeling for the maternal-fetal dyad has advanced from a niche technique to a powerful tool established in environmental toxicology and pharmaceutical research [1]. Being mechanistic in nature, PBK models can provide a causal and fundamental understanding the absorption, distribution, metabolism, and excretion (ADME) of drugs and xenobiotics in scenarios that are untested or untestable during (human) pregnancy.

In the field of clinical pharmacology, maternal-fetal PBK models are used for optimizing drug dosing regimens in pregnant patients while minimizing fetal risk and ensuring therapeutic efficacy for the mother and/or fetus. Importantly, predicting the transfer of drugs across the placenta has been a focal point of research [2]. Such predictions are facilitated by integrating experimental data obtained from different *in vitro* and/or ex vivo assays [3]. For example, data obtained from the ex vivo cotyledon perfusion experiment have been successfully leveraged in PBK models to predict fetal exposure to various drugs [4,5]. These examples can provide important insights and learnings on the generalizability and translatability of this approach to compounds with a similar physicochemical and pharmacokinetic profile.

Still, there is a considerable need for maternal-fetal PBK models that can reproduce in utero exposure during embryonic development – usually the most sensitive period to teratogenesis. This challenge is compounded by the lack of clinical data available for model building and validation. Hence, a systematic collection of (pharmaco)kinetic data is essential to improve the utility and confidence in these models [6]. Additionally, a consistent and structured assessment of placental transfer data from various sources, spanning *in vitro* systems, 3D bioengineered placental tissue, animal, and human studies, could help to advance the understanding of underlying processes driving transfer kinetics.

The fields of clinical pharmacology and (environmental) toxicology often overlap in their use of maternal-fetal PBK modeling to understand fetal exposure to various substances and chemicals. However, despite this overlap, synergies are not fully leveraged. Interdisciplinary communication and knowledge transfer could be improved by the use of open source modeling tools allowing researchers from both fields to share models, workflows, and modeling frameworks. Such a cross-disciplinary collaboration could lead to continuous model improvement, increased resource efficiency, and enhanced consistency by integrating diverse data sets and standardizing model applications across different disciplines.

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S20-04
Generic pharmacokinetic models for mother-to-offspring transfer of environmental chemicals

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In developing human health risk assessments, it is important to consider pregnant women, developing fetuses, and nursing children because chemical exposures experienced by these groups can lead to special types of adverse health outcomes (e.g., developmental effects) that do not typically arise in non-pregnant adults that experience similar exposures. Pharmacokinetic (PK) models, which are frequently used in chemical risk assessments, provide a means for estimating internal dose metrics from applied doses or environmental exposures based on mathematical descriptions of absorption, distribution, metabolism, and excretion (ADME). In addition to the ADME mechanisms commonly represented in adult PK models, PK models for pregnancy (and gestation) and the early postnatal period may include mathematical descriptions of transplacental and lactational transfer of chemicals from mothers to their offspring. We have developed two distinct PK models for human perinatal exposure scenarios that include mother-to-offspring transfer. The first model allows for simulations that quantify bioaccumulation of lipophilic persistent environmental chemicals (LPECs) in rats, mice, and humans, as well as transplacental and lactational mother-to-offspring transfer. The second is a physiologically based pharmacokinetic (PBPK) model that describes ADME in a pregnant human mother and her developing fetus during gestation. Both models are generic in the sense that they can be parameterized for many chemicals. We will provide details of the two generic PK models for mother-to-offspring transfer of chemicals, including a description of steps we took to develop and evaluate these models and a summary of their limitations. Then, we will provide examples of risk assessment applications of the models.

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S21 | Microphysiological systems as emerging tools in predictive toxicology

S21-01
Brain MPS for developmental neurotoxicity testing

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Recent advances in stem cell technology have piloted a new era of human brain modeling through the development of three-dimensional (3D) organoids derived from induced pluripotent stem cells (iPSCs) – brain Microphysiological Systems (bMPS). These models hold the promise of revolutionizing our understanding of neurodevelopmental disorders and rare diseases, such as autism, SYNGAP-ID, and leukodystrophies, by enabling detailed study of the mechanisms underlying these conditions in human-relevant and yet complex environments. We also address gene-environment interactions (GxE) using bMPS – a key hypothesis in understanding the recent increased prevalence of neurodevelopmental disorders and heterogeneity of the phenotypical presence of the disease in individuals with the mutation in the same gene. Our work leverages iPSCs to generate complex 3D brain models, which allow for the exploration of neural development, synaptogenesis, myelination, and neuroinflammation in a human-relevant context. This approach was enhanced in our laboratory by the integration of iPSC-derived microglia and the application of CRISPR/Cas9 technology for precise genetic editing, facilitating the study of specific disease signatures and the effects of environmental exposures on neural development. We generated CRISPR/Cas9- modified multi-fluorescent brain organoids to follow neural development (specifically oligodendrogenesis and synaptogenesis) and automate drug and chemical screening. Using patient-derived iPSC with mutation in autism high risk genes (CHD8, SYNGAP1 and 16.p.11.2 deletion) we study interplay between the genetic background and environment in context of Autism. We showed, that mutation in CHD8 synergize with exposure to organophosphate pesticide, chlorpyrifos. We currently expand the findng to further genetic backgrounds and exposures.

Recently, we developed 3D shell electrodes (brain organoid EEG) to record and characterized electrophysiological activity and neuronal plasticity in the bMPS. We also introduced the concept of Organoid Intelligence (OI) as a pioneering frontier in biocomputing, defining it as the intersection of advanced microphysiological systems, multi-electrode arrays as sensor systems, and artificial intelligence (AI). OI aims to harness biological learning mechanisms through *in vitro* brain-computer interfaces, offering a novel paradigm for studying biological and hybrid computing, as well as modeling complex cognitive functions. Our multidisciplinary research and development trajectory focuses on scaling up organoid production, integrating them with novel electrode arrays, and developing assays for learning and memory, addressing the functional endpoints in disease modeling.

This abstract encapsulates our vision for a collaborative effort across multiple disciplines, aiming to redefine the possibilities for disease modeling and neurotoxicity testing through stem cell-based models.

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S21-02
Heart-on-chip systems: next-generation microphysiological *in vitro* models for pharmacology and toxicology

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No abstract has been submitted.

S21-03
In vitro models for the detection of nephrotoxicity: implementing appropriate biomarkers in microphysiological systems

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Chemicals can lead to nephrotoxicity and drug-induced kidney injury (DIKI) can be a dose-limiting factor for medicines. Detection of kidney toxicity is difficult in the clinical setting and often relies on animal studies. Human kidney cell culture systems can complement or replace animal models for toxicity screening. Primary renal tubule epithelial cells (RPTEC) or cell lines (RPTEC/hTERT, ciPTEC, HK-2) are common-

ly used for drug toxicity screening [1]. However, renal tubule cell lines cultured under static conditions may not recapitulate tubular epithelial cell properties. Hence, several cell culture systems, from transwells to microfluidic devices are used to achieve better results, some of which are already considered robust [2]. Recently, kidney organoids from human pluripotent stem cells, containing a variety of cell lineages have been implemented and could be used for screening purposes [3,4].

The successful use of adequate cell sources and cell culture systems depends on the availability of suitable markers to interrogate the physiological and pathological effects of drug treatments. Many biomarkers have been proposed for the detection of nephrotoxicity, mainly *in vivo* and the first formal qualification of rodent-based safety biomarkers was a major milestone [5]. Cell culture systems also rely on biomarkers of cytotoxicity, molecular (mRNA and miRNA) and protein biomarkers (e.g. cytokines), as well as on functional tests (barrier function, protein uptake) and cellular morphological features (cilia, brush border).

The talk will focus on the detection of nephrotoxicity evaluated using two microfluidic devices and static culture conditions. The results obtained with drugs that induce DIKI such as colistin and cisplatin underline the need for fit for purpose models. Microscopy is a good indicator of toxicity and impairment of cellular function and imaging can be implemented manually or using automated, AI-supported image analysis systems. As an example, colistin-induced toxicity can be detected by imaging primary cilia (acetylated tubulin staining). Orientation and length of the cilia are also indicative of the profound morphological changes induced by medium flow. Release of cytokines (IL-6 and GM-CSF) can be also significantly modulated by flow and respond to drug treatment. Other biomarkers such as increased gene expression of HMOX-1 or release of miRNAs (e.g. miR34a, miR29a, and miR192) are indicators of toxicity, whereas KIM-1, an established biomarker in pre-clinical and clinical studies [6], does not seem an appropriate indicator of RPTEC damage *in vitro*, as also observed by others [2].

Finally, I will discuss the possibility of co-culturing RPTECs with liver cells to allow in-chip metabolism and hence a more systemic approach than with monocultures [7]. Co-culture of RPTECs and liver cells (HepaRGs or HepG2) is feasible and influences the toxicity of compounds undergoing hepatic metabolism such as Ifosfamide and Verapamil.

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S21-04
A microphysiological system of the liver that recapitulates the cellular composition and organization of the hepatic lobule

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Mimicking the complex biology and physiology of the liver *in vitro* is imperative to decipher the underlying mechanisms of liver toxicity and correctly predict the toxicological responses found *in vivo*. These models should not only represent the native tissue in its cellular composition and interactions, but also be suitable for the drug development industry by providing sensitive and predictive assays to study liver toxicity. Persistent efforts have been dedicated to developing various microphysiological systems that model the human liver. However, such systems are often incompatible with automated procedures and do not scale well for the drug development industry. Alternatively, scalable and automated models often compromise biological complexity and lack, therefore, the necessary cellular interactions to predict drug toxicity.

We developed a liver microphysiological system which shows unprecedented fidelity with the spatial organization and cellular interactions. The platform itself comprises 64 individual units, patterned underneath a microtiter plate and is therefore compatible with standard laboratory equipment. Despite its biological complexity, which includes polarized hepatocytes, functional vasculature with associated stellate cells and functional immune cells, we were able to fully automate all the necessary procedures to execute automated screenings. We developed steatosis and fibrosis assays, which we validated using sets of tool compounds in a fully automated workflow.

This system holds the potential for a groundbreaking progress in liver modelling which, besides including functional hepatic cells, also reflects the cellular organization and interactions found within the liver lobule. In conjunction with its high throughput capability, this has the potential to revolutionize pre-clinical drug development.

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S22 | What's new for addressing safety: a multi stakeholders' perspective

S22-01
Update on the science program of the international collaboration on cosmetics safety

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The International Collaboration for Cosmetic Safety (ICCS) was established in 2022 to accelerate the global acceptance of animal-free science for human and environmental safety assessment of cosmetics and their ingredients through Science, Education & Training, and Regulatory Engagement. This global organization brings together cosmetics manufacturers and suppliers, industry and research associations, and

animal protection organizations working under three pillars (science, education & training, and regulatory engagement), in order to build confidence in animal-free approaches.

This presentation will describe ICCS activities under the human health portion of the science pillar. ICCS maintains a balanced portfolio of 1) late-stage delivery projects to close research gaps and facilitate future validation and regulatory acceptance; and 2) method development projects that are closely linked to key regulatory challenges. Several ongoing projects are focused on toxicity endpoints nearing completion (e.g., eye irritation, skin sensitization, threshold of toxicological concern) and moving them towards education & training, and regulatory acceptance activities. Others are directed towards identifying NAMs and NGRA frameworks for systemic toxicity.

Accurate cosmetics safety assessments rely on exposure-led approaches, and both external and internal exposure are being addressed at ICCS Current projects are directed towards quantifying both toxicodynamic and toxicokinetic properties more effectively inform safety decisions. ICCS is also working to establish best practices guidance in read-across enhanced by NAMs. To ensure efficiency, avoid redundancy, and leverage expertise outside of the organization, ICCS frequently collaborates with external stakeholders and in fact many of our current projects involve partners from other organizations, service providers and/or academic research teams.

Collectively, ICCS seeks to develop universal confidence in robust animal-free NAMs and NGRA frameworks such that animal testing is no longer considered necessary for human and environmental safety assessments or registration of cosmetics and their ingredients. This presentation will provide an overview of the challenges in human health effects' assessment without animal testing and ICCS' ambition to address them.

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S22-02
Perspectives from regulators: progress from the EU PARC initiative

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Under the Partnership for the Assessment of Risks from Chemicals (PARC, https://eu-parc.eu), the overarching work package (WP) 2, "A common science-policy agenda" strives to ascertain that the innovation created by PARC meets the demands of the regulatory community and that it is taken up into regulatory practice, transitioning towards "Next-Generation Risk Assessment" (NGRA).

The presentation will start with a short overview from the regulatory perspective of the main challenges to chemical risk assesment addressed by PARC as well as the Partnership's progress in this regard two and a half years after its kickoff in May 2022. Subsequently, two activities under Task 2.2 ("Knowledge management and uptake into policy") will be presented and discussed in more detail:

- NGRAroute, a roadmap activity to establish NGRA as the default approach to chemical risk assessment in EU chemicals legislation,
- PARCopedia (https://parcopedia.eu), the knowlege management and social media platform for the chemical risk assessment community in and beyond PARC, which was launched in November 2023.

At the example of these two activities ideas will be presented and discussed for how to bring together the diverse compartments of the chemical risk assessment community in Europe and worldwide to create optimum synergies as well as to establish broad, multilateral stakeholder consensus to support the transition towards NGRA.

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S22-03
Where science meets technology: probabilistic risk assessment

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No abstract has been submitted.

S22-04
Building Confidence in 21st first-century approaches: perspectives from the OECD

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Many research initiatives aim to provide novel assessment frameworks and data generation tools for assessing chemical safety. In parallel, regulatory authorities are striving to integrate new methods to improve risk assessments, reduce reliance on animal testing, and address regulatory challenges. Similarly, the chemical industry invests in new chemistries and new testing strategies to evaluate chemical safety at the earliest stages and, for the successful new chemicals, address regulatory requirements. The Organisation for Economic Cooperation and Development (OECD) provides a forum for stakeholders to share experiences and develop common approaches for developing confidence in 21st-century approaches and their implementation in risk assessment.

OECD engages various stakeholders, including regulators, industry, animal welfare, and environmental organisations to exchange knowledge on existing and New Approach Methodologies (NAMs) for assessing chemical safety and, where possible, harmonise such approaches. For NAMs that may be used in combination to provide information on endpoints used in risk assessment, a way to establish confidence and robustness of such methods is needed. The multi-step process begins with sharing examples and experience on how such approaches are used in a regulatory setting. As NAMs are implemented, formats and templates for reporting the methods, results, and integration of multiple results can promote standardisation of new methods. As experience with use is gained, guidance documents can developed to advise stakeholders on the use, interpretation, and good/best practices for integrating emerging science in chemical assessment and advance global harmonization.

The presentation will provide an overview of OECD's efforts to use 21st-century approaches to address a regulatory need for better methods to address chemicals that may interfere with neurodevelopment. The experience gained through the Integrated Approaches to Testing and Assessment (IATA) case studies and the Developmental Neurotoxicity (DNT) *In vitro* Testing Battery (IVB) projects will be shared. Finally, the audience will be informed about the efforts to increase regulatory uptake of the DNT-IVB assays focused on organising a workshop that aims to leverage the learnings from the IATA case studies and elaborate aspects that can be standardised within IATAs.

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S23 | Bringing NGRA to life – a global joint effort for putting next-generation risk assessment into practice

S23-01
The ASPIS-initiated Safety Profiling Algorithm (ASPA): setting the stage for Next-Generation Risk Assessment

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The last decade has seen a significant shift in the development of scientific methodologies and frameworks for human health risk risk assessment of chemicals, offering a new era in the application of New Approach Methodologies (NAMs). Next-Generation Risk Assessment (NGRA) is considered a pivotal paradigm for integration of NAM-based data for toxicity, exposure assessment and kinetics, to enable regulatory decision-making whether a chemical is safe or not. Aiming for acceleration of the use of NGRA approaches for regulatory decision-making, a well-guided workflow for NGRA was developed under the Horizon2020 project RISK-HUNT*3R* in cooperation with the ASPIS cluster. This workflow, entitled 'ASPIS-initiated Safety Profiling Algorithm (ASPA) is intended for different types of end-users: end-users that need to compile a dossier and thus to generate data, and end-users that need to evaluate a dossier and thus need to interpret data. ASPA provides structured guidance and allows for parametrization of decision points and rule settings. Moreover, it strengthens the transparency and robustness of the decision-making and reporting. A selected set of case studies was conducted with the aim to determine the applicability domain for the use of ASPA, to identify gaps that require additional tools and approaches for further development within RISK-HUNT*3R* and at the same time to evaluate and refine the 'fit for purpose' aspects of the ASPA workflow enabling rapid modifications and improvements. The lessons learned have resulted in a new version of ASPA, which together with the first next steps will be presented in this lecture.

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S23-02
Assessment and modelling of metabolites: the conazole case study

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One of the main aims of the EU project, RISKHUNT3R (RH3R), is to develop a workflow for chemical risk assessment. Often, parent compounds or chemicals can form metabolites within the body, making it important to understand when metabolites are of concern in the overall risk assessment process. To address this, we have carried out a case study in RH3R using nine conazole compounds (difenoconazole, epoxiconazole, fenbuconazole, propiconazole, prothioconazole, cyproconazole, tebuconazole, itraconazole and ketoconazole). The conazoles can undergo metabolism within the body and form shared metabolites, such as 1,2,4-triazole from most of the selected conazoles in this case study, and hydroxymetabolites. Data from *in vivo* toxicity studies suggests that conazoles and 1,2,4-triazole have distinct effects. The conazoles and 1,2,4 triazole were also tested for toxicity using the *in vitro* test battery in RH3R. The virtual *in vitro* distribution model was used to convert nominal concentrations to the real concentrations causing toxicity in the *in vitro* assays. *In vitro* to *In vivo* Extrapolation linked Physiologically Based Kinetic (PBK) modelling was used to predict the kinetics of compounds within the body following external exposure to the parent compound. The absorption, distribution, metabolism and excretion data were predicted by both *in silico* and *in vitro* methods and a tiered approach was used to assess usefulness of these data in PBK modelling. The concentrations causing toxicity in *in vitro* were compared to lowest observed adverse effect level (LOAEL) -derived concentrations predicted using PBK modelling. The results and learnings from this tiered approach for kinetic modelling of parent and metabolites in risk assessment will be presented.

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S23-03
Prioritization and screening – which testing scope is sufficient?

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No abstract has been submitted.

S23-04
RapidTox: decision-based workflow environment to advance human health assessment

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Decision makers are commonly faced with addressing potential human health risks associated with exposure(s) to chemicals for which little-to-no hazard and dose-response data exist. In response, the field of toxicology is increasingly pivoting to an evolving and complex landscape of data streams to inform human health assessment. To expedite successful application of available traditional and new approach methodology (NAM) data across various decision contexts, knowledge delivery tools that can efficiently and rapidly provide chemical information are needed. RapidTox has been developed for such a purpose. This online tool represents a growing suite of decision support workflows for chemical problem formulations such as 'Emergency Response' and 'Human Health Assessment'. RapidTox workflows provide flexible modules that target the assembly of empirical and predicted physicochemical properties, existent human health toxicity values and effect level data, analogue chemical identification and read-across, and *in vitro* cell bioactivity data. Importantly, the workflow modules have been expertly scoped, designed, and curated such that the user can readily select qualitative hazard and quantitative dose-response data pertinent to a given decision context for single chemicals, or hundreds of chemicals in batch. Reducing the burden of data assembly on the end-user through knowledge delivery tools such as RapidTox may significantly advance more timely protection of human health.

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S23-05
PARCroute: a road from innovative science to regulatory risk assessment practice

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In this presentation by Task 2.2 ("Knowledge management and uptake into policy") of the European Partnership for the Assessment of Risks from Chemicals (PARC, https://eu-parc.eu) we present our activity "PARCroute" which aims to produce roadmaps promoting the uptake of New Approach Methodologies (NAMs) and Next-Generation Risk Assessment (NGRA) into regulatory risk assessment practice. As the first such roadmap, we are developing "NGRAroute", a concrete proposal for a roadmap towards the implementation of NGRA as the first line of risk assessment in all relevant European chemicals legislations. The history and main elements of a first conceptual draft of the roadmap will be presented, with a special focus on sharing our experiences about building a science-to-policy network of stakeholders from the research, regulatory and policy level in the process, including the efforts undertaken to integrate work on NGRAroute with that on the European Commission's "Roadmap for phasing out animal testing in chemical safety assessments" announced in 2023.

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S24 | New approach methods for risk assessment of thyroid disrupting chemicals

S24-01
Building an IATA-based testing strategy for thyroid hormone disruption resulting in developmental neurotoxicity

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Thyroid hormones (THs) are crucial for proper brain development. Deficiencies in TH levels in the brain during pregnancy are associated with lower IQ and delayed motor function in children. For example, genetic mutations of the gene encoded for the monocarboxylate transporter 8 (MCT8) might result in severe effects on IQ and motor function.

Exposure to endocrine disrupting chemicals (EDCs) targeting TH in the developing brain might result in neurodevelopmental disorders. The available *in vivo* studies in OECD test guidelines have many uncertainties regarding the sensitivity and the human relevance of the measured parameters. Therefore, there is a high need of developing New Approach Methodologies (NAMs) measuring the effect of TH disrupting chemicals in the developing brain.

Validated *in vitro* assays investigating TH-related molecular initiating events (MIE) are currently developed by EURL ECVAM and OECD. These assays, that for example measure sodium/iodide symporter (NIS) and Thyroid peroxidase (TPO) inhibition, are not specific for the brain. Therefore, brain specific NAMs need to be developed, guided by adverse outcome pathways (AOP) specific for TH disruption in the developing brain. Furthermore, AOPs need to be more specific for the brain and for later key events more mechanistic knowledge needs to be incorporated.

Based on thyroid-brain related AOPs, testing strategies can be developed by combination of individual NAMs in an Integrated Approaches to Testing and Assessment (IATA). To achieve this, validated assays are needed. Additionally, brain barriers play a crucial role in the regulation of TH levels in the brain and assays for these brain barriers should therefore be included in the IATA. We will discuss what is available for an IATA and what is further needed. Future research should focus on how thyroid related adversity in the developing brain can be measured and what NAMs have to be developed to accomplish this. This will help to improve proper Next Generation Risk Assessment for thyroid-mediated developmental neurotoxicity.

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S24-02
Biomarkers of neurodevelopmental effect can reduce uncertainty when assessing the risks of thyroid disrupting chemicals

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Various environmental contaminants can reduce serum thyroid hormones (THs) in laboratory animals and are correlated to thyroid disease in epidemiological studies. As THs control normal brain patterning and function, thyroid disrupting chemicals could also harm neurodevelopment. To address this risk to children's health, some standardized developmental and reproductive toxicity studies suggest or require serum thyroxine (T4) measures in pregnant, lactating, and developing rats. Any chemical capable of reducing serum T4 *in vivo* could therefore be considered a thyroid disruptor. However, the developing brain is not often examined concurrently by either histopathology or neurobehavior, making it is unclear when a serum T4 reduction is adverse. To address this data gap, we have worked to understand mechanisms of brain TH action to identify potential biomarkers of neurodevelopmental effect in the rat. In a series of publications, we have shown that abnormal cell migration and brain barrier disruption are two reproducible effects of TH interference *in vivo*. In these hypothesis-driven investigations, we demonstrated that targeted brain gene expression and histopathology assays, as well as serum microRNAs, could be used in toxicology studies as potential biomarkers of neurodevelopmental effect. Importantly, not only are these biomarkers rooted in the biology of TH action, but they can be assessed in young rats (postnatal day 0 – 14) using relatively rapid and cost effective methodologies. In all, this work suggests that directed evaluation of TH targets in the developing rat brain can strengthen the interpretation of serum T4 measures, thus improving chemical assessment. *This work does not reflect US EPA policy.*

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S24-03
Thyroid hormones quantitative systems toxicology platform (TH QST platform) in rat and human for cross-species NGRA

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Evaluating endocrine impacts, such as disruptions to thyroid hormones (TH), is challenging due to observed differences between human and animal (notably rats) responses [1–4]. Still, toxicity assessments chemicals rely on animal studies, highlighting the necessity to investigate the mechanisms behind species-specific responses. This study aims to develop a quantitative systems toxicology (QST) platform to elucidate TH regulation in both rats and humans, enabling the evaluation of species-specific impacts from TH-disrupting chemicals, including plasma protein binding and TH elimination pathways. The platform is validated by modeling the effects of Phenobarbital, a known TH axis disruptor [5].

An existing physiologically based kinetic (PBK) model of TH was utilized and enhanced by incorporating species-specific differences using the OSP suite (PK-Sim and Mobi) version 11.2 [6]. The original model included the pituitary gland synthesis of thyroid stimulating hormone (TSH), TH synthesis (T3 & T4) and induction by TSH as well as feedback loops. It was expanded to include UDPGT-mediated clearance of TH, TSH circadian rhythm, and the detailed binding of TH to key plasma proteins Albumin, Transthyretin (TTR), and Thyroid Binding Globulin (TBG). Thereby, a unified model framework was created for adult humans and rats. The model's accuracy and predictive capabilities were verified using existing literature and historical data of the UDPGT inducer Phenobarbital.

Albumin, TTR, and TBG were successfully integrated as endogenous large molecules in the model, with their species-specific turnover rates aligning with literature-reported values. Importantly, TBG, which significantly influences TH binding in humans, is absent in rats. The model accurately simulates the species-specific turnover of THs and their free fractions in plasma, drawing on existing literature. For rats, T3 elimination primarily involves glucuronidation by UGT enzymes (30%) and deiodination across all tissues, whereas in humans, UGT plays a minimal role in T3 clearance. T4 conversion to T3 (30%) and reverse T3 (30%), along with glucuronidation by UGT (25%) and sulfation were modeled.

This PBK research highlights how species-specific mechanistic detail can help elucidate variances in TH disruption following exposure to chemicals or drugs. The QST TH model serves as a flexible tool that can be integrated with any compound PB(P)K model to assess thyroid toxicity potential. Looking forward, ongoing efforts aim to expand the model to include the exposure and regulation of thyroid hormones in both maternal (human and rat mothers/dams) and offspring (fetuses and pups) contexts, offering a more comprehensive understanding of species differences in thyroid hormone dynamics across different life stages.

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S24-04
3D thyroid and liver models: a holistic *in vitro* approach for mechanistic investigations of agrochemicals

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Interferences with the thyroid hormone system are a relatively common drawback of exogenous substances under development, requiring thorough evaluation to ensure the safety of agrochemical products. New Approach Methodologies (NAMs) offer the opportunity to enhance mechanistic understanding while reducing animal usage. Advanced *in vitro* models closely mimic *in vivo* functions and physiology, enabling more relevant hazard identification of direct thyroid-perturbating effects or indirect liver-mediated impacts on thyroid function. The latter can arise from either chemical-induced alterations in hepatic thyroid hormone catabolism or the liver's production of thyroid active metabolites from the parent compound.

A holistic *in vitro* approach of functional 3D thyroid and liver models for both rat and human species will be presented that can be used individually or combined to investigate disruptions of the thyroid hormone homeostasis. Freshly isolated thyroid follicles exhibit distinct decreases in T3/T4 secretion when exposed to propylthiouracil or fluoride exceeding a specific concentration threshold. Human and rat hepatocyte-based liver spheroids respond with increased formation of glucuronidated thyroid hormone catabolites (gT4) to various compounds, showcasing species-specific reactions. Furthermore, our data demonstrate the limitations of using HepaRG cells as substitutes for primary human hepatocytes in this context.

Along a use case, we illustrate how integrating a rat 3D thyroid and liver model on a multi-organ-chip platform (HUMIMIC Chip2®) can enhance the identification of thyroid-related effects that elude early screens for endocrine disruption but exhibit severity in *in vivo* studies. Study results and the necessary stepwise approach to realize relevant multi-organ-chip assays will be discussed.

Future research will focus on refining the 3D thyroid and liver models with further use cases from the agrochemical development pipeline, with particular focus on identifying thyroid toxicity mechanisms arising from hepatic metabolites.

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S25 | Towards the implementation of virtual control groups – regulatory and scientific challenges

S25-01
Virtual Control Groups – roadmap into 3Rs

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The 3Rs have been the leading concept to solve the societal dilemma of need for and objection to animal experimentation. The R of Reduction, i.e., to obtain the same information with less animals, has argu-

ably been the weakest of the three, as today's animal tests are rather statistically underpowered with small group sizes. The enormous reductions seen with LD_{50} tests are certainly a thing of the past and most reductions are nowadays achieved by non-invasive (non-lethal) endpoints allowing animals to serve as their own control with often multiple measurements over time. A new approach of Virtual Control Groups (VCG) suggests using historical data and eliminate control animals. This could save 10–30% of animals – notably, the 30% refers to non-human primates (NHP) in experiments with recovery groups. Giving the spike in NHP prices due to shortages in supply, this is a strong value proposition.

Following a workshop on this topic based on earlier work of partners, a consortium of more than 20 partners has formed to further this concept. Data-sharing is envisaged to allow the analysis, model and implemen VCG settings. The project is a win/win for the 3Rs as it will either show a substantial reduction alternative or demonstrate the inconsistency of animal studies further opening the door for New Approach Methodologies.

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S25-02
ViCoG DB – a cross company control repository

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A large repository of control data is the backbone to support the generation of suitable virtual controls for studies that may use this information for evaluation of treatment related effects. It also serves as data source for AI solutions to generate synthetic data for control animals that may be part of control groups [1].

Therefore, a control database was created by integrating control data from five pharma companies, focusing on toxicology studies lasting 28 days or longer. The database follows CDISC SEND structure and employs standardized terminology. Data domains include demographics, body and organ weight, laboratory, macroscopic, and microscopic findings. Standardization and harmonization were achieved through robust curation processes. The database contains information on over 80k animals across various domains. It supports the generation of virtual control groups and the qualification of the virtual control group approach. Currently, it includes data on over 70k rats, 8k mice, 5.8k dogs, and 3.8k monkeys. The database provides valuable insights and potential to reduce animal use in *in vivo* studies [2].

First investigations on the possible way to the implementation and use cases on retrospective performance of virtual controls replacing concurrent controls could demonstrate that treatment related findings can be detected by using virtual controls thereby suggesting a great potential to reduce animal use in *in vivo* studies.

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S25-03
Analytical approaches to build and evaluate ViCoGs

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No abstract has been submitted.

S25-04
Into the SEND data-verse: with great virtual power comes great regulatory responsibility

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The replacement of animals from the control groups of *in vivo* toxicology studies with data from appropriately matched control animals from historical studies has been proposed to reduce animal use without sacrificing the scientific integrity of the studies. The existence of CDISC-SEND-formatted toxicology control data may be leveraged by proof-of-concept research into the feasibility of this approach, yet many questions remain with regarding the development and validation of a selection procedure to supply data from virtual control animals for a study. Greater than 13,000 SEND datasets have been submitted since CDER initiated a requirement for SEND electronic standardized study data. As analytical procedures for optimal selection of virtual control animals mature, external validation of these techniques can be performed via statistical simulations utilizing this database. The benefits and risks of full replacement, partial replacement, and augmentation of concurrent control groups with virtual control animals will also be explored via simulation experiments. In addition to these scientific concerns, many logistical issues will also need to be resolved to ensure that studies employing virtual control groups are conducted with integrity and in compliance with GLP regulations. Experimentation with virtual control groups in the low-risk contexts of use, e.g. non-GLP dose-range-finding studies, may build confidence in the scientific validity of the approach as well as experience handling logistical concerns. The availability of SEND datasets could revolutionize the ability to integrate historical toxicology study data into the analysis of current study results, and responsible implementation of virtual control groups may be among the first of many steps toward modernization of regulatory toxicology.

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S26 | Risk assessment under the real-life risk simulation (RLRS) approach – new technologies and mechanistic data

S26-01
Evidenced by experimental studies on real-life risk simulation supporting the need for new risk assessment evaluations

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In recent years, there has been growing scientific, regulatory, and public concern about exposure to chemical mixtures in everyday life. In reality, consumers are exposed to complex chemical mixtures by con-

suming food, water, and commercial products. Because risk is usually assessed on an individual basis, current regulation does not assess the overall risk of chemicals within the mix. In recent years, a large number of studies have been carried out with the aim of assessing the potential effects of long-term exposure to non-commercial mixtures of chemicals at low and realistic dose levels around the regulatory limits [1–10]. These studies have shown that long-term exposure to doses considered safe by regulatory authorities for individual chemicals produces non-monotonic effects and adaptive responses, organ toxicity, induction of oxidative stress, and biochemical and endocrine disruption [1–10]. These results highlight a need to move away from single chemical testing to a more complex approach that considers multiple stressors which may make it difficult to establish real safety levels.

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S26-02
The exposome approach to real-life risk assessment

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No abstract has been submitted.

S26-03
Challenges posed by real-life exposure scenarios: a systems toxicology approach to integrated assessment of multiple risks

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Real-world exposures are inherently complex as they involve dynamic interactions between chemicals, biological systems, and environmental factors. Traditional risk assessment methods are not best suited to assess this complex scenario, as they rely heavily on empirical data based on single chemical exposure and dose-response relationships. Such an approach often oversimplifies complex real-life scenarios by disregarding the interplay of exposure-related factors, such as temporal variations, cumulative effects, interactions among chemicals and differences in exposed populations. The Real-Life Risk Simulation (RLRS) approach intends to replicate real-world exposure scenarios, incorporating multiple chemicals, different adverse effects, and doses near or well below regulatory limits. By considering interactions and cumulative risks, RLRS provides a more accurate risk assessment. Systems Toxicology is another valuable and comprehensive framework aimed to evaluate multiple risks in a holistic manner. It goes beyond empirical data and incorporates mechanistic insights and novel technologies, thus becoming pivotal for addressing the complexities inherent in real-life exposure scenarios [1]. Implementation of New Approach Methodologies (NAMs) in modern risk assessment offers a deeper understanding of chemical-induced toxicity by mimicking human biology. While NAMs represent a crucial breakthrough, they fail to provide a structured framework for linking molecular events to adverse health outcomes. The Adverse Outcome Pathway (AOP) concept can successfully address this gap, especially AOP networks as they better represent the biological complexity where mixtures of stressors can trigger multiple effects. The integration of classical toxicological approaches for risk assessment with novel methodologies and mechanistic data has become pivotal for comprehensively assess risks associated with real-life exposure scenarios [2]. This presentation addresses the challenges posed by real-world exposures and explores how RLRS and the Systems Toxicology approach enhance our understanding and management of chemical risks.

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S26-04
The concept of risk assessment evaluations in the 21st century

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Sources of exposure to environmental chemicals of concern are ubiquitous, and people of all ages and at all stages of development are exposed to a cocktail of xenobiotics through all possible routes every day of their lives. To date, only one chemical has been the subject of long-term toxicity assessments, which are used to set appropriate reference doses and presumed safe limits. The likelihood of predicting a safe threshold for individual chemicals is low by applying the classical long-term safety assessment due to the scale of real exposure to many environmental chemicals, the complexity of mixtures and non-linearity. This presentation will provide an overview of the current regulatory approaches to the assessment of the toxicity of mixtures and to analyze the new proposed methodologies that can better predict the toxicity of a chemical in the context of a real exposure scenario [1–7]. There is a clear need to move from assessing the risks posed by individual chemicals to assessing cumulative risks to achieve better protection of the population.

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S27 | Interindividual variability in toxicokinetics and toxicodynamics in chemical safety assessment

S27-01
Toxicokinetic and toxicodynamic uncertainties in chemical safety assessment

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Chemical risk assessment aims at identifying levels of exposure to a chemical that should not result in adversity. For decades, the establishing of such health-based guidance values has used data mostly derived from *in vivo* animal toxicity studies, using traditional endpoint measurements and identifying critical points of departures such as NOAELs or BMDLs. To extrapolate from experimental animals (typically rodents) to humans an uncertainty factor of 10 is applied to account for differences in toxicokinetics and toxicodynamics between the test species and humans. An additional uncertainty factor of 10 is then applied to account for the inter-individual variability in the human population. Whereas the uncertainty factor of 10 used for the extrapolation from the test species to humans is supported by considerable empirical evidence in the species differences in toxicokinetics (4.0) and toxicodynamics (2.5), the uncertainty factor of 10 used for human inter-individual variability rests on the default factors of 3.2 for both toxicodynamics and toxicokinetics. It is only in recent years that progress has been made to identify more precisely the true variability for the different metabolic pathways, and also how the toxicological response to a chemical varies between individuals. The aim of this session is to discuss recent advances in our understanding of inter-individual variability in toxicokinetics and toxicodynamics.

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S27-02
Human interindividual variability in toxicokinetics

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Human interindividual variability in toxicokinetics (TK) is a complex and multifaceted issue that significantly impacts the assessment and management of risks associated with toxic substance exposure. By recognizing and studying the sources of this variability, we can improve public health outcomes, enhance the precision of medical treatments, and develop more effective regulatory policies.

The variability in TK necessitates the consideration of sensitive subpopulations in risk assessments. Regulatory agencies often adopt default safety or uncertainty factors (UF) to account for this variability, which are generally considered as conservative, ensuring protection for the most vulnerable individuals. The 100-fold default UF has been rationalized to account for interspecies differences (10-fold) and human variability (10-fold) which is further subdivided into two equal default uncertainty factors ($10^{0.5}=3.16$) to allow inter-individual differences in TK and TD dimensions. Are they actually protective? Ideally they should be replaced by chemical-specific adjustment factors (CSAF) or pathway-related ones. CSAFs are derived using chemical-specific TK data using physiologically-based TK (PBTK) models describing ADME processes from external to internal exposure. In order to address these issues, an EFSA funded Project was aimed to i) quantify human variability in toxicokinetics (TK) through extensive literature searches (ELS) and meta-analyses using a hierarchical Bayesian model for a number of metabolic enzymes (e.g. CYP3A4, UGTs, GST) and transporters (e.g. Pgp, BCRP and OAT) and ii) produce data for specific chemicals (e.g. phosmet, microcystins) to be used in PBK models allows to

refine methods applied in chemical risk assessment through integrating mechanistic understanding and implementation of New Approach Methodologies (NAMs).

The results of the project showed that the identification of variability related to age and inter-phenotypic differences in world populations potentially allows for: 1) the application of pathway-related UFs in the risk assessment of compounds for which *in vitro* metabolism evidence is available without the need for animal data; 2) the integration of related variability distributions with *in vitro* metabolism data into PBK models for quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) and 3) the estimation of UFs in chemical risk assessment using variability distributions of metabolism. It was also highlighted the still relatively limited human data available for a majority of enzymes and transporters. The experimental produced data underlined the relevance of generating *in vitro* isoform-specific kinetic information for improving human risk assessment of single chemicals (and their mixtures), describing potential inter-individual differences and supporting the development of robust QIVIVE and PBK models to face a risk assessment in an animal free environment.

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S27-03
Population variability of toxicodynamics driving adverse responses

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Understanding the variability across the human population with respect to toxicodynamic responses after exposure to chemicals, such as environmental toxicants or drugs, is essential to define safety factors for risk assessment to protect the entire population. Activation of cellular stress response pathways are early adverse outcome pathway (AOP) key events of chemical-induced toxicity and would elucidate the estimation of population variability of toxicodynamic responses. We aimed to map the variability in cellular stress response activation in a large panel of primary human hepatocyte (PHH) donors as well as human peripheral blood mononuclear cells (PBMC) to aid in the quantification of toxicodynamic interindividual variability to derive safety uncertainty factors. For this purpose we used high-throughput transcriptomics to assess the toxicodynamics of a panel of toxicants with diverse yet well-defined mode-of-action, including oxidative stress response (OSR), DNA damage response (DDR), unfolded protein stress response (UPR) and inflammatory signalling response (ISR). Using a population mixed-effect framework, the distribution of benchmark concentrations (BMCs) and maximum fold change were modelled to evaluate the influence of donor panel size on the correct estimation of interindividual variability for the various stimuli. So far, we have assessed the variability in 50 PHH donors [1]. We identified that the average of transcriptional BMCs had a maximum difference of 864-, 13-, 13-, and 259-fold between different PHHs for UPR, OSR, DDR, and ISR. Estimated toxicodynamic variability factors of stress response activation in PHHs based on this dataset ranged between 1.6 and 6.3. In a current EFSA-supported project, TD-TRAQ, we extend our work on PHH and focus on assessing the interindividual toxicodynamics variability in freshly isolated human PBMCs from in total 200 healthy donors with different age and ethnicity. We use a similar strategy and apply high-throughput transcriptomics to define the concentration response-dependent cellular stress response activation in relation to onset of cytotoxicity for eight different compounds with diverse modes of action. The current status and findings of this project will be discussed. Our overall findings will provide critical scientific insights in the relevance and acceptance of the current traditional toxicodynamics safety factors used in risk assessment.

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S27-04
Pharmacogenetics testing in personalized medicine to prevent drug-induced adverse reactions

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Patients respond highly variable to drugs both with regard to efficacy and adverse drug reactions. About 50 years ago it has been discoverd that drug reponse is a heritable trait and variations in genes encoding proteins involved in drug transport, drug metabolism and drug targets partly explain variabilty in individual response to drugs. More recently, it has been discoverd that patients carrying specific variants in the HLA-gene are at risk for specific idiosyncratic reactions, especialy drug allergies.

The Dutch Pharnacogenetics Working Group (DWPG) has drafted more than 100 clinical guidelines on gene-drug interactions and these recommendations have been incorporated in drug prescribing systems and medication surveillance systems used by physicians and pharmacists nationwide in The Netherlands. There is ample evidence for a series of single gene-drug combinations showing that pharmacogenetic testing and -guidance is beneficial for patients. In the recent years pretherapeutic testing has been implemented for several gene-drug combinations and have become standard of care. Besides clinical benefit, several studies have been published showing cost-effectiveness of pretherapeutic testing.

The actionable gene-drug combinations that have merged from the DWPG guidelines are related to about 40 drugs and 50 genetic variants, and were defined as the ‘pharmacogenetic passport’. This passport was implemented in 7 EU countries involving a total of 6,900 patients in a

randomized trial. This so-called PREPARE study showed a 30% reduction of clinically relevant adverse drug reactions of pharmacogenetically guided prescribing as compared to standard-of-care treatment.

The next step may be pre-emptive screening of patients e.g. as part of the heelprick and/or repurposing sequencing data of individuals.

In conclusion, the use of pharmacogenetics makes drug therapy safer and more efficacious.

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S28 | HOT TOPIC: how to build trust in artificial intelligence for toxicology?

S28-01
Garbage In, Garbage Out: Mitigating the Ethical Risks of Artificial Intelligence and Machine Learning

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Artificial intelligence (AI) and machine learning (ML) are enabling advances in understanding the Earth and its systems at all scales and are increasingly being used in diverse health and societal applications to address urgent issues including climate change and natural hazards. Moreover, the recent release of powerful large-language models including Chat GPT is creating radical change in scholarly publishing, education, and beyond. To address the urgent need for guidance on the ethical use of AI and ML in earth, space, and environmental science-focused research, a recent multi-disciplinary community report facilitated by American Geophysical Union and funded by the U.S. National Aeronautics and Space Administration introduces a framework of principles and responsibilities for using AI and ML in Earth, space, and environmental science-focused research. These principles are largely adoptable by most disciplines and important to incorporate into the research ecosystem and fundamental expectations such as an ethical Code of Conduct for research. In this talk, the process for developing the Ethical framework for AI and ML will be shared along with an overview of the principles for organizations and researchers.

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S28-02
AI in chemical risk assessment: opportunities, challenges, solutions

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This contribution will discuss opportunities offered by AI to facilitate chemical risk assessment and to support the transition to NGRA. This talk would not be complete if it did not also cover challenges encountered while (trying) to implement AI based tools in everyday risk assessment practice.

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S28-03
Do machine learning systems that support public decision making undermine legitimacy?

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Machine learning algorithms are increasingly used to support decision-making in the exercise of public authority, for example when evaluating whether a product is safe or unsafe. Here, I argue that an important consideration has been overlooked in previous discussions: whether the use of ML undermines the democratic legitimacy of public institutions.

From the perspective of democratic legitimacy, it is not enough that ML contributes to efficiency and accuracy in the exercise of public authority, which has so far been the focus in the scholarly literature engaging with these developments. According to one influential theory in political philosophy, exercises of administrative and judicial authority are democratically legitimate if and only if administrative and judicial decisions serve the ends of the democratic law maker, are based on reasons that align with these ends and are accessible to the public.

These requirements are not satisfied by decisions determined through ML since such decisions are determined by statistical operations that are opaque in several respects. However, not all ML-based decision support systems pose the same risk, and I argue that a considered judgment on the democratic legitimacy of ML in exercises of public authority need take the complexity of the issue into account.

This talk will outline considerations that help guide the assessment of whether a ML undermines democratic legitimacy when used to support public decisions. I argue that two main considerations are pertinent to such normative assessment. The first is the extent to which ML is practiced as intended and the extent to which it replaces decisions that were previously accessible and based on reasons. The second is that uses of ML in exercises of public authority should be embedded in an institutional infrastructure that secures reason giving and accessibility.

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S29 | Integration of developmental neurotoxicity data across adverse outcomes for improved safety assessment of chemicals

S29-01
Identification of phosphoproteomics changes for adverse outcome pathway development in neurotoxicant risk assessment

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The information in this Abstract has been reviewed by the Center for Computational Toxicology and Exposure and approved for presentation. Approval does not imply that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Adverse Outcome Pathways (AOPs) offer a structured framework for understanding the sequence of events connecting chemical exposure to adverse health effects, aiding regulatory decision-making. While

genomics, proteomics, and metabolomics have been employed in AOP development, the global analysis of post-translational modifications (PTMs), such as protein phosphorylation, remains limited. Phosphorylation, a crucial PTM, is essential for dynamic changes in gene and protein expressions and toxicity pathways underlying phenotypic changes induced by environmental chemicals. It represents a critical non-genomic signaling cascade, contributing to adverse outcomes in AOP development.

This presentation explores phosphoproteomics strategies and methods for identifying phosphorylated proteins in rat brain tissues and cortex cells exposed to neuron and brain developmental toxicants. We emphasize critical changes linked to modes of action in target tissues and cells, as well as key events in AOPs. In an exploratory study, we measured 167 phosphoproteins critical to neurodevelopment using targeted phosphoprotein arrays (Full Moon Biosystems, Sunnyvale, CA) in the cortex tissues of male offspring of Pregnant Long-Evans rat mothers exposed to Propylthiouracil (PTU) at 3 and 10 ppm from gestational day (GD) 6 to postnatal day (PN) 30, via drinking water. A total of fifty-four and fifty-two differentially phosphorylated proteins (>50% increase or decrease in phosphorylation levels), respectively, were identified compared to control rats ($p<0.05$). Examples include Disabled-1 (Dab1), regulating cell positioning in the developing brain and adult neurogenesis, and Tau (Tyr729), involved in synaptic impairment and neurofibrillary tangle formation. Twenty-five proteins showed altered phosphorylation levels at both concentrations, with twenty-two proteins exhibiting dose-response relationships. These include members of the Calcium/calmodulin-dependent protein kinase (CaMK) family and NMDA receptor (NMDAR) family, known to regulate synaptic transmission and plasticity in the mammalian nervous system.

Previous studies indicate synaptic transmission and plasticity impairments in neonatal rat brains due to PTU treatment, potentially contributing to learning deficits in developmental hypothyroidism. Changes in phosphorylation within critical signaling pathways may drive altered synaptic plasticity in PTU-exposed rats, suggesting a role in adverse outcome pathways for PTU-induced neurodevelopmental effects on learning and memory. Additionally, this study also demonstrates the effectiveness of high throughput phosphoproteomics profiling in investigating adverse responses to neurotoxicants. The presentation will also discuss its application *in vitro* and for extrapolating neurotoxicity data across models.

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S29-02
Development of AOP-informed IATA for developmental neurotoxicity (DNT) risk assessment of deltamethrin and flufenacet

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New approaches in toxicology including the adverse outcome pathway (AOP) and integrated approach to testing and assessment (IATA) concepts, the use of *in vitro* human stem cell-derived neuronal models, QSARs and read across used in an integrated manner, may pave the way to a more efficient and predictive assessment of developmental neurotoxicity (DNT), solving various regulatory challenges.

Recently, international efforts have led to the development of the Developmental Neurotoxicity *In vitro* Battery (DNT IVB) based on which a Guidance Document (GD) has been published under the umbrella of the OECD, EFSA and US EPA. This GD provides information on the regulatory use of DNT *in vitro* assays (https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf) based on fit for-purpose applications.

To assess the applicability of the DNT IVB in the context of the European pesticide regulations (EU) 283/2013 and 1107/2009 (European Commission, 2009, 2013), adverse outcome pathway (AOP)-informed integrated approach to testing and assessment (IATA) has been developed by the EFSA DNT Working Group in support of DNT hazard identification and characterisation based on two pesticide active substances deltamethrin (type II pyrethroid) and flufenacet (a herbicide).

The AOP concept was applied to integrate information from human epidemiological studies, animal data (including in-vivo regulatory studies), *in vitro* and zebrafish data. Systematic literature review and critical appraisal of all the evidence was performed, including New Approach Methodologies (NAMs) for *in vitro* studies, high-throughput testing from IVB and zebrafish studies.

This stepwise approach resulted in the development of an evidence-based AOP network for deltamethrin with a probabilistic quantitative estimation of the weight-of-evidence using a Bayesian network analysis that allowed the quantification of the uncertainty in the postulated AOP. The AOP network consisted of two MIEs that triggered cascade of key events leading to altered behavioural function defined as the adverse outcome (AO).

This approach allowed to conclude with an acceptable level of certainty that deltamethrin could trigger DNT effects in contrast to flufenacet (non-neurotoxic), supporting the importance of the mechanistic understanding of possible DNT effect and at the same time, increasing scientific confidence in decision-making process.

These case studies showed the applicability of the DNT-IVB for hazard identification and characterization and illustrated the usefulness of an AOP-informed IATA for regulatory purposes.

The overall knowledge integrated in AOP-informed IATA led to improved interpretation of human epidemiological data by providing a plausible mechanistic link to adverse outcomes, supporting the risk assessment of pesticides.

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S29-03
GENESIS: Generative Exploration of NEurotoxicity Targets through SImilarity Assessment of SMILES

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An important principle in developing Adverse Outcome Pathways (AOPs) and quantitative AOPs (qAOPs) models is to provide an agnostic assessment of biological perturbations. Even though the focus is on the underlying mechanisms of toxicity, the chemical space of AOPs/qAOPs, which reflects the type of chemicals and structural alerts that initiate molecular events (MIEs) and key events (KEs) within an AOP, helps define the applicability domain of AOPs. Current approaches to neurotoxicity prediction deal with data scarcity, besides other limitations in data generation that impede learning the chemical space for human and environmental safety assessment. Thus, there is an utter need for new computational strategies, particularly for endpoints such as neurotoxicity, to train models for MIEs/KEs-predictions effectively.

The objective was to explore the development of a Siamese Neural Network (SNN) for similarity assessment of potential neurotoxicants for three known MIEs. The MIEs were: inhibitors to acetylcholinesterase (AChE), binding of antagonists to N-methyl-D-aspartate receptors (NMDAR), and binding to thyroid receptors alpha (THRA) and beta (THRB).

The SNN was explored to learn how two molecules are related based on their Simplified Molecular Input Line Entry System (SMILES). It was composed of two identical sub-networks with the same structure, parameters, and weights to determine the input similarity. A transformer-Convolutional Neural Network (CNN) model was trained to canonize SMILES as a Sequence-to-Sequence (Seq2Seq) problem. Data augmentation of molecular structures using the SMILES chemical language was applied during the training. The similarity score was determined by the distance between the feature vectors of each of the sub-networks, and the subtraction was fed into the active/inactive target prediction. The model represented an adapted workflow of Zhang et al (2023) [1] applied to a classification task instead. The Layer-wise Relevance Propagation (LRP) method, which splits the overall predicted value into a sum of contributions of individual neurons, was scrutinised for model interpretability. The SNN model was validated against a list of compounds with known human and *in vitro* evidence for neurotoxicity.

We anticipate the preliminary results helping link molecular substructures with neurotoxicity targets. In addition, the SMILES-based similarity assessment has the potential to enhance the applicability domain of AOPs/qAOPs for read-cross and grouping tasks and, overall, improve our understanding of chemical-target interactions within the context of AOPs for neurotoxicity.

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Short Orals Sessions

OS01 | Short Orals Session 1

OS01-01
Exploring extracellular and Intracellular interactions and effects of advanced materials on reconstituted primary human bronchial epithelial cultures and connecting their potential impacts on lung health

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While the respiratory tract possesses inherent mechanisms for defense and self-clearing, specific classes of dust particles that enter the lung at high rate or persist and accumulate in the distal airways and alveoli can lead to pulmonary lung diseases. For example, crystalline silica or asbestos inhalation is linked to lung silicosis or asbestosis, respectively. Exposure to multi-walled carbon nanotubes (MWCNTs) has been associated with lung fibrosis. However, some of the underlying mechanisms of how materials interact with bronchial cells and induce ciliary dysfunction remain to be investigated. Our study explores the impact of advanced materials, silicon carbide nanowires (SiC) and graphene nanosheets on reconstituted primary human 3D bronchial epithelial cultures (HBE, MucilAir™, Epithelix, Switzerland). We also included silica quartz (DQ12), silica dioxide nanoparticles (NM203, JRC) and MWCNTs (NM401, JRC) as a reference to compare the biological effects of advanced materials with well-studied micro- and nanoscale particles. The particles were first characterized for physicochemical properties using zeta-sizer (hydrodynamic size and zeta potential), SEM (morphology and size distribution) and Raman spectroscopy (chemical properties). Next, the biotransformation on the particle's surface was studied with mucus (collected from human bronchial lung cultures) by measuring changes in surface charge and morphology. HBE cultures were then repeatedly exposed to selected particle types (dose range: 1–50 µg/cm^2) for four days under semi-air-liquid interface conditions. Following exposure, ciliary interactions, cellular uptake and intracellular distribution of advanced materials were investigated by applying high-resolution imaging techniques, including Raman confocal microscopy, SEM-EDX and TEM. LDH release from the cells and transepithelial barrier resistance (TEER) were measured to assess acute cytotoxicity and epithelial barrier integrity, respectively. No significant ($p>0.05$) acute cytotoxicity (LDH release) or loss of barrier integrity was observed either on day 2 or day 4 at all exposed concentrations. Advanced materials, especially SiC, significantly ($p<0.05$) reduced the cilia beating frequency (CBF), and this potentially caused further impairment of ciliary functions. However, active cilia area or mucociliary clearance (MCC) was not significantly ($p>0.05$) affected after exposure. To further understand the molecular basis of impaired cilia functions at the cellular level, we analyzed cytokine-chemokine responses including effects at transcriptional levels for the genes involved in maintaining ciliary functions in the lungs. Overall, the results from this study will fill a gap in understanding potential extracellular changes to consequent intracellular response in the lungs after exposure to advanced materials.

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OS01-02
New strategy of genotoxicity test using organoids in the 3-dimensional tissue culture system

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A DNA double-strand break (DSB) is considered one of the most cytotoxic forms of DNA damage, as unrepaired DSBs can lead to chromosomal abnormalities and cell death. Currently, genotoxicity tests *in vitro* are conducted using 2-dimensional (2D) cell culture systems, as 3-dimensional (3D) tissue models have not yet been established. This study aims to develop a new genotoxicity test that enables the analysis of DSBs in organoids. Both 2D placental cells and 3D placental organoids were treated with different types of DNA damaging agents, including bleomycin (BLM), which causes DNA strand scission, mitomy-

cin C (MMC), which causes interstrand crosslinks, and camptothecin (CPT), which inhibits topoisomerase I. The study analysed the effects of etoposide (VP-16), gemcitabine (dFdC), and 5-fluorouracil (5-FU) on the inhibition of topoisomerase II, DNA synthesis, and accumulation of DSBs using pulsed-field gel electrophoresis. In 2D cell cultures, treatments with BLM and VP-16 resulted in higher accumulations of large fragments of broken DNA, while treatments with MMC, CPT, and dFdC showed slight accumulations of small fragments of broken DNA. However, the treatment with 5-FU did not result in the accumulation of DSBs. In contrast, treatment with BLM led to the accumulation of large fragments of broken DNA in organoids, while treatment with VP-16 did not. These results suggest that the genotoxic effect and/or transportation ability to target cells of VP-16 differed between 2D cell cultures and organoids. Only treatment with CPT induced the accumulation of small fragments of broken DNA. The formation of DSBs after treatment with DNA damaging agents in organoids differed from that in 2D cell cultures.

Based on these results, we concluded that we have developed a new technique that can detect DSBs in organoids. This technique will enable us to analyze not only the genotoxicity of the subject compound but also its ability to transport to the target cells in the organoid.

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OS01-03
Toward standardization of testing strategies for *in vitro* hepatic metabolism studies

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In vitro biotransformation studies are a relevant tool to obtain insights into species specific toxicokinetics. In Europe, comparative *in vitro* metabolism (CIVM) studies are required for pesticide active substances, as described in Commission Regulation (EU) No 283/2013. A Scientific Opinion published by EFSA's Scientific Panel on Plant Protection Products and their Residues (PPR Panel) on testing and interpretation of CIVM studies, provided suggestions for a setup of such studies. The PPR Panel highlights how unique human metabolites or disproportionate human metabolites can be identified, i.e. metabolites formed in incubations with human hepatocytes in amounts at least fourfold higher than in incubations with animal hepatocytes. It is of importance for regulatory applications that the data generated are consistent, reliable and reproducible requiring standardized protocols and testing strategies for CIVM studies, which to date are not available. For *in vitro* biotransformation studies, the applied concentration of cells/enzymes, incubation period, and concentration of substrate play an important role in the outcome, and these factors are preferentially optimized for each chemical. Therefore, the aim of this study was to perform CIVM studies for rats and humans using primary hepatocytes and liver S9 fractions as the two metabolic test systems to determine: 1) disproportionate human/rat metabolites and 2) unique human/rat metabolites and 3) to compare S9 metabolism studies with hepatocyte metabolism studies. First, the liver S9 fractions and primary hepatocytes were characterised for their phase I and phase II metabolic activity using reference compounds for the following (human) enzymes: CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 and the overall glucuronidation and sulfonation enzymes. Second, the solubility in the *in vitro* system and non-specific binding was assessed for the chosen test substances (chlorpropham, imidacloprid and thiabendazole). For the primary hepatocytes, effects of the test substances on cell viability were also determined. Subsequently, CIVM studies were performed at non-cytotoxic concentrations of 1, 10 and 100 μM for all three test substances and the selected metabolites were quantified using LC-MS/MS. Unique human/rat metabolites were identified using high resolution (HR)-MS. The results of the CIVM studies show that there is substantial disproportion in metabolite formation between human and rat observed in both metabolic test systems. A set of unique human and rat metabolites were detected and identified by HR-MS. Hence, employing standardized testing strategies serves as a valuable tool for effectively conducting CIVM studies. This case study corroborates that comprehensive CIVM studies are required to obtain reliable insights in possible species specific toxicokinetics.

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OS01-04
Review of New Approach Methodologies for application in risk assessment of nanoparticles in the food and feed sector: status and challenges

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New Approach Methodologies (NAMs) show great potential in advancing risk assessment (RA). However, their regulatory implementation is lagging behind compared to their scientific development. The EFSA Guidance on RA of nanomaterials suggests nano-specific RA is best achieved through Integrated Approaches to Testing and Assessment (IATA) with NAMs as the first choice to generate new information. Integrating NAMs in RA promises several advantages such as a better human focus, a stronger emphasis on molecular mechanisms and a higher efficacy. However, applying NAMs to nanomaterials (NMs) also poses considerable challenges such as issues related to dispersion stability and dosimetry. Significant efforts are being undertaken to establish nano-specific NAMs. Within the EFSA-funded project NAMS4NANO we have conducted a review of NAMs which are potentially useful for application in RA of NMs in the food and feed sector.

Our review covers nano-specific NAM-frameworks and individual NAMs. Specifically, for NMs we emphasize physicochemical characterization methods for their inclusion as NAM based approaches. A particular focus was on initial screening methods according to the EFSA framework, i.e., on NM degradation/dissolution, genotoxicity, cytotoxicity, oxidative stress, inflammation, and barrier integrity. In total, we identified 242 relevant individual NAMs, including 24 NAMs for genotoxicity, 27 for cytotoxicity/ cell viability, 16 for reactivity/ oxidative stress and 8 for inflammation and barrier integrity. In addition, 37 NAMs for physicochemical characterization and 23 for dispersion are includ-

ed in the review. In summary, various nano-specific NAMs could be relatively mature and therefore very promising to be further explored for RA, especially in integrated approaches, along with conventional animal and human data (where existing).

To further advance the regulatory application of NAMs two general recommendations are proposed. Firstly, it is considered important to discuss selected NAMs that are deemed to be mature with regulatory and validation experts. Secondly, such NAMs need to be practically tested in nano-specific RA case studies to better explore their potential along with remaining challenges and uncertainties. Several of the identified issues are currently addressed within the umbrella of the EFSA-funded NAMS4NANO projects.

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OS01-05
Visualization of polystyrene particles in Calu-3 cell cultures by stimulated Raman spectroscopy

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The presence of microplastic and nanoplastic particles (MNPs) in food, drinking water and the environment has raised intense health concerns. Humans are ubiquitously exposed to a diverse class of microplastic compounds via ingestion but also inhalation of particles in air. For the risk assessment of inhaled microplastics, information on acute local toxicity as well as intracellular uptake is required. It is expected that the accumulation of MNPs in cells impairs their biological functions, with largely unknown consequences for human health. Quantifying the uptake of MNPs is necessary to assess the potential hazard to human health. Current research on clarifying this question is hindered by the lack of analytical methods for the detection and quantification of true, label-free MNPs in human cells and other biological matrices. Fluorescence microscopy is currently mainly used for the detection of MNPs in biological matrices, but it only works for colored MNPs and is therefore unsuitable for quantifying the actual particle uptake by humans under real-life conditions. We aimed to develop a label-free method for the visualization of polystyrene particles in cultures of human airway epithelial Calu-3 cells.

Calu-3 cells were cultured on transwell inserts with 0.4 µm pore size and 0.9 cm^2 growth area for 8 days under submerged conditions. The cultures showed good barrier formation, as judged by transepithelial electrical resistance (TEER) values >1000 Ω cm^2 on day 7. On day 8, the cultures were apically treated with 2 µm fluorescent polystyrene (PS) microspheres at 10, 100, 500, 1000 µg/mL. The absence of cytotoxicity was indicated by WST-assay and IL-8 release. Paraformaldehyde-fixed cell monolayers were subjected to scanning electron microscopy (SEM), confocal microscopy, and stimulated Raman spectroscopy (SRS). SRS is a resonantly enhanced process, and its signal is several orders of magnitude higher than that of a spontaneous Raman scattering.

In addition, Calu-3 cells were detached with accutase and the cell suspension was analyzed by flow cytometry to confirm PS particle uptake SEM analyses confirmed the presence of spherical PS particles on the cell surface. Confocal microscopy revealed that PS particles were present on the cell surface and throughout the complete Z-stack, suggesting cellular uptake. Using SRS, the PS signal could be discriminated from the cell signal and the 3D volume distribution of PS particles in the cell compartment could be visualized. Employment of machine learning approaches enabled the determination of particle numbers and relevant particle characteristics, e.g. diameter, surface area, volume, and intensity. Flow cytometric measurements confirmed that nearly 100% of live cells were positive for PS microspheres.

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OS01-06
Human-based New Approach Methodologies for developmental and adult neurotoxicity testing *in vitro*

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Developmental (DNT) and adult neurotoxicity (ANT) testing are both currently performed in rats according to OECD or US-EPA *in vivo* guideline studies. These are enormously resource-intense and not suited for testing large numbers of chemicals and therefore, insufficient to protect human health appropriately. Therefore, regulatory agencies like the European Food Safety Authority (EFSA) and the US-Environmental Protection Agency (EPA) have strongly been promoting the set-up of *in vitro* methods, i.e. the DNT-*in vitro* battery (IVB), that allow faster and more cost-efficient neurotoxicity testing.

As an integral part of the DNT-IVB we set up the Neurosphere Assay consisting of 3D primary human neural progenitor cells (hNPC). This test system covers the neurodevelopmental processes hNPC proliferation, neuronal, astrocyte and oligodendrocyte differentiation and migration as well as neurite outgrowth. In addition, we set up a human cell-based assay for neural network formation (hNNF) comprised of hiPSC-derived glutamatergic excitatory and GABAergic inhibitory neurons, as well as primary human astroglia on 48-well microelectrode arrays (MEAs). For studying ANT, we established a 3D BrainSphere method that allows the analysis of acute effects on neurotransmitter receptors on 96-well MEAs using spike sorting.

Our results show the basic set ups of the neurosphere multi-endpoint evaluations and indicate that these DNT-IVB assays can be used for compound screening and mode-of-action (MoA) analysis. Results can be embedded into AOPs for facilitating regulatory application of DNT-IVB data. For ANT, we initially characterized the BrainSphere method by directly targeting neurotransmitter receptors for glutamate, GABA, dopamine, and serotonin as well as indirectly for acetyl choline by inhibition of acetylcholinesterase with this human multi neurotransmitter receptor (hMNR) assay. A case study indicates the ability of the method to specifically identify ANT MoA.In summary, the described DNT and ANT *in vitro* test methods have been and will contribute to the establishment and acceptance of DNT and ANT *in vitro* testing batteries. By creating Adverse Outcome Pathway (AOP) networks regulatory acceptance will be facilitated.

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OS01-07
European Commission Roadmap towards phasing out animal testing for chemical safety assessments

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The European Commission supported by the European Agencies (ECHA, EFSA and EMA) is working towards a roadmap for replacing animal testing for chemical safety assessments in Europe. This follows a commitment to develop such a roadmap in the Commission's response to the European Citizens' Initiative 'Save cruelty free cosmetics' of July 2023.

The roadmap is foreseen to spell out the critical needs necessary to transit to an animal free system for all chemical safety asssessmens required by EU-legislation. This should include for example the ability to derive toxicological reference values from molecular data as opposed to from adverse effects observed *in vivo*. The roadmap should also highlight areas where methodological developments of animal-free NAMs are still needed to achieve the desired end-goal and it should point out where elements of the current horizontal system may need to be adjusted in order to allow the use of NAM-data (e.g. hazard classes).

European general legislation on chemicals is based on a horizontal generic systems of generating information on chemicals, while putting the burden of proof on industry. This creates a higher demand for universally applicable animal-free NAM solutions. The in-going assumption in the roadmap development is that the current horizontal generic system would be largely maintained, i.e. the identification of hazards through information requirements in the REACH Regulation and classification of substances based on adverse effects, by applying specific criteria agreed at EU and international level (GHS).

At the time of abstract submission, the Commission is organising how to harness the experience and possible solutions of a very varied group of interested stakeholders via specific working groups addressing certain aspects in the roadmap. A presentation coudl highlight the progress achieved towards the roadmap and its critical milestones and actions.

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OS01-08
Assessing the protectiveness and utility of a new approach methodology-based toolbox for systemic toxicity

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For many years, a completely non-animal method that allows systemic toxicity safety assessments to be conducted without generating new animal test data seemed out of reach. In recent years, a combination of scientific advances and the use of human-relevant and exposure-led safety assessment frameworks has challenged this perception. Several different research groups and regulatory authorities are trialling the use of physiologically-based kinetic (PBK) modelling to provide internal exposure estimates which can be compared with points of departure (PoDs) from a variety of *in chemico* and *in vitro* assays to determine if specific exposure scenarios associated with chemical uses are safe. Such approaches are already revolutionizing toxicological safety assessment, and the pace of change will only increase. To manage this transition to animal-free safety assessments responsibly, it is important to ensure that the level of protection offered by a safety assessment based on New Approach Methodologies is at least as high as that provided by a safety assessment based on the traditional animal studies. To this end, we have developed an evaluation strategy to assess both the level of protection and the utility offered by a new approach methodology (NAM)-based toolbox intended to protect our consumers and workers from systemic health effects. The toolbox consists of PBK models to predict internal (consumer or worker) exposure, and bioactivity NAMs designed to give broad biological coverage across many different toxic modes of action. These NAMs include *in vitro* pharmacological profiling to screen for specific protein interactions which can lead to adverse health effects, a panel to screen for general stress-mediated toxicity, and high throughput transcriptomics across the entire human genome, representing a non-targeted approach to detect many possible biological activities. The output of this workflow is a bioactivity:exposure ratio (analogous to a margin of internal exposure), which can be used to inform decision-making. We have tested this approach with 48 chemicals and 94 exposure scenarios and found that for the majority of these (>90%) the NAM-based workflow is protective of human health, enabling us to make animal-free safety decisions for systemic toxicity and preventing unnecessary animal use. We have also identified critical areas for improvement to further increase our confidence in the robustness of the approach.

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OS01-09
Incorporating expert knowledge in the Skin Allergy Risk Assessment (SARA) Model: an integrated approach to testing and assessment (IATA) demonstrated in case studies

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There has been a successful effort to develop non-animal methods (NAMs) for skin allergy, combine these in defined approaches for hazard and categorical potency assessment and implement them in the context of flexible frameworks for Next Generation Risk Assessment (NGRA) (OECD, 2023; Gilmour *et al.*, 2020). The SARA Model (Reynolds *et al.*, 2022), which employed a Bayesian statistical model to define a human-relevant point of departure, the ED_{01}, and the estimated risk associated with a consumer exposure, integrated data from the human repeat insult patch test (HRIPT), local lymph node assay (LLNA), direct peptide reactivity assay (DPRA), KeratinoSens™, h-CLAT, and U-SENS™. Here we share a significant development to the SARA Model which, though an expanded database, addition of further data sources and integration of expert information on chemical reactivity and sensitisation potential, has allowed increased utility of the model as an integrated approach to testing and assessment.

The database was expanded from 81 to 428 chemicals. Additional data types, including historical published human maximisation test and kinetic DPRA were added. Reactivity classifications were defined by expert chemists who reviewed database chemicals and the respective outputs from four *in silico* tools (Toxtree, OECD QSAR Toolbox, TIMES, and DEREK): Non-reactive, Non-reactive (autoxidation possible), Reactive, or Reactive (High Potency Category). These classifications bias estimates of the ED_{01} toward typical values associated with

the assigned class. Low quantiles of these distributions were used to define exposure-based waiving (EBW) thresholds. *In vivo* data for database chemicals were reviewed, and expert-defined sensitiser/non-sensitiser classifications were assigned. A decision model was defined: to conclude low risk, the probability of low risk is >0.95; to conclude high risk, probability of high risk is >0.05; otherwise, the call is inconclusive.Distributions of sensitiser potency for reactivity classes were reflective of published potency ranges. The updated model, using reactivity only, had improved overall classification performance (70%) against benchmark exposures when compared with published EBW thresholds (64%). Given all available data, the updated model correctly classified 83% of the risk benchmarks versus 64% with the previous version. The updated model was applied to the case studies described in Gilmour *et al.* (2022), where it provided substantially higher confidence levels in the same conclusion versus the previous version. For example, using the same data, the previous lactic acid risk metric for a face cream exposure was 0.9, with the updated model this was 0.99 with a probability of non-sensitiser calculated to be 0.91 (91%). This version of the SARA Model is a significant improvement on the earlier version, allowing PoD and risk metrics to be defined whilst ensuring systematic inclusion of expert reactivity classifications in risk decision making.

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OS01-10

Development of a QSAR model for predicting PPARα activation by PFAS based on human *in vitro* data

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Per- and polyfluoroalkyl substances (PFAS) are synthetic chemicals extensively employed across various industries and numerous consumer goods. The group of PFAS contains ca. 10,000 substances and increases continuously. The utilization of many legacy PFAS is declining due to their high persistence and hazardous effects. Novel PFAS are increasingly used for numerous industrial applications, even though currently there is limited or no data available on their toxicity.

Many PFAS have been shown to activate the nuclear receptor PPARα, which plays an essential role in lipid metabolism and is being activated by fatty acids. However, the large number of PFAS makes it impossible to perform hazard characterization on all PFAS based on experimental data. To this aim, leveraging *in silico* models can offer valuable hazard insights and subsequently guide experiment prioritization.

In the present study, an *in vitro* assay was employed to investigate the potential of a list of PFAS to activate PPARα. Subsequently, these data were used to develop a Quantitative Structure-Activity Relationship (QSAR) model, which enables predicting the potential of PPARα activation by PFAS. In total, 34 different PFAS compounds were selected for *in vitro* testing. The list contains a number of perfluoroalkyl sulfonic acids (PFSA) and perfluoroalkyl carboxylic acids (PFCA), including the well-characterized perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), as well as the subgroup of mono- and polyether PFAS, with both linear and branched structures.

A luciferase-based reporter gene assay was performed with the selected PFAS to examine their potential to activate PPARα. Benchmark dose modelling was applied to determine the benchmark dose lower bound (BMDL), upper bound (BMDU) and confidence interval (BMD CI). System-specific parameters, like PFAS concentration, were combined with computational descriptors and used as input in the modelling pipeline. The feature preprocessing step involved scaling followed by feature selection. The complete dataset was split into training and testing datasets, cross-validation was employed for hyperparameter tuning and model selection. Different machine learning algorithms were tested and model performance was evaluated by multiple goodness-of-fit metrics. The best-performing model during cross validation was selected, subsequently its predictive capabilities were evaluated on the test set. State-of-the-art methodologies were employed for deriving feature importance and for defining the applicability domain of the model.

The developed QSAR model facilitated the screening of a larger set of PFAS, which identified a number of PFAS with high potential to activate PPARα. The rigorous definition of the model's applicability domain enhanced confidence in our predictions. Specifically, we applied a selective approach, restricting our predictions to only those PFAS congeners that fall within the defined applicability domain.

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OS01-11

A new device for temperature-controlled e-liquid testing for toxicological assessment

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Electronic cigarettes (e-cigarettes) have gained widespread popularity as an alternative to traditional tobacco smoking, with e-liquids being vaporized during operation.

E-liquids primarily contain nicotine, flavorings, and a base liquid comprised of propylene glycol and/ or vegetable glycerin.

To comply with regulatory standards, E-liquid manufacturers are committed to notify the list of all ingredients contained in e-liquids, the emissions resulting from the use in the e-cigarette as well as the respective toxicological data.

At present, it is not regulated how the emissions from the use of liquids in e-cigarettes have to be generated. Manufacturers currently use non standardized e-cigarette devices for vaporization and analytical testing.

Emission may vary depending on many factors (e.g. type of coil, voltage, resistance). This is especially problematic if substances are present which decompose to more critical compounds at high temperatures which are not reached with the chosen device.

These combustion products will thus not be detected and are therefore not part of the toxicological evaluation.

To address this problem, we developed a new device for standardized e-liquid testing at defined, controllable and reproducible temperature conditions. Test liquids with known composition were vaporized using the device and the resulting emissions were analyzed by GC-MS.

It could be shown that with the developed device, e-liquids can be vaporized under stable and reproducible conditions in a temperature range between 100 and 500°C. Decomposition products of e-liquids containing temperature-sensitive components have been detected by GC-MS showing the suitability of the device for the desired application.

Our intention is to generate emissions for the toxicological assessment of e-liquids being relevant independently from the device used.

With this project, we hope to achieve a better safety for the consumer by detecting and thus allowing to eliminate toxicological relevant substances.

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OS01-12
A computational toolbox supporting the development of Safe and Sustainable by Design chemicals and materials

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The Safe and Sustainable by Design (SSbD) concept has been introduced to integrate the safety and sustainability aspects of chemicals and materials in a holistic way as early as possible, considering their entire life cycle. The SSbD framework addressed by the EC [1] describes a five-step approach to the assessment of the safety and sustainability of a chemical or material, which is linked to the innovation process through the stage gate model [2]. The five-step approach of the framework is organized so that the initial three steps relate to hazard and risk assessment, while the last two steps relate to environmental and socioeconomic sustainability assessment, respectively. The current paper presents the development and testing process of the alpha version of the SSbD toolbox under the auspices of the EU partnership on chemical risk assessment (PARC) project. The PARC toolbox aims to provide an innovative toolbox that facilitates the operationalization of the EC SSbD framework. The testing process of the alpha version of the toolbox was conducted by implementing a detailed case study, both in the early and late innovation stages. The aim of this case study was to evaluate the efficiency and complete potential of the toolbox, as well as identify any inconsistencies in the results obtained from the different tools, in both innovation stages. The case study included the assessment of Bisphenol-A (BPA) in two different applications: the replacement of BPA in polycarbonate bottles and epoxy resin paints, using Bisphenol-AP (BPAP) and Isosorbide as alternatives. The only information considered during the early stages was the structure and the potential application of the chemical. A wide range of tools used including Quantitative structure-activity relationship (QSAR) models (VEGA, OECD QSAR toolbox, Janus, Oncologic, Mistra SafeChem in silico Toolbox, and Danish (Q)SAR database) for Step 1, models such as ECETOC TRA, ProScale, ART, Stoffenmanager, INTEGRA, ConsExpo, SimpleBox, CEM, and Vermeer FCM for Steps 2 and 3, and GaBi LCA for a preliminary/prospective Life Cycle Assessment (LCA) in Step 4. Based on the results, significant differences were observed in the outcomes when applying the models of Steps 2 and 3 in both innovation stages. These variations may arise due to the type and quality of input data (e.g., QSARs) used in both cases. Therefore, the evaluation of the reliability of predictions resulting from QSARs, as well as an uncertainty analysis of the results are of great importance. Moreover, the integration of New Approach Methodologies (NAMs) for hazard predictions during the middle innovation stages is highlighted. Additionally, the data scarcity during early innovation contributes to uncertainty in LCA results. In summary, this work has established a basis for the further development of the SSbD toolbox, aiming at improving its effectiveness and applicability.

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OS02 | Short Orals Session 2

OS02-02
Establishing scientific confidence in physiologically based kinetic (PBK) models for quantitative *in vitro*-to-*in vivo* extrapolations (QIVIVE) in the absence of in vivo kinetic data

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The translation from external exposures to internal concentrations is a crucial step in next generation non-animal risk assessment (NGRA) to obtain a dose metric that can be used for quantitative *in vitro*-to-*in vivo* extrapolations (QIVIVE). Physiologically based kinetic (PBK) models, developed based on *in vitro* and in silico input data, allow for estimating such internal concentrations. However, currently, PBK model simulations are generally considered in a regulatory context only when there are available *in vivo* animal or human kinetic studies to evaluate the models' performance. For many substances, such validation data do not exist, and generating these data is not feasible. Therefore, an alternative approach is required to establish scientific confidence in PBK model predictions. We explored means to establish scientific confidence in PBK model predictions without support of *in vivo* data, using the sunscreen agent, BP-4, as case study. The key steps applied included: 1) defining a core PBK model containing state-of-the-art minimal required *in vitro* and in silico input for liver metabolism, fraction unbound, plasma protein binding, and partition coefficients, 2) running sensitivity and uncertainty analyses, and 3) demonstrating the effect of additional model assumptions, particularly the influence of transporter kinetics, on the estimates of plasma concentrations. Additionally, the use of different PBK software approaches to integrate the input

data to simulate plasma concentrations contributed to obtaining scientific confidence. While the PBK model results obtained could not be evaluated against *in vivo* data, conclusions regarding the protectiveness of the predicted plasma concentrations could be drawn. Sufficient confidence was therefore obtained that the predicted plasma concentrations would yield a conservative safety assessment when applied in an NGRA.

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OS02-03
Using a systems biology approach to construct adverse outcome pathway networks aligned with the FAIR principles

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Adverse Outcome Pathways (AOPs) serve as frameworks connecting molecular initiating events to adverse outcomes through key events (KE), which are essential for understanding the link between chemical exposure and adverse health effects. Current graphical representations of AOPs are being adapted for machine-readability, aligning with FAIR principles (Findability, Accessibility, Interoperability, and Reuse of digital assets). The Systems Biology Graphical Notation (SBGN) elements enhance AOPs with standardized graphical notation, improving visualization and interpretability, as introduced by Mazein and collaborators (2023). In addition, artificial intelligence (AI)-based systematic review, data screening and curation have been performed by van Ertvelde and collaborators (2023) to accelerate the building of large AOP networks. The KE descriptor concept overlaps with the biology enrichment present in Mazein's work, linking biological entities to biological activity (as KE) and ultimately enhancing their mechanistic representation and relevance. This work aims to strengthen the bridge between toxicology and systems biology by proposing a semi-automated workflow for AOP network development.

In this sense, we developed a pilot study on liver steatosis, addressing challenges and opportunities in developing SBGN-based AOP networks and proposing scalable solutions that can be adjusted to meet various demands. This approach integrates different data acquisition methods with automated network construction, followed by manual graphical improvements. Our workflow consists of: a) data checking, annotation and disambiguation; b) automated data processing; c) conversion into a CellDesigner SBML network (Funahashi *et al.*, 2008), using R scripts applying functions from the minervar package (Gawron *et al.*, 2023); d) manual design editing for improved layout and human interpretability. The MINERVA platform (Hoksza *et al.*, 2020) was used for automated annotation, sharing, visualization, and exploration of the network. This approach was applied to extended case-studies with different data acquisition methods (i.e., AI-driven data extraction, literature review and integration of AOPs from AOP-Wiki) highlighting its versatility.

The proposed approach leverages established standards and automated methods to expedite machine-readability and ensure FAIR principles compliance in AOP networks. Utilizing the proposed workflow in constructing AOP networks not only boosts reproducibility and interoperability but also facilitates the development of more accurate and biologically relevant networks. The incorporation of KE descriptors and biology enrichment simultaneously expands mechanistic relevance, improving the overall accuracy and comprehensiveness of the AOP networks.

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OS02-04
Using a machine learning framework to improve the efficiency of mitochondrial toxicity screening by guiding compound selection

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Mitochondrial dysfunction plays a major role in the onset of off-target drug effects, including hepatotoxicity and cardiotoxicity. Early identification of potential mitochondrial toxicants during drug development is essential to prevent these off-target toxicities. Despite the utilisation of established assays for mitochondrial function measurement, these methods usually lack efficiency and intent, reflected in their indiscriminate approach to compound screening selection. Therefore, harnessing machine learning (ML) workflows may provide avenues to improve the screening proficiency of mitochondrial toxicants.Active learning (AL), a ML framework, was employed to guide compound selection and improve screening time to demonstrate the strengths of this hybrid screening approach. An initial screen, conducted on 1520 compounds from the Prestwick Chemical Library using an ATP cell viability assay in metabolically-switched HepG2 cells, revealed over 100 mitochondrial toxins. These results were then used to iteratively train the AL model, demonstrating a 2-fold improvement in identifying true positives and true negatives compared to random selection when only half of the library was used in the training set. Although promising, here AL was employed retrospectively, incentivising incorporation of this technique during real-time screening. An in-house pesticide library was selected to assess the utility of this AL workflow. Each screening

round was guided by AL, enabling the verification of the majority of mitochondrial toxic compounds present in the library, despite only screening half of all compounds – confirmed via screening the entire library post-AL prediction. Therefore, integrating AL-enhanced mitochondrial toxicity screening may reduce screening time whilst preserving true positive identification for drug safety assessment.

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OS02-05
In silico NAMs for nanoforms: how far we are?

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Replacing animal testing with single *in vitro* or *in silico* studies is not feasible. Thus, the evolution of New Generation Risk Assessment (NGRA) methods requires novel approaches that integrate various methodologies, such as Integrated Approaches to Testing and Assessment (IATAs) and Adverse Outcome Pathways (AOPs). Efficient testing strategies should include using *in silico* NAMs, whenever possible, to reduce the time and cost of the experimental work. The reports released in 2023 by EUON/ECHA [1,2] and the recently launched *NAMs Network* database [3] show that over 200 different *in silico* NAMs have been developed for nanomaterials. However, how many of them are relevant for the regulatory purpose? Moreover, how many of them are of enough quality, and how should this "quality" be defined? In the context of *in silico* nano-NAMs, defining "minimum quality criteria" for evaluating the applicability of computational tools and models in a regulatory setting is essential. This brings us to another question: Does the OECD QSAR Assessment Framework, published last year, adequately evaluate predictive nano-related models, such as nano-QSARs? Are there any additional considerations to be made for the specificity of nanostructures [5,6]?

The lecture will attempt to answer these challenging questions and prompt discourse on the applicability of currently available and newly developed computational NAMs for regulatory risk assessment of substance nanoforms.

The discussion will be exemplified through three case studies: (i) a new model for predicting water solubility of nanoforms based on criteria outlined in the recently published IATA, which supports grouping and read-across of nanomaterials in aquatic systems [7]; (ii) a set of models for predicting genotoxicity (through comet and micronucleus tests) of metal oxides nanoparticles in line with the EFSA guidance [8] that have been implemented into the new *nQTb* software [9]; and (iii) novel models for predicting points of departure in AOP173 [10] that enable grouping of multiwalled carbon nanotubes based on their potential for inducing lung fibrosis.

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OS02-06
Leveraging *in silico* binding affinity, *in vitro* bioactivity, and chemical structure to develop machine learning models for predicting *in vivo* toxicity

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Traditional toxicity testing methods, particularly OECD test guidelines, are time-consuming and costly due to their complexity. Hence, machine learning emerges as a promising alternative for evaluating the toxicity of chemicals [1]. In the field of toxicology, quantitative structure-activity relationship (QSAR) approaches are widely employed for toxicity prediction models [2]. These models operate under the assumption that chemicals with similar structures will exhibit similar toxicity profiles. Despite the prevalence of QSAR-based models, many toxicity prediction models exhibit suboptimal performance. Therefore, there is a pressing need for more efficient methods to enhance model performance. In response to this challenge, we propose integrating mechanistic data into model development, rather than relying solely on chemical structure as a data feature. We represent chemicals based on their binding affinity with target receptors, bioactivity in ToxCast assays, and chemical structure descriptors. Under the assumption that model performance will improve when descriptors associated with the target endpoint are used, we also utilized a set of ToxCast bioactivities correlated to *in vivo* toxicity from the ToxRef DB in our previous study. Subsequently, we employ supervised machine learning techniques to predict *in vivo* toxicity. We train classifiers using six machine learning algorithms (MLP, GBT, Random Forest, kNN, Logistic Regression, Naïve Bayes), incorporating various combinations of MACCS, in silico binding affinity descriptors, ToxCast bioactivity descriptors, and hybrid descriptors. We evaluate the predictive performance of our classifiers using 5-fold cross-validation testing and in-loop validation. Our analysis reveals that the hybrid classifier yields the best F1 score for predicting toxicity. Additionally, employing feature selection methods allows us to identify the most important features, providing valuable insights into potential toxicity mechanisms underlying the toxicity. Overall, our findings underscore the utility of in silico molecular docking analysis and high-throughput assays for characterizing rodent toxicity, as well as the benefits of using hybrid representations that integrate bioactivity and chemical structure.

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OS02-07
Virtual cornea: a computational approach for predicting corneal injury and recovery from chemical exposures

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Introduction: Accurately predicting corneal injury and recovery following chemical exposures is crucial for risk assessment and regulatory decision-making. The dynamic and spatial nature of corneal wound healing following injury lends itself to mechanistic multiscale, multicellular computer simulation, known as Virtual Tissue (VT) modeling. We present progress being made in Virtual Cornea, an agent-based model that aims to simulate the cellular interactions and processes underlying corneal homeostasis, injury, and recovery, offering a new approach methodology for risk prediction. Chemical or physical injury to the cornea invokes complex autocrine or paracrine interactions relating biophysical, electrophysiological, and physiological cues which can be difficult to recapitulate *in vitro* or extrapolate from data-based Machine Learning or molecular-level computer simulations. Our ability to predict eye irritation is dependent on the time of recovery from Draize rabbit eye test data, which has limitations in predicting human responses accurately due to variability, subjectivity, and anatomical differences between rabbit and human eyes. Virtual Cornea aims to address these challenges by providing a mechanistic understanding of the processes underlying corneal injury and recovery.

Methods: Virtual Cornea is a two-dimensional agent-based model developed in CompuCell3D and Tissue Forge. It represents the cornea's layers, including tear film, epithelium and its basement membrane, stroma with keratocytes, fibroblasts, myofibroblasts, and extracellular matrix. The model simulates cell behaviors such as proliferation, differentiation, migration, and signaling molecule secretion in response to cell-cell and cell-matrix interactions, aiming to accurately model the corneal recovery process.

Results: Virtual Cornea has successfully reproduced the organization of the corneal epithelium and stroma under homeostatic conditions and during recovery from mild to moderate injuries. The model incorporates detailed simulations of basement membrane regeneration, collagen organization, and chemical damage modes, utilizing computational toxicology approaches. Notably, it has achieved homeostasis restoration within 21 days after moderate injuries and accurately replicated instances of ectopic fibrosis and fibroblast accumulation in severe damage scenarios, demonstrating its potential to predict injury severity and recovery outcomes.

Conclusion: Virtual Cornea offers a promising new approach for predicting corneal injury and recovery, providing predictive insights into injury depth, recovery patterns, and the risk of persistent opacities. This computational model represents a significant advancement in chemical risk assessment and regulatory evaluation, with the potential to refine predictions of injury severity and recovery timelines by integrating further data and biological knowledge.

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OS02-08
Small RNA-sequencing based discovery of microRNAs for use as non-invasive biomarkers of CNS-injury

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As neurotoxicity poses substantial risks across pharmaceuticals and environmental exposures, the search for novel biomarkers is paramount for enhancing safety evaluations, drug development, and environmental monitoring. MicroRNAs (miRNAs) hold great promise as biomarkers due to their disease relevance, cell-type specificity, and extracellular presence in biofluids where they are stabilized by incorporation into protein complexes or extracellular vesicles. The Innovative Medicines Initiative TransBioLine (TBL) consortium aims to explore the pivotal role that miRNA biomarkers may play in advancing our understanding and management of CNS toxicity, driving transformative progress in neuroscience and public health.

We hypothesized that miRNAs enriched in the CNS and consecutively in cerebrospinal fluid (CSF) may transfer into peripheral blood during CNS injury. Therefore, we screened and compared miRNA levels between CSF and serum. Matched CSF and serum samples were obtained from 27 patients with multiple sclerosis (MS) and 13 patients with no confirmed CNS disorder (NCD). A small RNA-sequencing-based assay for absolute quantification of miRNA biomarkers in biofluids was used to analyze all 80 samples and identified >1000 miRNAs. Of these, 25 miRNAs showed consistently higher concentrations per microliter in CSF than serum (EdgeR, FDR<0.05). CSF enrichment was consistent between MS and NCD patients.

To confirm these findings, we developed an RT-qPCR assay that enabled cross-species (human and rat) detection of the 7 miRNAs with the highest CSF enrichment, including miR-124-3p and miR-9-5p. The RT-qPCR assay performed comparable to the NGS assay when applied to a subset of samples previously analyzed by NGS.

To explore the utility of the identified CSF-enriched miRNAs as biomarkers for neurotoxicity, serum samples collected from rats exposed to known toxicants with histologically confirmed damage to CNS and/or PNS tissues were analyzed, along with their respective control groups (n=4 per group). Cq-values from RT-qPCR were standardized to an internal spike-in control and non-parametric analysis (Kruskal Wallis H, $p<0.05$) was performed. Serum miR-124-3p levels increased significantly on day 2 (>10-fold) and day 3 (>100-fold) after treatment with 750 mg/kg 2-chloropropionic acid (CPA) as well as on day 15 after treatment with 8 mg/kg Doxorubicin (5-fold). miR-9-5p serum levels increased significantly after CPA (day 3, 30-fold), 10 mg/kg kainic acid (day 2, 5-fold), and Doxorubicin (day 15, 2-fold) treatment. In conclusion, small RNA-sequencing of paired CSF and serum samples enabled the identification of CSF-enriched miRNAs, which can be robustly detected in serum by RT-qPCR and represent potential candidates for further evaluation as neurotoxicity biomarkers.

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OS02-09
Spatial transcriptomics reveals proximal tubular ER stress in alcohol-induced renal toxicity

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Alcohol overconsumption negatively impacts numerous organs besides the liver, including the heart, brain, and pancreas. A severely understudied consequence of alcohol-induced end-organ damage is kidney injury. Indeed, acute kidney injury (AKI) is commonly observed in patients with end-stage alcohol-associated liver disease (ALD). To reveal key mechanisms of alcohol-induced renal injury, we employed a well-defined Lieber-DeCarli model of chronic alcohol consumption and carbon tetrachloride (CCl4) treatment. This paradigm resulted in the elevation of multiple kidney damage markers when compared to alcohol consumption alone, validating our findings with previously published work. Importantly, significantly elevated Kim-1 was identified in the urine of mice consuming alcohol with CCl4 treatment in as early as 3 weeks of exposure. A cutting-edge tissue spatial transcriptomics analysis was utilized to identify specific gene expression alterations across kidney morphology. Using a publicly available scRNA-seq dataset, gene lists for different renal cell types were aligned to identify mRNA variances across specific cell types. Our analysis also enabled the generation of spatially resolved gene cluster analysis. The combination of alcohol and carbon tetrachloride yielded a significant reduction in gene expression related to proximal tubule cells and an increase in regions with a non-signature for a specific renal cell type. A decrease in the expression of genes involved in alcohol metabolism (Adh1, Cyp2e1, Catalase, Aldh2) was localized to the renal cortex. Only alcohol and CCl4 exposure combined resulted in increased expression of renal damage marker Kim-1 in the cortex. Expression of the AKI biomarker Lcn2, macrophage marker Cd68, and fibrosis marker Timp1 were increased across the kidney. Interestingly, the Unfolded Protein Response was induced across the proximal tubules and was morphologically associated with gradients of alcohol metabolism. Here, key genes including HSPA5, HSPA8, PDIA6, and numerous RPL/RPS were directly altered due to alcohol metabolism across renal proximal tubules. Furthermore, epigenetic alterations in regulatory marks such as H3K9ac, H3K14ac, H4K12ac, H4K16ac, and H4K20me3 were found associated with alcohol metabolism. Collectively, the application of tissue spatial transcriptomics to a well-defined model of alcohol toxicity reveals dynamic morphologic changes in gene expression across specific renal cell types due to alcohol alone and alcohol with CCl4 exposure. Further analysis is ongoing to relate these alcohol-induced renal gene expression changes to the clinic through the utilization of immunohistochemical and proteomic techniques to define novel epigenetic alterations across renal cell subtypes.

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OS02-10
Enhancing toxicological insights through multi-omics: a case study on direct and indirect thyroid toxicity

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Integrating multi-omics data is a comprehensive method for understanding cellular or organismal responses to chemical exposure. In Canzler *et al.* (2020) [1], we described best practices for conducting multi-omics studies. Here, we present a multi-omics investigation following these best practices integrating clinical, histopathological, and six layers of omics data (long and short transcriptomics, proteomics, tissue and plasma metabolomics, and phosphoproteomics). Utilizing the well-studied compounds Phenytoin and Propylthiouracil (PTU) in a rat toxicity study over 28 days with an additional 14-day recovery period, we aimed to explore mechanisms of direct and indirect thyroid toxicity.

Our findings demonstrate that multi-omics approaches significantly surpass single-omics analyses in identifying regulatory pathways and molecular effects relevant to toxicology. For instance, the multi-modal data elucidated complex responses to PTU and Phenytoin, both at the transcript and protein levels and in metabolomic shifts, offering insights into the perturbations of thyroid hormone biosynthesis and liver metabolic pathways, respectively. Also, we found the combined interpretation of omics-, clinical, and histopathological parameters particularly beneficial. Importantly, the simultaneous data integration reveals intricate interplays between different omics layers, highlighting how individual and combined data layers uniquely contribute to understanding toxicological outcomes. Furthermore, grouping approaches focusing on common molecular effects benefit substantially from multiple omics layers.

This study emphasizes the superiority of multi-omics in detecting molecular responses to toxicants, deepening our understanding of mechanisms of action and enhancing the predictive capabilities of toxicological assessments. Our work emphasizes the value of integrated multi-omics strategies in advancing the field of toxicology towards more holistic and mechanistically informative evaluations, potentially informing regulatory decision-making and risk assessment processes.

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OS02-11
Metabolic disturbances linked to metabolic disorders in *in vitro* and *in vivo* models exposed to PFOA: an integrated multi-omics analysis

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Per- and polyfluoroalkyl substances (PFASs), notably perfluorooctanoic acid (PFOA), are environmentally persistent and bioaccumulative, found in numerous consumer products. Research has highlighted their role in multi-organ toxicity and the dysregulation of metabolic processes, particularly affecting lipid pathways, raising serious health concerns regarding their link to metabolic disorders progression. This study compiles the multiomics analysis outcomes following the exposure of *in vitro* and *in vivo* models to environmentally relevant concentrations of PFOA. Hepoid-HepaRG cells underwent a 14-day treatment with 100pM, 100nM, and 10µM PFOA, while EndoC-βH1 cells were exposed to 1, 10, and 100 nM PFOA for 72 hours, following established culturing procedures previously described by Ravassard *et al.* (doi: 10.1172/JCI58447). Additionally, 5-day post-fertilization Danio rerio embryos, each weighing approximately 0.025 grams, were exposed to 10µM PFOA. Differentially expressed genes (DEGs) were identified using Agilent microarrays, while differentially expressed metabolites (DEMs) were through untargeted metabolomics using Reversed Phase (RP) and Hydrophilic Interaction (HILIC) Liquid Chromatography in positive and negative ionization modes. Data pre-processing, cleaning, and statistical analyses were performed using limma R-package for transcriptomics, xcms, IPO, CAMERA, and xMSannotator for metabolomics, and MetaboAnalystR for joint pathway analysis. Exposure of HepaRG cells to PFOA at concentrations of 100pM, 100nM, and 10µM resulted in the identification of 121, 19, and 20 DEGs, respectively, alongside 49, 44, and 40 DEMs. A joint pathway analysis identified 42, 25, and 17 disrupted metabolic pathways for each exposure level, with glycerophospholipid metabolism consistently affected across all conditions (p-value$<$0.05). Similarly, exposing EndoC-βH1 cells to 1nM, 10nM, and 100nM PFOA led to the detection of 22, 157, and 382 DEGs, and 83, 120, and 128 DEMs, respectively. This analysis highlighted 24, 40, and 58 affected metabolic pathways, with 23 common pathways including glycerophospholipid metabolism across all exposure levels. Moreover, *in vivo* multi-omics study confirmed the significant perturbation of glycerophospholipid metabolism (p-value$=$0.021) due to altered levels of phosphatidylethanolamine, phosphatidylcholine, and 1-Acyl-sn-glycero-3-phosphocholine. Such disturbances in glycerophospholipid metabolism are linked to metabolic disorders by impacting insulin signalling, mitochondrial functionality, and inducing systemic inflammation. In conclusion, this multi-omics study utilizing both *in vitro* and *in vivo* models offers mechanistic insights into the disruption of glycerophospholipid metabolism following exposure to PFOA. These findings have significant implications for the development of adverse outcome pathways (AOPs), enhancing our understanding of PFOA's health impacts.

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OS02-12
Contextualising *in vitro* NAMs on a network of AOPs to improve immunotoxicity safety assessments

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Perturbations of immune system function and immunotoxicity can have significant and varied toxicological consequences, so it is important that the risks posed by these interactions are considered in safety assessments. However, the immune system is complex, and it can prove challenging to define and effectively test perturbations using traditional approaches. To aid in these assessments, the OECD have published a detailed review paper (Series on Testing and Assessment No.360) outlining a tiered approach to ensure correct assessment of potential immunotoxic effects of compounds. The first two tiers of this system define the cytotoxic effects on the immune system, myelotoxicity or leukotoxicity, whilst the final tier aims to elucidate mechanisms of concern that occur at cytotoxic doses. Whilst this approach provides some structure to immunotoxicity assessment, multiple tests will be required for an evaluation where assay choices will be key. Assessors will also be required to efficiently evaluate results to determine data coverage and required next steps, in order to present findings to regulatory authorities in a framework that can be understood, and mutual agreement can be reached.

Adverse outcome pathways (AOPs) present an ideal framework for the organisation, contextualisation, and rationalisation of evidence from these NAMs, alongside other knowledge and evidence to ensure appropriate assessment. A preliminary network of AOPs relating to immunotoxicity has previously been developed and encoded into software allowing for future expansion. In this work all of the assays listed within the OECD detailed review paper were associated with this network, using literature searches each assay was associated with the appropriate key events (KEs) and grouped into their respective tier.

Through the inclusion of the assays proposed within this tiered approach, alongside other assays and expert rule-based structural alerts already associated with the AOP network, we aim to aid in the contextualisation of data generated to make more confident decisions. The combination of the OECD defined tiered approach and an AOP framework, can aid in the expert review of weight-of-evidence (WoE) approaches to immunotoxicity assessments, such as those required as part of an ICH S1B addendum. An AOP framework can be used to identify where effective areas for mechanistic testing should be performed, as well as allowing for the identification of areas that may be beneficial for additional testing to increase confidence in assessments.

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OS03 | Oral Session 3

OS03-01
Utilization of human cardiac microtissues for 3D modelling of myocardial hypertrophy

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Translation of safety and efficacy findings from healthy to a diseased state remains a challenge in drug discovery. *In vitro* modelling of cardiovascular disease and drug-induced cardiotoxicity utilizing 3D multi-cellular models shows promise in studying the intra- and inter-cellular signalling perturbations and their influence on cardiac phenotype. In this context, patient-centric *in vitro* 3D models which can recapitulate the molecular and phenotypical hallmarks of pathological hypertrophy allow identification of targets and drugs that may reduce hypertrophy and are useful to understand drug-induced perturbations that may lead to pathological hypertrophy induction or exacerbation. Cardiovascular comorbidities are prevalent within patient populations and are not incorporated into early safety assessments, however, an earlier assessment and understanding of the molecular mechanisms of pathophysiological changes and the responsible targets may reduce drug attrition at later clinical stages. Therefore, this study aimed to develop a 3D human cardiac microtissue (huCMT) model of cardiac hypertrophy. In order to establish such a model, huCMTs containing human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs), primary human ventricular cardiac fibroblasts (NHCF-Vs) and primary human cardiac microvascular endothelial cells (HMVEC-Cs) were formed at a cell ratio of 4:2:1. To induce hypertrophy the huCMTs were exposed for 5 days to a range of concentrations of known hypertrophy inducers including endothelin-1 (ET-1) and isoproterenol (ISO). The stimulated huCMTS were assessed by real-time qPCR for the expression of hypertrophy and cardiac cell-type specific markers, including NPPB, NPPA, SPP1, COL1A, VIM, MKI67, PECAM1, and TNNT2. Cellular supernatants were analyzed to determine the levels of NT-proBNP protein by MSD (Meso Scale Discovery) ELISA. In addition, the size and appearance of the huCMTs were characterized by bright-field microscopy. The results showed that after 5 days ET-1 induced the over-expression of NPPA, NPPB, SPP1, COL1A1 and VIM (1.7-fold, 2.3-fold, 6.5-fold, 2.8-fold and 3.2-fold, respectively) in treated huCMTs compared to vehicle control. Minimal changes were observed for TNNT2 and PECAM1 (0,77-fold and 0.79-fold) that are markers for hiPSC-CMs and HMVEC-Cs, respectively. ISO treatment did not result in changes in gene expression compared to vehicle controls. Both ET-1 and ISO treatment increased the production and secretion of NT-proBNP protein. No changes in the size of huCMTs were observed with either ET-1 or ISO stimulation. In conclusion, the present study shows that the treatment of huCMTs with known hypertophy inducers determined the up-regulation of known and relevant molecular markers (mRNA and protein level) of cardiac hypertrophy. Next steps involve further characterization of the model with tool compounds known to induce pathological hypertrophy in the presence and absence of prior treatment with ET-1.

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OS03-02
To Accept or Not To Accept a Read-Across Adaptation: A Systematic Analysis of 15 Years of Testing Proposal Decisions by the European Chemicals Agency

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An important element of the EU REACH regulation is the assessment by ECHA of testing proposals submitted by registrants in their dossiers. From 2008 to 2023, Testing Proposals have been submitted to ECHA on 2,630 substances of which 1,538 had a decision published. Testing Proposals may contain adaptations to standard data requirements such as the use of read-across to an analogue. Where an adaptation has been proposed, a rationale must be provided, and this is assessed as part of ECHAs evaluation. Read-across is a common adaptation proposed, and previous studies showed that it is often rejected. In this study, we systematically evaluated all publicly accessible Testing Proposals as of August 11, 2023. We extracted information on what test(s) was missing, whether read-across or other adaptations were proposed, and whether ECHA accepted or rejected proposed read-across. Information on read-across hypotheses was standardized into 17 assessment elements; each submission was classified as to which elements were used in submission or decision. Data were analyzed for patterns and associations to determine if any trends (over time, available data types, guideline test(s) in question, etc.) were discernable. Of 1,538 Testing Proposals, 353 (23%) had adaptations; analogue (167) or group (137) read-across were most common. Among submissions containing read-across, 64 analogue (38%) and 85 group (62%) hypotheses were accepted. Similarity (or lack thereof) in structure/physico-chemical properties was the most common assessment element for supporting read-across invoked by either registrants or ECHA. Toxicokinetic considerations and availability of other toxicological data (bridging studies) were considered by ECHA to be the most informative for both accepted and rejected read-across decisions. Overall, this analysis provides an unbiased overview of 15 years of experience with read-across adaptations by both registrants and ECHA and should inform future submissions based on the outcomes of past evaluations.

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OS03-03
Plasticheal risk evaluation and categorization framework (PlasticRiskCat)

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A lot of studies have been published on the environmental, health and safety concerns of micro- and nanoplastics (MNPs) in recent years and it has become increasingly difficult to interpret the reported findings. We have developed a systematic evaluation and categorization tool

called PlasticRiskCat that can support researchers, companies and regulators in their first-tier assessment and communication on what they know about the hazards of 1) pristine MNPs of different sizes and shapes, 2) different commercially used MNPs and their additives or 3) unintentionally produced MNPs and their hazardous contaminant. PlasticRiskCat has been developed as part of the EU funded project PLASTICHEAL that aims at developing innovative tools to study the impact and mode of action of MNPs on human health. The final outcome of PlastRiskCat is communicated in the form of a radar plot. Each line on the radar plot presents a given intentionally produced or unintentionally produced MNPs and can furthermore also represent a given MNP size and shape, known additives or hazardous contaminant depending on the preferences of the users of PlasticRiskCat. The category labels of the radar plot include carcinogenicity, mutagenicity, reproductive toxicity (CMR), cardiovascular effects, *in vivo* effects, immunotoxicity, respiratory effect, gastrointestinal effects, oxidative stress, and cytotoxicity. The axis of the radar plot illustrates the strength of evidence ranging from "Very strong", "Strong", "Moderate", "Weak" to "Very weak" following the guidance provided by the European Environment Agency. In this paper, we first introduce the criteria used to evaluate the MNP hazards and assign these in the PlasticRiskCat radar plot and second, we present the results of applying PlastRiskCat to five different MNPs that have been studied extensively by PlasticHeal, namely e.g., 50, 200 and 500 nm polystyrene (PS), 200–600 nm polylactic acid (PLA), 150–300 nm PLA, 2–6 µm PLA, 2–6 µm Polyethylene Terephthalate (PET) and <500 nm NANOPET. Overall, we find that there is moderate strength of evidence for PS when it comes to CMR, especially based on *in vitro* studies. We furthermore find that there is strong evidence of reproductive toxicity *in vivo* for PS and moderate evidence for PET *in vivo*.

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OS03-04

Integration of New Approach Methodologies (NAMs) data into hazard assessment of chemicals: a case study on three Azole Fungicides- Tebuconazole, Propiconazole, and Cyproconazole regulated under K-BPR

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New Approach Methodologies (NAMs) encompass various non-animal testing methods such as in silico, in chemico, and *in vitro* approaches. However, due to concerns about their reliability, regulatory decisions often don't heavily consider NAMs data. This study aims to explore how NAMs can contribute to the hazard assessment of fungicides chemicals, which are being evaluated under the Consumer Chemical Products and Biocides Safety Act (K-BPR). We systematically analyzed ToxCast bioactivity and literature on the toxicity of three azole fungicides – tebuconazole, propiconazole, and cyproconazole – focusing on human toxicity endpoints regulated by K-BPR. Comparing the hazard assessment results from the EU-BPR report (where three azole fungicides were previously evaluated) with NAMs data collected from literature and the ToxCast database revealed several discrepancies. While the EU-BPR deemed three azole fungicides non-carcinogenic and non-genotoxic, several *in vitro* studies showed positive DNA damage. Additionally, developmental toxicity concerns identified by the EU-BPR were confirmed by positive cytotoxicity in human placental cells. Notably, despite no observed neurotoxicity in rat studies, positive cytotoxicity emerged in human neuroblastoma cells. In conclusion, this case study demonstrates the value of leveraging NAMs data to prevent the underestimation of chemical hazard assessments and offers a perspective on accelerating the use of NAMs data in chemical risk assessment.

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OS03-05

Exploring the impact of inhaled (nano)particles on the blood-brain barrier (BBB) through interdisciplinary investigation

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The increasing prevalence of human-produced (nano)particles in our environment has become a pressing concern in recent years, with mounting epidemiological evidence linking air pollution to a range of human neurological diseases including Alzheimer's disease, Parkinson's disease etc. It is postulated that inhaled (nano)particles could have negative effects on the brain either directly, by entering the brain via the olfactory nerve or through the blood circulation, or indirectly, i.e. that chemical messengers released in the lung when exposed to air pollution particles then reach the brain and induce a range of deleterious effects including promoting oxidative stress, neuro-inflammation etc. This study is employing an interdisciplinary approach, integrating cellular models, advanced analytical techniques, and state-of-the-art facilities to gain a deeper insight into how inhaled (nano)particles could affect the blood-brain barrier (BBB).

By utilizing ambient particulate matter (PM) and samples from specific sources such as diesel exhaust particles (DEPs), the study exposed a BBB cellular model composed of brain endothelial cells to PM. The interaction between particles and the BBB were investigated using the Zeiss Crossbeam 550 focused ion beam scanning electron microscope (FIB-SEM) in conjunction with Confocal microscopy (Central Laser Facility). Unlike a typical scanning electron microscope (SEM), which uses a single electron beam, the FIB-SEM incorporates a second beam, the ion-beam, for material cutting while the SEM performs high-resolution imaging. This combination allows for high-resolution (10–20 nm), material-sensitive ultrastructural 3D imaging of cellular volumes. Initial observations indicate that FIB-SEM effectively visualises the electron-dense carbon-based particles in biological samples, enabling direct observation of nano-sized particles derived from PM. Images collected show that particles (individuals/clusters) adhere to the plasma membrane or are taken up by cells inside lysosome-liked vesicles, particularly the nano-sized particles.

To further probe the interactions between (nano)particles and plasma membrane lipids, quasi-elastic neutron scattering (QENS) experiments were conducted at the OSIRIS spectrometer (ISIS Neutron and Muon Source). QENS was employed to measure the scattering signal arising from the dynamics of intra-cellular water, which serves as an indicator of lipid membrane behaviour. Prior to exposure to the neutron beam, cell samples were rinsed with deuterated PBS to eliminate any signal from extracellular water. Initial findings suggest that QENS

experiments offer a promising approach for examining how the dynamics of lipid membranes are impacted by particle entry and traversal of barriers in heterogeneous biological models, such as the BBB.

Further analysis is ongoing, and the results will be presented in the conference.

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OS03-06
Aggregated exposure to perfluorooctane sulfonic acid coupled with PBPK model and parameter optimization using a human study for validation

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"Forever Chemicals", per- and polyfluoroalkyl substances (PFAS), are used in a wide range of consumer products and have been detected in air, water, soils, sediments, and in rain. Several PFASs, including Perfluorooctane sulfonic acid (PFOS), have been characterized as *p*ersistent, *b*io accumulative and *t*oxic (PBT) and can cause various adverse health effects. [1] PFOS is also ubiquitous and the widespread exposure is raising public health concerns. Various sources contribute to the blood levels of PFOS in the general population, with the food as the main contributor. PFOS is also found in personal care products (PCP), but the contribution to the internal exposure is not known. An external probabilistic exposure assessment was performed using data on food consumption and PCP use from the EuroMix human biomonitoring study (HBM) conducted in Norway [2], combined with PFOS concentration data in food and PCP from literature. The probabilistic external individual exposure estimates were used as input values to a physiologically based pharmacokinetic (PBPK) model to predict internal aggregated exposure. A PBPK model by Loccisano in 2011 [3] was further refined by incorporating the dermal exposure pathway, removing the fat compartment and some changes in parameters related to the kidney and faecal excretion. The model parameters were selected from human studies. There is high uncertainty and variability in human parameters data, especially in different human subpopulations with different demographic, exposure, and toxicokinetic properties. The model parameters and their uncertainty were assessed using Bayesian analysis with Markov chain Monte Carlo (MCMC) simulation to improve the model reliability, while the model's performance was validated with the human study. [4] The aggregated internal exposure estimated by using the PBPK model for the EuroMix population, shows that the main contribution for PFOS is the diet and that PCP have only small contribution, but the estimates are uncertain. The internal exposure estimates from the PBPK model were compared with measured serum concentrations from the HBM study. The internal exposure estimated of PFOS by using the lower bound (LB) external exposure estimates, was in the same range as the measured serum concentrations. Additional studies on exposure to PFOS and possibly other PFAS from PCP would be of importance. The improved PBPK model with optimized parameters can advance knowledge on PFOS exposure and risk assessments.

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OS03-07
Epigenetic transgenerational effects of environmental pollution: assessing the impact of the herbicide linuron on *Xenopus tropicalis* Frogs

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Unsustainable human activities are driving the ongoing decline in biodiversity. Amphibians are particularly affected; almost half of the species are threatened with extinction. Environmental pollution plays a significant role among the causes of the decline in amphibian populations. Water and soil contamination with pesticides are common in areas with intensive agricultural activity, which often include amphibians' habitats. One such pesticide, linuron, has been found in water sources near agricultural areas at concentrations up to 100 µg/L. We have recently shown that linuron can cause endocrine disrupting effects in *Xenopus tropicalis* frogs, and that these effects can be transmitted across generations, even to offspring that were never exposed to the contaminant. However, the mechanisms underlying these transgenerational effects require further investigation. Here, we conducted a study examining the transgenerational effects of linuron on DNA methylation patterns in the brain, testis and pancreas of *X. tropicalis*. Tadpoles were exposed to an environmentally relevant concentration of linuron during development (45 µg/L) until metamorphosis, and adult males were then mated with naïve females to obtain the F1 generation frogs. Adult males from linuron lineage F1 were mated with control F1 females to obtain F2 generation and follow the paternally inherited transgenerational effects. Reduced representation bisulfite sequencing (RRBS) was used to assess DNA methylation patterns of the adult male F2 generation. Our analysis identified multiple differentially methylated regions (DMRs) in the brain (3060 DMRs), testis (2551 DMRs) and pancreas (1117 DMRs). Gene sets were over-represented in several pathways, including pathways related to synaptic plasticity in the brain, epigenetic regulation in the testis and calcium signalling in the pancreas. Brain DMRs were present in key genes involved in somatotropic (*igfbp4*) and thyrotropic signaling (*dio1* and *tg*). The levels of methylation in those regions correlated with alterations in body size, weight, hind limb length and plasma glucose levels. In the testis, DMRs

were present in essential genes for spermatogenesis, meiosis and germ cell development (*piwil1*, *spo11* and *tdrd9*) and their methylation levels correlated with the number of germ cells nests per seminiferous tubule, an endpoint of disrupted spermatogenesis. In the pancreas, DMRs were present in genes that are important for pancreatic function, including the lipase *pnliprp2*, and the genes linked to type 2 diabetes *tcf7l2* and *adcy5*, suggesting a potential disruption of normal pancreas functioning. The results suggest that the DNA methylation changes could be potential mediators of the transgenerational effects of linuron. Our study can help to better understand the link between environmental pollution and amphibian decline, and also indicate potential implications for human health, given the similarity between *X. tropicalis* and human genomes.

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OS03-08
Comprehensive chemical and toxicological screening of e-waste plastic chemicals

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This study presents a comprehensive chemical and toxicological screening of chemicals extracted from WEEE (waste from electrical and electronic equipment) plastics. Chemical identification was conducted through suspect and target screening methods, revealing a diverse array of hazardous compounds including polycyclic aromatic compounds (PACs), organophosphate flame retardants (OPFRs), phthalates, benzotriazoles, and others. Toxicological endpoints included cell morphological phenotypes, inflammatory response, aryl hydrocarbon receptor (AhR) activation, activation of estrogenic receptor, and anti-androgenic activity. Results demonstrated that WEEE plastic chemicals significantly altered cell morphological phenotypes, particularly affecting the cytoskeleton, endoplasmic reticulum (ER), and mitochondrial measures. Moreover, WEEE chemicals induced inflammatory responses in resting human macrophages and altered ongoing inflammatory responses in lipopolysaccharide (LPS)-primed macrophages. Furthermore, WEEE chemicals exhibited potent AhR agonistic activity, activated estrogen receptor-α (ERα), and inhibited androgen receptor (AR) activation. The findings suggest that WEEE plastic chemicals exert their effects through multiple modes of action, targeting various subcellular sites. Thus, a combined approach utilizing non-target and target screening tools is essential for comprehensively assessing the toxic effects and health hazards associated with WEEE plastic chemicals.

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OS03-09
When a model gives you trouble: handling hazard assessments for thousands of predicted (bio)degradation products

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A case study is presented in which the (environmental) degradation products of a chemical compound were predicted using a rule-based computer model. The goal of this modeling exercise was to obtain a list of potential (bio)degradation products (BDPs) that may form in the environment in case of emissions of the parent compound. The persistency, bioaccumulation and toxicity (PBT) profile of the predicted BDPs would then be examined and compared with the PBT profile of the parent compound. However, due to the complex structure of the parent compound and the conservative nature of the computer model, several thousand potential BDPs were predicted, rendering an individual hazard assessment for each product practically infeasible.

The structures of the predicted BDPs were analysed, whereby the structural similarities were assessed in terms of functional groups as well as based on different structural similarity scores. In addition, other chemical characteristics such as molecular weight and the octanol-water partition coefficient were explored for potential use as part of a more general grouping approach within the context of BDPs. The results of this were further analysed to assess the extent to which the BDPs could be grouped to allow for a more pragmatic assessment of their PBT profile.The discussion of this study will focus on the use of the different grouping approaches and their value when assessing the hazard profiles of a multitude of (potential) degradation products of single and/or more complex (e.g., multi-constituent or UVCB substances) substances, as well as on the extent that a more hazardous profile than that of the parent compound may be expected.

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OS03-10
Early biomarkers of cancer susceptibility in benzene-exposed workers: integrated analysis of gene expression, microRNA alterations, and DNA methylation datasets

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Benzene, an airborne pollutant found in gasoline combustion byproducts, industrial emissions, and tobacco smoke, poses a significant occupational hazard. Industries such as petroleum, coke production, shoemaking, painting, rubber, and plastic manufacturing continue to utilize benzene, resulting in daily worker exposure. Inhalation is the primary route of exposure, with potential for skin absorption as well [1]. The International Agency for Research on Cancer (IARC) classifies benzene as carcinogenic to humans, prompting stricter regulations such as the European Union's recent revision of the limit value to 0.2 ppm. Evidence links benzene exposure to various cancers, including acute myeloid leukemia in adults, and suggests associations with other malignancies such as non-Hodgkin lymphoma, chronic lymphoid leukemia, multiple myeloma, chronic myeloid leukemia, acute myeloid leukemia in children, and lung cancer [2]. Benzene's harmful effects stem from its metabolic activation, which generates electrophilic metabolites inducing oxidative stress, DNA damage, chromosomal alterations, immunosuppression, and hepatotoxicity [3]. While evidence suggests that benzene exposure influences gene expression, comprehensive studies on genetic and epigenetic alterations, including DNA methylation and microRNA expression pat-

terns, are lacking [4]. This study aims to identify specific genetic and epigenetic modifications indicative of both benzene exposure and blood cancer development in workers. Integrated analysis of gene expression, microRNA expression, and DNA methylation datasets retrieved from the GEO DataSets database identified putative biomarkers associated with benzene exposure and cancer transformation. Additionally, prediction tools such as g:Profiler, STRING and miRNet, elucidated the functional roles of these biomarkers. Overall, the study revealed benzene's ability to modulate gene expression and to induce epigenetic changes in healthy, occupationally exposed workers compared with non-exposed controls. These findings offer valuable insights into the early identification of biomarkers for blood cancer susceptibility in these populations. Ultimately, characterizing this panel holds promise for enhancing monitoring protocols for at-risk workers and guiding the development of cutting-edge preventive strategies.

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OS03-11
Exposure to PFAS and associated toxicity in workers exposed to hexavalent chromium – a cross-sectional study within the SafeChrom project

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Background: This study aimed to investigate the exposure to perfluoroalkyl substances (PFAS) and cancer-related toxicity in workers in different Swedish industry sectors with exposures to hexavalent chromium (Cr(VI)).

Methods: The study consisted of 111 exposed workers and 72 controls. The exposed workers were performing manufacture/processing of metal products (n=55), working in steel production (n=31), bath plating (n=17), and others (n=8). The PFAS exposure was assessed by the determination of ten PFAS in serum samples by LC-MS/MS. Cr was measured in red blood cells (RBC) by ICP-MS, as a long-term marker for Cr(VI) exposure. Telomere length and DNA methylation of lung cancer-related genes in DNA from peripheral blood were measured by qPCR and pyrosequencing, respectively.

Results: Significant differences between workers with Cr(VI) exposure and controls were found for the PFHpA and PFOA ($P<0.001$, linear regression analysis adjusted for age). There were significant differences between occupational groups where the bath platers showed significantly higher levels of PFHpA, PFHPS, PFPeS, PFOS, PFDS, and PFHxS compared with the other occupational groups. The highest serum concentration was found for PFOS: 700 ng/ml in a bath plater, to be compared with the median concentration of 3.82 ng/ml among the controls. PFOA and PFHPS correlated positively with RBC-Cr ($r_S=0.27$ and 0.35 respectively) in exposed workers. PFOA was associated with shorter telomere length after adjustment for age. No clear associations was found for DNA methylation in cancer-related genes. Ongoing analysis will assess mixture effects on toxicity biomarkers.

Conclusion: The considerably high PFAS exposure in Cr(VI) bath platers can be explained by the former application of PFAS as mist suppressants in electroplating baths but further sources of exposure should be examined. The association between PFAS and some cancer-related biomarkers warrants further examination, including potential mixture effects.

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OS03-12
Mixture toxicity in risk assessment under EU legislation

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There is growing concern on the cocktail of different chemical substances humans are exposed to everyday. Currently, exposure via food is acknowledged as being an important source, for example due to the cocktail of pesticide residues that could have been used on different crops. The European Commission has been focusing on this relevant topic for years, nevertheless in EU legislation the risk assessment is mainly focused on the evaluation and analysis of individual substances without specifically considering exposure to a mixture of chemicals, intentional or unintentional. Scientific evidence demonstrates that in some cases the risk of mixtures may exceed the risk of each individual component, consequently mixture toxicity represents an important subject to be addressed and a great challenge for the improvement of the risk assessment.

The aim of this research was to understand if mixture toxicity represents an important and relevant matter of safety concern for both human and the environment, and how it could be assessed. Literature and legislation was studied to provide a general overview about the currently available methods and tools for the evaluation of mixture toxicity. The efficacy and the applicability of methods and tools such as the Mixture Assessment Factor (MAF), the WHO/ICPS Framework, but also the use of Adverse Outcome Pathways (AOP) and Threshold of Toxicological Concern (TTC) are discussed. A comparison between different legislations and regulations was done in order to evaluate how mixture toxicity is effectively implemented, focusing on REACH, the Plant Protection Product Regulation (PPPR) and the Biocidal Product Regulation (BPR). In addition a critical analysis of different experimental studies and case studies was performed to examine the relevance of toxic effects posed by mixtures of chemicals on both humans and environment, and the usefulness of available tools to implement the assessment of mixture toxicity in legislation.

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OS04 | Oral Session 4

OS04-01
Sexual dimorphism in lung immune adaptations in fetuses exposed to alcohol in pregnancy

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Current fetal alcohol spectrum disorders (FASD) studies primarily focus on alcohol's actions on the fetal brain although respiratory infections are a leading cause of morbidity/mortality in newborns. The limited studies examining the pulmonary adaptations in FASD demon-

strate decreased surfactant protein-A and alveolar macrophage phagocytosis, impaired differentiation, and increased risk of Group B streptococcal pneumonia with no study examining sexual dimorphism in adaptations. We hypothesized that developmental alcohol exposure in pregnancy will lead to sexually dimorphic fetal lung morphological and immune adaptations. Pregnant rats were orogastrically treated once daily with alcohol (4.5 g/kg, gestational day [GD] 4 to 10, peak BAC, 216 mg/dl; 6.0 g/kg, GD 11 to 20, : peak BAC, 289 mg/dl) or 50% maltose dextrin (isocalorically matched pair-fed controls) to control for calories derived from ethanol. Male and female fetal lung RNA from a total of 20 dams were assessed using the TapeStation (Agilent) and Qubit RNA broad-range assay. Samples with RINs >8 were prepared using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB), xGen Broad-range RNA Library Prep (IDT), and xGen Normalase UDI Primer Plate 2 (IDT). Final libraries were checked for quality and quantity by Qubit hsDNA and LabChip. The samples were sequenced on the Illumina NovaSeq S4 Paired-end 150bp. Fetal lung tissue were analyzed for morphological and immunological analysis. Mean fetal weight and crown-rump length of the alcohol-administered rats were ~9% and ~8% lower ($P<0.05$) than the pair-fed control pups. Differentially expressed genes indicated a sex-linked gene regulation dichotomy with a significantly higher number of genes altered in the female fetal lungs compared to the male. Network analysis plot of downregulated genes in the females exposed to alcohol in utero showed a negative impact on T cell activation and regulation, T cell differentiation, decrease in CD8+ T cell number etc. The most altered genes were CD8b, CCl25, CD3e, CD27, CD247, Cd3d, CCR9, CD2, CD8a and were decreased by a log2fold change of >2 ($P<0.05$) in the female fetal lungs. KEGG analyses showed that male and female fetal lungs had downregulated genes associated with development and mitosis, whereas the females alone showed dysregulation of T cell genes. Males and females both showed an increase in alcohol metabolism gene expression. RT-PCR data validated RNA-seq. Histology validated the effect on lung development showing stunted differentiation, were relatively hypoplastic, and displayed diminution of alveolar size and air spaces, in both male and female. These data provide novel information in a growing area focused on alcohol effects on the offspring lung and its influence on appropriate fetal/neonatal immune responses and highlights the importance of examining sexual dimorphism in developmental adaptations. NIH HL151497, AA23520, AA23035 (JR)

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OS04-02
Development of an innovative human-based testing battery for assessing thyroid hormone mediated developmental neurotoxicity

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Background: Thyroid hormone (TH), the collective name of the prohormone T4 and biologically active hormone T3, is crucial for proper neurodevelopment, and deficiencies in TH levels during pregnancy are associated with lower IQ and delayed motor development in children. Endocrine disrupting chemicals (EDCs) are associated with disrupting TH balance and neurodevelopmental effects during pregnancy. Current *in vivo* animal data has limited relevance for toxicity prediction in humans, especially considering species-specific issues of age, sex and exposure in different life-stages. The aim is to develop a human-based innovative testing strategy to assess the safety of chemicals for developmental neurotoxicity mediated by disruption of TH signaling.

Method: We developed an Adverse Outcome Pathway (AOP) network on brain development based on human physiology including cell specificity, barriers and knowledge from clinical studies. Critical molecular initiating events (MIEs) and key events (KEs) have been identified and used as a basis for selecting parameters for an *in vitro* testing battery. The first part of the testing battery consists of human cell models representing neurons (SK-N-AS), oligodendrocytes (MO3.13) and astrocytes (H4). TH uptake and metabolism by the cell lines was tested using radiolabeled TH. T3-receptor activation was measured using qPCR of the T3-responsive gene KLF9.

Results: The MIEs shown in the AOP are related to TH availability in the brain via transport, metabolism and receptor activation. Partial inhibition of T3 and T4 uptake by silychristin, an inhibitor of the TH-specific transporter MCT8, was observed in all cell models. Endogenous deiodinase activity in a whole-cell metabolism assay showed inhibition of deiodinase type 3 (D3) by iopanoic acid in MO3.13 and SK-N-AS cells. Preliminary data indicate that EDCs can exert negative effects on transport and metabolism. All cell types showed increased KLF9 expression in response to T3.

Conclusion: The selected cell lines are suitable models to test the effect of compounds on uptake, metabolism and action of TH. Future experiments will include testing of multiple EDCs on receptor activation and whole-cellmetabolism assays. Furthermore, advanced stem-cell models will be incorporated into the testing battery to study whether, and to which extend, developmental neurotoxic compounds disrupt the TH balance in the developing brain.

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OS04-03
Unravelling the impact of endocrine disruptors on human neurodevelopment through the lens of brain organoids

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Endocrine-disrupting chemicals (EDCs) are a class of widespread environmental pollutants that interfere with hormonal physiological functioning, leading to various adverse health effects. A growing body of evidence linked early life EDCs exposure with developmental neurotoxicity, yet the molecular mechanism of action of such chemicals in this domain remains to be fully characterized. Moreover, EDCs risk assessment is mainly performed on single compounds, while complex chemical mixtures are almost not covered by the current regulatory frameworks.

The advent of induced pluripotent stem cells (iPSCs) allows for the *in vitro* reproduction of human developmental processes. IPSCs-derived Cortical Brain Organoids (CBOs) provide a three-dimensional *in vitro* model that closely mimics the complex cytoarchitectural and gene expression landscape of the developing human brain.

Previous studies investigating EDCs neurological effect lacked the integration of epidemiological evidence and longitudinal clinical profiling, failing to link the exposure of *in vitro* systems to real-life population-based exposure levels.

To overcome these limitations, we explored the neurodevelopmental effects of EDCs by exposing CBOs to 7 single chemicals and their

weighted mixture associated with developmental neurotoxicity in the Swedish SELMA pregnancy cohort. Furthermore, to dissect the endocrine mode of action of these environmental pollutants, we established an atlas of hormonal impact on neurodevelopment by selective modulation of 7 hormonal pathways known to be relevant for EDCs effects during CBOs differentiation.

By integrating bulkRNA-seq, scRNA-seq, targeted stereoidomics, and immunohistochemistry we charted a unique map portraying how hormonal signaling and its disruption shape early cortical formation with unprecedented granularity.

Our preliminary results show that both single endocrine disruptors and their mixture alter cell type proportions and transcriptome in a biological model that recapitulates the developing human brain. Functional characterization of gene signatures modulated in response to EDCs exposure also highlights transcriptional programs associated with various neurodevelopmental disorders. We then pinpoint multiple hormonal pathways through which EDCs exert their biological effects.

Taken together our research sheds light on cellular and molecular alterations induced by EDCs at epidemiologically relevant concentrations, reinforcing organoids as a valuable tool to decipher environmental toxicants' deleterious impact on a human-relevant biological model.

Moreover, these resources contribute to the ENDpoiNTs and REMEND consortia, EU-funded projects aimed at providing regulatory agencies with new tools for risk assessment of endocrine disruptors.

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OS04-04
Multi-behavioral fingerprints in larval zebrafish can identify neuroactive environmental chemicals and underlying mechanisms

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Exposure to some environmental chemicals is associated with neurodevelopmental disorders. There is a lack of alternative test methods that encompass complex endpoints present in rodent test guidelines, such as learning and memory. To fill this gap, we devised a visual and acoustic motor response (VAMR) new approach method (NAM) in 5-day post-fertilization (dpf) zebrafish. This method aligns with the principles of the 3Rs concept. The VAMR NAM is comprised of 25 sequential automated behavior-based endpoints that measure a range of visual motor responses, visual and acoustic startle responses, habituation learning, and memory retention. To increase confidence in the use of the VAMR NAM to detect human-relevant effects, a neuroactivity fingerprinting system was established using concentration-response profiles from 55 reference chemicals and five predicted negative chemicals that were inactive in cellular neurotoxicity NAMs. Reference compounds that target specific neurotransmission pathways (dopaminergic, glutamatergic, gabaergic receptors, serotonin reuptake), neurodevelopmental signaling (EFGR, PDGFR, GSK-3, mTOR, PKC, SRC, AKT, PI3-kinase, RyR, CREB, COX-2, NOS, EP1-4, PORCN, BMP, NO-cGMP), protein synthesis (brefeldin a), and toxicologically-relevant pathways (ER, AHR, PPAR, RXR, TR) were evaluated in the VAMR NAM following 60 min exposure in 5 dpf larvae. Larvae were exposed to six concentrations of each reference chemical, and significance was determined relative to vehicle control (0.4% DMSO). To build the fingerprints, hierarchical clustering of compound- and concentration-specific profiles was based on effect sizes (SSMD, strictly standardized median difference) across all endpoints. We identified a diverse array of toxicity fingerprints, including compounds that reduced or accelerated habituation learning (MK-801, SC79, LY294.002) or caused changes in visual (GW7647, T0070907, ODQ) and/or acoustic (CP-673451, celecoxib, MHY1485) startle responses. Notably, exposure to GABA receptor (GABAR) modulators (picrotoxin, bicuculline, CGP13501, isoguvacine) exhibited altered dark-phase motor activity, increased activity in visual and acoustic startle response endpoints, and seizure-like activity in the acoustic interstimulus interval, indicating a potential mechanism-behavior relationship. To explore the hypothesis that GABAR modulators trigger a specific behavior signature, the US EPA CompTox database and rodent studies were mined for environmental chemicals that interact with GABAR in cellular assays. Exposure to the known GABAR antagonists dieldrin or lindane significantly altered behavior in GABAR-related endpoints. Interestingly, exposure to tetrabromobisphenol A (TBBPA) also modified behavior in GABAR-related endpoints with altered directionality. Pharmacological co-exposure experiments confirmed that dieldrin and lindane act as GABAaR antagonists in zebrafish and identified a novel mechanism for TBBPA acting as a GABA agonist.

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OS04-05
Addressing uncertainties in the DNT *in vitro* battery (DNT IVB) by incorporating glial models and increasing human relevance

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From the earliest stages of embryogenesis through adolescence, the human brain undergoes a series of highly orchestrated events called key neurodevelopmental processes (KNDPs), including neurogenesis, gliogenesis, migration, synaptogenesis, and neuronal network formation. Each KNDP is critical for the proper formation and function of the brain, with perturbations resulting in neurodevelopmental deficits. A DNT *in vitro* battery (DNT IVB) has been established under the guidance of the European Food Safety Authority (EFSA) with *in vitro* test methods modeling relevant KNDPs and screened for a plethora of environmentally relevant chemicals. However, uncertainties remain that need to be addressed to promote regulatory confidence in the applicability of the test results.

Here, we describe ongoing efforts to improve the effectiveness of the battery, focusing on underrepresentation of key neural cell types and species-related uncertainties. To incorporate KNDPs related to glial cells, we developed assays to evaluate chemical effects on the differentiation and functionality of astrocytes and radial glia. Here, neural progenitor cells (NPCs) differentiate into astrocytes in the presence of BMP2 and CNTF, which respond to TNFα exposure with inflammatory activation. In addition, effects on growth factor (EGF and FGF)-dependent migration and proliferation of NPC-derived radial glia are assessed based on immunochemical (ICC) stainings for nuclei (Hoechst) and the proliferation marker KI67. To address the involvement of the immune system, we are incorporating human induced pluripotent stem cell (hiPSC)-derived microglia in several human-based KNDPs of the DNT IVB, highlighting the need to better assess their contribution to KNDPs. The DNT IVB primarily assesses chemical effects on proliferative processes during the fetal phase, potentially leaving uncertainty at critical embryonic stages. We now established a proliferation assay using hiPSC-derived NPCs, which more closely recapitulate the embryonic developmental stage, to address chemical sensitivities at different developmental windows. Finally, we addressed species-related uncertainties, as some KNDPs are still modeled using rat-based *in vitro* systems. Due to the peculiarities of human brain development, we developed human-based assays to assess neural network formation and synaptogenesis based on hiPSC-derived NPCs using multi-electrode array recordings and ICC stainings for synaptic markers, respectively.

Significant advances have been made in alternative DNT testing, demonstrating the applicability of the DNT IVB for chemical hazard assessment as well as screening and prioritization. Ongoing efforts to refine glial-related endpoints and incorporate human-based neurotransmission and neural network assays will increase confidence in the predictivity of the test results and promote confidence in the use of the DNT IVB for regulatory purposes.

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OS04-06
Juvenile age is a vulnerable period for the risk of future male reproductive disorders with fluoxetine use in a rat model of post-traumatic stress disorder

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PTSD can occur in children after even one traumatic event, but repeated or prolonged trauma increases the risk [1]. Fluoxetine is still one of the most common antidepressants used in childhood for PTSD [2]. Nevertheless, results on fluoxetine use and its side effects (including negative effects on the male reproductive system) are mainly based on clinical studies involving adult patients [3]. This means that there is insufficient data on not only the effectiveness of treatment but also a large gap in knowledge about the toxicological consequences of fluoxetine use in childhood and adolescence, especially its long-term effects, exists.

The aim was to investigate the effects of fluoxetine on parameters characterizing the male reproductive system of juvenile male rats under PTSD simulation.

Male rats, 44 days old (corresponding to adolescence in humans), were subjected to combined stress followed by repeated stress and fluoxetine 10 mg/kg for 21 days. Animal groups (control; PTSD and PTSD+fluoxetine) consisted of 14 animals each. The adequacy of PTSD simulation was assessed using common behavioral tests. The effect on the biochemical parameters of serum, which characterize metabolic processes in the organism, was studied. The morpho-functional state of the gonads and the testosterone level in serum were evaluated. Assessment of the activity of rats in the Porsolt test demonstrated an increase in the total time of immobility in the PTSD group, which indicates the presence of behavior similar to depression and is a consequence of experienced traumatic events. Given that PTSD is associated with a significantly increased risk of metabolic syndrome, we examined biochemical parameters characterizing carbohydrate, lipid, protein, and purine metabolism. No differences in serum glucose, cholesterol, triglycerides, creatinine, and urea were found. However, we observed a clear tendency for an increase of uric acid levels in rats with PTSD with its significant rise in animals that were administered fluoxetine. In rats treated at a juvenile age with fluoxetine a decrease in the mass of the epididymis by 12% was found as compared with both control and PTSD groups. Simultaneously decrease in the number of sperm from the tail part of the epididymis by 25% was noticed. Taken together such results evidenced the negative effect of SSRI on spermatogenesis, which could be especially vulnerable to fluoxetine at that age. It should be noted that the level of testosterone in serum was significantly lower (2 times), both in the PTSD group and with the administration of fluoxetine.

Thus, studies of the effects of fluoxetine on the model of juvenile PTSD indicate a risk of disruption of metabolic processes in the body of rats during puberty and disruption of the normal formation of the spermatogenesis process. The obtained results prove the need for a comprehensive evaluation of the effectiveness and long-term safety of fluoxetine used in childhood for the treatment of PTSD.

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OS04-08
The Nanopig™ – the other non-rodent

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The majority of drug development programs require the use of nonrodents as part of the safety testing needed to move a drug into clinical trials, and to continue the development through marketing. The majority of drugs in development are either small molecules or biologics, with the remainder being other modalities. For small molecules, the default nonrodent has been the dog while for biologics the default has been the non-human primate (NHP). There has been growing pressure to reduce the use of NHPs in nonclinical studies, and their cost has increased substantially. In addition, there has been public pressure to reduce the use of dogs as they are companion animals. The minipig is another nonrodent species but has historically been limited to programs involving dermal delivery of the test article due to the minipig skin being the best model for human skin. Since pigs are food animals, there is not the same pressure to limit their use in safety testing for pharmaceuticals.

For small molecules, one of the key features for the selection of the nonrodent is the metabolism of the test article with early testing often including metabolic profiling using NHP and dog microsomes or hepatocytes. Data has shown that the metabolic processes of the minipig have a high degree of similarity to humans, indicating the minipig is a potential option as a non-rodent species for small molecules. For

biologics, the nonrodent species needs to be pharmacologically relevant, and the minipig contains many orthologs of common targets for biologic test articles, again indicating it is a viable option as a non-rodent species.A common concern when discussing minipigs is their size, as many minipigs can reach 40kg or more raising a concern over the amount of test articles that would be required for studies in minipigs, particularly for chronic studies. With selective breeding and diet management, the Sinclair Nanopig™ is comparable to beagle dogs, even for chronic studies. Taken together the Nanopig™ can represent a cost-effective and scientifically justified nonrodent species for use in safety studies for small molecules and biologics and uses a comparable amount of test articles to beagle dogs.

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OS04-09

Alkylated naphthalene toxicity varies across alkyl carbon number and position: insights from embryonic zebrafish exposures and transcriptomics

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Polycyclic aromatic hydrocarbons (PAHs) are a concern to environmental and human health due to their ubiquitous presence in the environment and adverse biological activity. There are over 10,000 PAHs in the environment that occur in complex mixtures, however, our knowledge of their activity is primarily based on unsubstituted PAHs. Understanding the toxicity of alkylated PAHs is important for a comprehensive understanding of the hazard potential, as they often occur in higher abundance and are often more biologically active than the parent PAHs. Based on previous screening results and chemical fate quantification, we know that compounds in the naphthalene parent group are volatilizing from the media in our standard 96-well plate exposure method. To overcome this challenge, we developed a methodology for testing volatile compounds in early life stage zebrafish. Pools of 8 chorionated embryos were loaded into 2mL glass vials with minimal head space, exposed to chemical at 6 hours post fertilization, immediately capped, and mixed. Exposure vials were continuously mixed overnight at 28°C. Individual embryos were plated into 96 well plates with chemical free embryo media at 24 hpf. At 120 hpf embryos were evaluated for morphological and behavioral effects. Using this method, we exposed a series of 22 naphthalenes at 5 concentrations n=32 (3.125–50μM). The morphological dose response curves had 4 distinct shapes, steep curves starting at 12.5 μM or 25μM, gradual curves, or no response (groups A, B, C, D respectively). Benchmark dose 50% (BMD50) values ranged from 13–47 μM and 7 compounds did not have any morphological activity including naphthalene and singly methylated naphthalenes. All the naphthalenes with 3 methyl carbons were in group A, while naphthalenes with 2 methyl groups were in groups B, C and D depending upon alkyl position. For example, 1,7-dimethylnaphthalene had a BMD50 of 20 μM while 2,7-dimethylnaphthalene had no morphological effects. Based on these results 20μM was chosen for targeted transcriptomic sequencing exposures. At 48 hpf 6 embryos were pooled in their vial groups for RNA extraction n=4 for each chemical. Targeted sequencing for >3000 toxicologically relevant genes was preformed using the Tempo-Seq™ Bio Spyder platform. In addition to demonstrating a new method for aquatic exposures to volatile compounds, these results provide additional evidence that alkylated PAHs are often more toxic than their parent compounds and that toxicity varies based on alkyl position and amount. This research was supported by the NIEHS of the National Institutes of Health under Award Number P42 ES016465, P30 ES030287, and T32 ES007060.

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Poster Presentations

P01 | *In vitro* methodologies & screening

P01-01
A review of *in silico* and *in vitro* methods for use in a risk assessment of a substance acting via oestrogen or androgen modalities

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The testing of cosmetics on animals is banned in many countries, but manufacturers are responsible for demonstrating the safety of constituents for users. Of concern are those ingredients with endocrine disrupting properties. Therefore, the cosmetics industry requires an approach for conducting risk assessments for substances with potential endocrine activity which does not require *in vivo* animal testing, instead, demonstrating their safety using *In vitro* to *In vivo* Extrapolation (IVIVE) approaches. These approaches rely on the use of *in vitro* bioassays supported by predictive *in silico* data. Whilst there are many *in silico* models and *in vitro* methods which are used to predict the endocrine activity of chemicals, most have been optimised with a focus on classifying substances with endocrine activity rather than consideration of their biological relevance. A review of the available *in silico* and *in vitro* methods was therefore conducted to assess which were most suitable to support a human risk assessment for a substance acting via oestrogen or androgen modalities. The review considers several aspects including the relevance of the assay for humans (tissues and receptors) and for predicting effects in the whole organism, the sensitivity and specificity of the assay, applicability domain of the methods, as well as regulatory acceptability and commercial availability. An issue with extrapolating effects to humans is that metabolism is not considered within the assays. Therefore, the review also considers the possibility of incorporating metabolizing systems (e.g., S9) in the *in vitro* assays. We also considered whether these assays had already been used in IVIVE approaches and if so, whether the extrapolations were realistic.

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P01-02
In vitro identification of the possible immunotoxic potential of RNA drugs focusing on four specific stress pathways

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In the last few decades, the treatments of human disorders are focused on personalizing therapies. This evolution is unfolding due to the identification of the molecular target involved in the development of diseases with the consequent development of high specificity drugs as treatments disorder. The great power and flexibility of nucleic acids candidate these molecules in medicine with a wide range of possible applications, and the possible adverse effects must be taken into consideration.

This research aims to establish a platform by using new approach methods (NAMs) to evaluate the potential toxicity of RNA drugs, in view of their potential immunogenicity and off-target effects.

To reach this goal, human peripheral blood mononuclear cells (PBMCs), obtained from buffy coats from healthy male and female donors were used and four toxicity pathways, known to be activated by several chemicals, were selected: I) oxidative stress, II) endoplasmic reticulum stress III) mitochondrial stress, and IV) autophagy. For each pathway, a specific positive control was used: tert-Butyl hydroperoxide (tBHP) for the oxidative stress, thapsigargin (TG) for endoplasmic reticulum stress, rotenone (Rot) for mitochondrial stress, and rapamycin (Rapa) for autophagy. Cell viability was assessed through lactate dehydrogenase release and apoptosis. The release of IL-6, IL-8, IFN-g, TNF-a was analyzed performing ELISA test on supernatants. Furthermore RNA-seq was performed on the positive controls to generate information on target genes to be selected as representative of the main stress pathways and indicators of potential toxicity.

The results obtained on the experiments performed on the positive controls allowed the selection of their maximum concentration that determines cell viability higher then 80%: 250 nM for tBHP, and 1mM for TG, Rot and Rapa. An increase in the release of TNF-a was observed after exposure of PBMCs to tBHP, while TG was able to increase the release of IL-6, IL-8 and TNF-a. Data derived from RNAseq based on the four positive controls selected are already ongoing.

The next steps will be focused on the study of several RNA drugs already selected: Morpholino ASO C9, siRNA RyR2, mRNA vaccine anti-Rida, mRNA vaccine anti-Sars-Cov-2. In particular, through the Real-Time PCR, the modulation of the selected genes derived from the

RNAseq analysis will be analyzed in PBMCs exposed to the selected RNA drugs.

The research has the potential to develop an *in vitro* method in the screening of safer RNA drugs allowing the selection of more promising drug candidates, thereby enhancing drug safety.

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P01-04

Extracts from different next generation product platforms show less potential to induce oxidative stress than extracts from cigarettes in human coronary artery endothelial cells (HCAECs) *in vitro*

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Smoking is a cause of serious diseases in smokers, including heart disease; this is attributed to the high levels and numbers of harmful chemicals present in cigarette smoke. Previous studies report that chemicals in cigarette smoke interact with endothelial cells in the cardiovascular system, triggering oxidative stress and inflammation which can lead to atherosclerosis. Generation of aerosols from next generation products (NGP) such as heated tobacco products (HTP) and electronic vapor products (EVP) does not involve burning tobacco and therefore these contain fewer and lower levels of harmful chemicals compared to cigarette smoke. The present *in vitro* study investigated induction of oxidative stress in human coronary artery endothelial cells (HCAECs) by bubbled medium extracts (bMED) from different products. A HTP (Pulze & ID™) and two EVP (blu 2.0™ and blu bar disposable™ device) products were compared to the 1R6F Reference Cigarette.

HCAECs were treated for 2 or 24 hours with the different extracts and were subsequently stained to detect the generation of reactive oxygen species (ROS), the depletion of reduced glutathione (GSH) and the induction of DNA damage. Furthermore, the specificity of effects was tested by identical cell treatment in the presence of the ROS scavenger N-Acetylcysteine (NAC).

The results indicated that oxidative stress could be induced with bMED from all platforms when measured by GSH depletion, however, at much higher concentrations of the NGP bMED than the 1R6F bMED. In the absence of NAC, a 5-fold, 43-fold and 61-fold higher concentration (measured on a nicotine basis) was needed to reduce the GSH levels beyond the median absolute difference for HTP, blu2.0 and blu bar bMED extracts respectively. The GSH-depletion effect was reduced in the presence of NAC when combined with bMED from 1R6F or HTP but not when treated with bMED from the EVP products. Reproducible statistically significant ROS induction was only seen with the bMED from 1R6F with a reducing effect by NAC. DNA damage was induced in the 24hour treatment with bMED from 1R6F and HTP in the absence of NAC, with 10-fold higher concentrations needed for HTP bMED to induce the damage (measured on a nicotine basis). The bMED obtained from EVP did not induce any reproducible DNA damage.The results obtained under the conditions of this study indicate that extracts from the different NGP platforms induced less oxidative stress in the HCAECs than the extracts generated from 1R6F smoke thereby supporting the reduced harm concept of the NGP platforms which deliver nicotine without tobacco combustion leading to reduced number and levels of harmful chemicals in the aerosol.

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P01-05

Investigating the genotoxic effects of the *Alternaria* toxin tenuazonic acid in human cells

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Human exposure to mycotoxins is pervasive worldwide and, due to climate change, will likely increase in the future. This urges adequate an up-to-date science-based regulation to prevent human exposure and thus protect public health. Tenuazonic acid (TeA) is a mycotoxin produced by black moulds of the genus *Alternaria*, which occurs ubiquitously and affect a broad spectrum of plant-based food commodities including grain and grain-based products, apples, oilseeds, sunflower oil and tomato products. Despite the recognition that most mycotoxins are hazardous to human health, especially regarding their chronic effects, the existing toxicity data is insufficient to perform risk assessment for most of the *Alternaria* toxins [1]. In the European Partnership for the Assessment of Risks from Chemicals (PARC; https://www.eu-parc.eu/), *Alternaria* toxins have been underlined as unknown hazards and, thereby, a project aiming at investigating their toxicological effects, is underway. Concerning TeA, the few published studies have reported that it was not mutagenic in bacteria and did not induce DNA damage (as assessed by the comet assay) or double-strand breaks in DNA (γH_2AX assay) in intestinal or in liver cells, respectively [2]. Since genotoxicity is a key toxicological endpoint driving decisions in risk assessment and the existent data on TeA is insufficient to draw a firm conclusion, further genotoxicity assessment was undertaken in PARC, concomitantly with endocrine disruption and immunotoxicity assessment, using the same toxin batch.

In the present study, the genotoxicity of TeA was investigated through the *in vitro* micronucleus assay (OECD TG 487) [3] in liver cells (Hep G2). To set a concentration-range for further genotoxicity assays in HepG2 cells, a preliminary cytotoxicity assessment was performed using the MTT assay, after 48h exposure to TeA. The results showed a dose-dependent cytotoxicity above 50 µM, with a clear toxicity above 200 µM. The *in vitro* micronucleus test comprised a short HepG2 cells exposure to TeA (3h), with and without metabolic activation (S9 fraction), and a longer exposure (48 h), to allow the detection of clastogenic and aneugenic effects.

This work provides toxicological data on TeA, mainly contributing to its hazard identification and characterization. These data, together with data from other toxicological endpoints, will be used for an integrated hazard assessment of this toxin that will support risk assessment and regulation by competent EU authorities.

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P01-06
Validating multi-ion channel inhibition assays following best practice considerations

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Purpose: As ICH E14/S7B Q&As was adopted in February 2022, the pharmaceutical industry paid more attention to the best practice considerations, as well as CiPA activities. When sponsors are using hERG data to support interpretation of clinical QT data in specific scenarios, and when using Cav1.2 and Nav1.5 to support a proarrhythmia assessment, best practice aspects should be considered as described in the E14/S7B Q&As. More and more sponsors request the multi-ion channel inhibition assays following best practice considerations for submission. Considering this trend and the ICH guideline adoption, we have initiated and completed the validation for hERG, Cav1.2, peak and late Nav1.5 currents following best practice considerations using manual patch-clamp technique.

Method: Manual patch-clamp system was used as the platform. The recording temperature was near physiological temperature (35–37°C) for the multi-ion channel inhibition assays.The compounds from the low-, intermediate-, and high-risk categories of the "training drugs" from the CiPA in silico model were selected respectively. The experiments were conducted on the following three cell lines: CHO-hERG, CHO-Nav1.5, HEK293-Cav1.2. CiPA recommended voltage protocols were applied for recording hERG, Cav1.2, Peak and Late Nav1.5 currents. The stimulation frequency of 0.2 Hz was applied for all ion channel inhibition assays. A full blocker was added at the end of the test article perfusion. E4031 (1 μM), verapamil (100 μM); and TTX (30 μM) were used for hERG, Cav1.2, Nav1.5 assays, respectively. Current amplitude, input resistance, holding current for individual cells were monitored continuously during perfusion.

Results: The IC_{50} values of hERG block for Moxifloxacin, Ondansetron, Dofetilide, Verapamil and Cisapride were 67.40±9.66 μM, 1.47±0.76 μM, 27.31±3.66 nM, 0.23±0.13 μM and 23.87±6.21 nM (mean±95% CI). For Cav1.2 assay, the IC_{50} values of Verapamil, Cisapride, and Quinidine were 3.69±2.55 μM, 2.08±0.76 μM, and 22.57±21.27 μM. Ranolazine, Tetracaine and Flecainide were tested in both peak and late Nav1.5 assays. The IC_{50} values of peak sodium current inhibition were 334.10±247.42 μM, 33.16±13.47 μM and 15.95±9.58 μM, and the IC_{50} values were 22.83±6.52 μM, 0.54±0.41 μM and 1.37±0.34 μM respectively in the late Nav1.5 assay. All the results were comparable with the data reported in the literatures, which indicates these multi-ion channel inhibition assays were well validated. The CiPA multi-ion channel inhibition assays following best practice considerations can minimize the inter-lab variations, help evaluate potential effects on the cardiac action potential and estimate the proarrhythmic risk in patients more accurately.

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P01-08
Combining *in vitro* and *in silico* tools for assessing the inhalation hazard of sodium dodecyl sulphate aerosols

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Aerosols generated during the spray application of various consumer spray products can potentially be inhaled by the users and bystanders. Inhaled aerosol droplets that reach the alveoli can interact with the lung surfactant; a complex mixture of surface-active phospholipids and associated proteins present as a thin film at the air-liquid interface of the fluid lining the alveolar surface. These components enable effortless breathing by lowering the surface tension to near zero values to prevent alveolar collapse at the end of expiration.

Constrained drop surfactometer, a cell-free model, was employed to investigate the effect of aerosolized sodium dodecyl sulphate (SDS, a surfactant used safely in consumer spray products), on lung surfactant function inhibition. This model has previously been used to assess chemicals, where lung surfactant function inhibition was determined by using a minimum surface tension of 10 mN/m as the pre-determined cut-off point, above which the alveoli in the intact lungs will collapse. To refine the determination criteria, we used a mathematical model developed to measure changes in the viscoelasticity of the surfactant and to quantify the effect of SDS on surfactant rheology – the Fourier Mode Dynamic Tensiometry Method. Inhibition was correlated to the rate of exposure, calculated as the concentration of SDS multiplied by the infusion rate. This novel method of assessing the effect of aerosols on lung surfactant function and rheology could provide additional information for consumer safety risk assessments, when combined with information from the toolbox of non-animal approaches currently available.

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P01-09
Isolation of adult cardiomyocytes from mouse heart by a novel, semi-automated perfusion technology

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Background and Aims: Primary adult cardiomyocytes represent a major tool in biomedical research. To date, different protocols for cardiomyocyte isolation, such as the Langendorff perfusion system, have been introduced, all requiring highly trained staff to avoid considerable fluctuations in quality and yield. We aimed at establishing an easy and user-independent workflow for the isolation of primary cardiomyocytes.

Method: We recently introduced a new semi-automated perfusion technology, which is suitable for the gentle, rapid, and efficient generation of single-cell suspensions from rodent liver tissue [1]. We have now adapted this procedure for adult mouse heart. Tissue is clamped into an adjusted disposable and enzymatically digested using optimized reagents. Afterwards, single cells are liberated by a short mechanical

disruption of the perfused tissue and the sample is loaded onto a strainer (MACS SmartStrainer) to remove any remaining larger particles from the single-cell suspension. Cardiomyocytes are then enriched by a low spin centrifugation step. Yield, viability and purity of cardiomyocytes were determined, immunofluorescence analysis and functionality tests were performed.

Results: The new perfusion technology is efficiently generating single-cardiomyocyte suspensions from mouse heart. We have designed a new tube format that is operated in combination with optimized reagents and newly designed perfusion sleeves on the instrument (gentleMACS Octo Dissociator with Heaters, Miltenyi Biotec) for optimal isolation of cardiomyocytes. Nested processing allows simultaneous handling of up to 8 samples. The protocol does not require inconvenient steps such as ligation of the aorta to a syringe needle under a microscope which is part of the Langendorff procedure. The yield of isolated cardiomyocytes from one adult mouse heart is $>1 \times 10^6$ with >80% viability. Microscopic analysis revealed that the cells show the characteristic rod-shaped appearance and can be stained for characteristic cardiomyocyte markers.

Conclusion: The novel perfusion technology allows for the simple and reliable isolation of primary adult mouse cardiomyocytes. Disposable (gentleMACS Perfusers 2, Miltenyi Biotec) and appropriate reagents (Heart Perfusion Kit, Miltenyi Biotec) have been optimized for mouse cardiomyocytes but can be adapted to non-parenchymal cells. The semi-automated workflow enabling heart perfusion in a closed system is easy to apply and helps to implement the 3R principle of animal experimentation as the failure rate is drastically reduced.

Conflicts of interest: Carsten Poggel, Timo Adams, and Olaf Hardt are employees of Miltenyi Biotec B.V. and Co. KG.

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P01-10
Cell painting PLUS: an enhanced multiplexed phenotypic assay for chemical hazard screening

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Morphological changes of cells can be used to identify potentially harmful environmental chemicals. In high-throughput / high-content phenotypic screenings using the Cell Painting (CP) method [1], hundreds of features describing cellular morphology are extracted from images. Changes in those morphological profiles can be correlated with organelle-specific, toxicologically-relevant substance activities. CP has already demonstrated its applicability for robust and cost-effective *in vitro* bioactivity screening of environmental chemicals [2] and, thus, supports the identification of substances that pose potential risks to human health, while limiting the need of animal testing. However, in the original CP method, the total number of dyes that can be applied to the same cell are limited. Therefore, depending on the technical specifications of the microscope setup, multiple dyes are often combined in the same imaging channel, compromising profile specificity.

To overcome this constraint, we developed the novel Cell Painting PLUS (CPP) assay, which includes a novel iterative staining-elution cycle that allows subsequent imaging of at least seven fluorescence dyes in separate channels to distinguish at least nine distinct subcellular compartments. This includes the plasma membrane, actin cytoskeleton, cytoplasmic RNA, nucleoli, lysosomes, nuclear DNA, endoplasmic reticulum, mitochondria and the golgi apparatus. This way, the CPP method improves the specificity of compound activity profiles, e.g., by distinguishing strong Actin effects and mild Golgi effects of cytochalasin D across different cell types. Furthermore, CPP expands the multiplexing capacity of the original CP method, e.g., by inclusion of an additional dye staining lysosomes, which have a crucial role in various cellular responses [3]. Therefore, CPP provides important information about compound-induced changes in lysosome morphologies and, thus, helps to identify lysosome-disrupting compounds. The CPP assay has already been applied to different cell lines including RPTEC-TERT1 (kidney), HepG2 (liver), U2OS (bone) and MCF-7 (breast) without any further adaptation needed, demonstrating its wide applicability. Using CPP in these cell lines, we already screened multiple compound libraries in the context of EU and national projects to support chemical hazard evaluation. We therefore see diverse areas of application for the novel CPP assay in the future not only in toxicological risk assessment, but also in basic research and drug discovery.

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P01-11
Germline integration assessment in preclinical gene therapy studies

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In the last few years, a significant number of gene therapy products have advanced in development from preclinical to clinical with increased chances of approval for curing genetic disorders. It is critical to determine that the gene product will not be transmitted to the offspring of treated patients, and thus new approaches have been developed to investigate the potential for germline integration. Essentially this is to prove a negative in terms of DNA integration and a risk for germline modification. In preclinical studies using adult nonhuman primates, genomic analysis is performed on oocytes (females) and semen (males). In male animals, semen is collected at several time-points during the study. The studies typically are 6–12 months of observation after the gene product has been administered to the animals. In females, ovaries are harvested at necropsy for oocyte isolation. To facilitate qPCR analysis in female animals, the ovaries are subjected to manual disruption with subsequent mechanical denudation to aid in removal of extraneous cellular/tissue material for 'cleaning' of the oocytes. Under microscopy, approximately 100 follicular oocytes per ovary were isolated. Between 50 and 100 oocytes were established to

be adequate to extract DNA for genomic analysis. In male animals, the collected semen samples were subjected to wash steps using various combinations of buffers. In a recent experiment, DNA isolation from semen samples gave a yield of >120 ng/uL. In conclusion, the *in vivo* methods and DNA isolation techniques will facilitate analysis of the potential for germline integration.

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P01-12
Rapid assessment of pulmonary genotoxicity induced by pollutant mixtures *in vitro* at the air-liquid interface using a semi-automated methodology

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In the fields of Defense and Aerospace, combustion of solid composite propellants is essential to ensure rapid and efficient propulsion of missiles and rockets. However, this process releases combustion aerosols containing alumina nanoparticles (Al_2O_3 NPs) and hydrogen chloride (HCl_g) in substantial quantities. Therefore, workers may be exposed on an acute or daily basis to these pollutant mixtures mainly by inhalation, triggering potential pulmonary adverse events. Such occupational exposures may consequently have implications on the health of individuals.

Based on our previous studies and literature, acute submerged exposures *in vitro* to Al_2O_3 NPs or Al_2O_3 NPs/HCl mixtures could induce genotoxicity and specifically DNA double-strand breaks (DSB) [1,2]. Immunolabeling of γ-H2AX emerged as the most expeditious and precise technique for detecting DSB following exposures. Considering the importance of rapid and accurate assessment of DSB in predicting the genotoxicity of pollutant mixtures, we aimed to develop a reliable semi-automated methodology after air-liquid interface (ALI) exposures on an alveolar-capillary barrier (ACB). Image analysis automation will allow to minimize human error and to generate standardize measurements.

To mimic the ACB configuration, a co-culture of cells was established within bicameral chambers. This co-culture included type I pneumocytes (hAELVi line) in ALI in the apical compartment and pulmonary microvascular endothelial cells (HPMEC-ST1.6R line) in the basal compartment. Subsequently, these cells were exposed a single time in a cloud system (Vitrocell®) to aerosols, of sterile water (control), Al_2O_3 NPs (primary nanoparticle size 13 nm, at concentrations of 1 mg/mL and 0.05 mg/mL), HCl1.37 mM), and a combination of Al_2O_3 NPs and HCl. DSB were evaluated 24h after exposure. To validate the methodology, two positive controls were applied to the co-culture for respectively 24h or 1h: etoposide (10µg/mL, submerged interface) or γ-radiation (1Gy at 0.5 Gy/min at the ALI). Images were analyzed with the Fiji Software, the surface area of the nuclei was determined using the StarDist plugin [3,4], mathematical morphology and the surface area of the foci was obtained by thresholding.

Exposures to positive controls (etoposide and γ-radiations) increased the genotoxicity (DSB) after 24h and 1h respectively, validating the employed methodology. In our experimental context, pollutants of interest (Al_2O_3 and/or HCl) did not modify cell viability nor induced genotoxicity. However, this methodology holds a potential for extension to the toxicity assessment of other pollutants at the ALI. Implementation of this method could enable rapid and reliable assessment of the genotoxic potential of inhalable pollutant mixtures, such as various environmental exposures, which allows us to assess potential risks to humans and reduce animal testing.

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P01-13
Inhibition of lung surfactant function *in vitro* and breathing pattern changes in mice after exposure to plant protection products

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Currently, testing of acute inhalation toxicity in animals is required for regulation of pesticide active ingredients and formulated plant protection products. The main outcome of the regulatory tests is the concentration that will kill 50% of the exposed animals i.e. “lethal concentration 50” (LC50). However, work is ongoing to identify New Approach Methods (NAMs) to replace animal experiments. To this end, we studied 11 plant protection products sold in the European Union (EU) for their ability to inhibit lung surfactant function *in vitro* in the constrained drop surfactometer (CDS). Subsequently we monitored changes in breathing patterns of mice exposed to the same products. Six of the eleven products inhibited lung surfactant function, and eight changed breathing patterns in mice. Most changes in breathing indicated sensory irritation (6), three plant protection products caused changes indicating pulmonary irritation and two reduced tidal volume. The results from the *in vitro* inhibition of lung surfactant function predicted changes in respiration of exposed mice with a sensitivity of 65% and a specificity of 66%. None of the products was labelled as lung irritants, albeit two products were labelled as “harmful if inhaled”. Both these inhibited surfactant function *in vitro* and changed breathing patterns in mice. Lung surfactant function inhibition *in vitro* predicted changes in breathing to a lesser degree than for previously tested substances. This could owe to the requirement for rigorous testing of plant protection products prior to approval that might have sorted out lung surfactant inhibitors, e.g. due to severe adverse effects during inhalation.

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P01-14
In vitro bioaccessibility of mycotoxins co-occurrence in infant formulas and complementary foods

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Mycotoxins, produced naturally by fungi in food, pose significant health risks to humans and animals, particularly to children and newborns. The co-occurrence of multiple mycotoxins in food presents challenges

as their combined toxicity is not always predictable based on individual toxicity. Scientific interest in understanding the combined toxicity of mycotoxins is growing rapidly, but the combined effect of regulated and other mycotoxins is poorly documented, especially in infant foods. In this study, *in vitro* bioaccessibility studies were conducted using an established digestion model under conditions described by Versantvoort *et al.* (2005) and Assunção *et al.* (2016). Briefly, this model consisted of a short incubation (5 min) of sample with saliva, addition of gastric juice followed by a 2-h incubation, and addition of duodenal and bile juice followed by a 2-h incubation. Thus, the method includes three sequential steps: oral, gastric and intestinal phases, using a-amylase to simulate the salivary fluid (SSF) (pH 7). For the gastric and intestinal phases, a simulated gastric fluid (SGF) (pH 3) and a simulated intestinal fluid (SIF) (pH 7) were used, respectively. Samples underwent sequential digestion steps and were analysed for mycotoxin concentrations. The bioaccessibility (%) of combined mycotoxin were calculated taking into account the different dilution processes during sampling preparation. The determinations were performed considering a pool of mycotoxins standards, including aflatoxin (AF) B_1, fumonisin (F) B_1, ochratoxin (OTA), zearalenone (ZEN), deoxynivalenol (DON) and patulin (PAT). The obtained bioacessible fractions values in cereal based infant food were 67%, 51% 16%, 11% and 7.5% for ZEN, DON, OTA, FB1 and PAT respectively. For fruit puree, bioaccessible values for OTA, DON, PAT and AFB_1 were 93, 51, 42 and 14% respectively. For infant formula, the highest bioacessibility was observed for AFB1 (87%), followed by ZEN (19%) and PAT (7%). The study demonstrated elevated bioacessible fractions of AFB1 in infant formulas available on the Brazilian market. OTA DON and ZEN also presented high percentages in infant cereal and fruit purees samples. Considering the frequency of consumption, these results suggest that the exposure of infants to mycotoxins through the diet may increase and represent a health concern. Therefore, the results of this study provide valuable insights into the potential health risks posed by infants' exposure to mycotoxins globally, which underscores the importance of implementing quality control measures in the production of infant formula and complementary foods. Furthermore, these findings enhance the understanding of the bioavailability of regulated mycotoxins in Brazil and contribute to the assessment of contamination risk in foods commonly consumed by young children.

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P01-15
Two-image paired inference model for automatic classification of growth inhibition in the Ames test

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The Ames test is a commonly used bacterial bioassay to assess the mutagenicity of chemicals. In the Ames test, test substances can induce not only reverse mutations, but also dose-dependent cytotoxicity which can hinder the accurate assessment of mutagenicity. To measure cytotoxicity, it is necessary to record bacterial growth inhibition (GI). This is typically done by quantifying thinning of the background bacterial lawn through stereomicroscopic observation by trained laboratory researchers. This process places a heavy burden on research staff and periodic checks are required to prevent differences in classification criteria between observers. It is therefore desirable to improve the efficiency of the process and to ensure objectivity in GI classification.

In a previous study, we built an inference model for automatic GI classification of *Salmonella* Typhimurium TA100 using a machine learning platform, DataRobot (DataRobot, Boston, United States). This model achieved an average inference accuracy of 97.6% in GI classification in 5 severity levels (Kum *et al.*, "Examination of automated growth inhibition classification in the Ames test by machine learning", The CORESTA Smoke Science and Product Technology Conference, Cancun, Mexico, Oct 2023).

In this study, we applied the same method to another bacterial strain typically used in the Ames test, TA1535, and found the accuracy of the inference model to be lower (85.1%) compared to strain TA100. To examine the cause of this decrease in accuracy, we compared the background lawn between strains TA100 and TA1535. For strain TA100, the background lawn on the solvent control (SC) plate changed little between test batches. In contrast, the background lawn on the SC plate of TA1535 was markedly different in each test batch.

To address this heterogeneity in background lawn with strain TA1535, we built a new 2-image paired interference model, with Auto-tuned K-Nearest Neighbors Regressor, using 1,157 pairs of images of SC plate and test sample plate as training data. Using this new 2-image paired inference model, the average accuracy for TA1535 was increased to 92.6%. In addition, comparing the 2-image paired inference model and the previous model (1-image model) for strain TA100 yielded similar average accuracy scores. For both types of inference model, Auto-tuned K-Nearest Neighbors Regressor gave the best results based on Root Mean Squared Error analyses.

In conclusion, we have developed a new 2-image paired interference model for Ames test strain TA1535. Training data based on images of SC plates improved the versatility of the inference model for TA1535. This method could be useful for building inference models for other bacterial strains used in the Ames assay.

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P01-16
A miRNAs-basedapproach to assess occupational allergic asthma mechanisms: from patients to an*in vitro*3D system

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Asthma is a heterogeneous and chronic disease of the lower airways that affects around 300 millions people worldwide. The most common and well-studied form of asthma is the allergic type affecting both children and adults. The main pathological feature of allergic asthma is due to the complex interactions between immune cells and immunological mediators. Recent evidences reported alterations in miRNA expression in a variety of lung diseases, including allergic asthma. Several miRNAs have been associated with asthma and airways inflammation but target identification remain not yet determined for the majority of the studies.

The specific aim of this study, entirely based on human cells, is to identify miRNA patterns and consequently which specific targets are involved in the occupational allergic asthma disease. miRNA expression through microRNA panels is performed on human biological samples from healthy and asthma donors, both serum and EBC, collected in collaboration with Dott. Liviero, Occupational Unit of Padova University. For the *in vitro* approach the human lung epithelial cells, namely Calu-3, are exposed to 5 respiratory sensitizers (hexamethylen, methylene diphenyl, and toluene diisocyanate; ammonium hexachloroplatinate; trimellitic anhydride), 1 skin sensitizer (2,4-dinitrochloro-

benzene) and 1 irritant (sodium dodecyl sulphate) through a liquid aerosols system (Cloud Alpha System – VITROCELL® Systems). The miRNAs expression is than compared between the two systems, human and 3D *in vitro* assay. The cell system will be also useful for further investigation on specific target involved in the occupational allergic asthma. The translational potential of the project is due to the use of a primary cell culture model as well as human samples. Furthermore, implementation of miRNA patterns could be useful as prevention tool (biomarkers for diagnosis) of allergic asthma.

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P01-17

Cell-free systems to synthesize and characterize novel nanopore applications

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Pore-forming toxins (PFTs) are a large family of toxins which can be found in various pathogens. Some well-known representatives of such pathogens are *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli*. Pores formed by PFTs insert into target cells' membranes forming open holes within the cell membrane. These holes allow a free exchange of ions and small molecules, causing an osmotic lysis in the process.

Today, PFTs are important for the development of biological nanopores, an emerging technology for biosensing and sequencing. They are used for DNA- and protein sequencing, disease marker screening as well as water purification. In order to build a functional nanopore based on a PFT, the pore must be characterized and subsequently adapted. This is commonly done by either adding additional proteins like a helicase for additional functionality or by systematically mutating PFTs to alter their pore specific behavior.

Due to their toxic nature, expression using cell-based systems can be difficult and requires optimization of the producing organism. These difficulties become even more important when screening a variety of different mutants. Cell-free protein synthesis can be used to circumvent these issues. In comparison to cell-based production, cell-free protein synthesis does not require living cells. Therefore, toxic properties of PFTs are not affecting the protein synthesis. Additionally, protein coding templates can be used freely without preceding cloning procedures as linear DNA templates such as PCR products can be used.

Using cell-free protein synthesis, we have synthesized and characterized different PFTs, namely alpha-Hemolysin from *S. aureus*, Cytotoxin K from *B. cereus*, Outer membrane protein G from *E. coli* as well as Perfringolysin O from *Clostridium perfringens*. Protein activities were analyzed qualitatively for hemolytic activity using blood agar plates. Quantitative characterization was assessed using electrophysiological analysis using an artificial lipid bilayer system. We were able to show, that active and characterizable proteins were produced using cell-free protein synthesis. Further, we were able to show indications of differing multimerization behavior due to point mutations. In addition, two novel PFTs, namely Delta-Actitoxin-Aeq1a (*Actinia equina*) and Delta-Stichotoxin-She4b (*Stichodactyla helianthus*) were expressed and characterized. Our data show that cell-free protein synthesis systems are a valid tool to establish novel biological nanopores and nanopore applications based on PFTs.

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P01-18

Potential of cell-free systems for the synthesis, characterization and application of ribosome-inactivating proteins

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Ribosome-inactivating proteins (RIPs) are ubiquitously found, especially amongst bacteria. RIPs are known to act enzymatically upon the eukaryotic 60S ribosomal subunit or translation elongation factor 2. Thus, RIPs are potent inhibitors of eukaryotic protein translation. This led to great scientific interest due to their potential harmful nature to human health but also pose a possibility in biomedical research as anti-cancer pharmaceuticals. Since the synthesis of RIPs is rather limited, their functional characterization is challenging. In this work we present cell-free protein synthesis (CFPS) as a platform for the synthesis, characterization and application of RIPs. CFPS is especially valuable for the synthesis of toxins as a lysate rather than viable cells as the toxic nature of the protein cannot harm the cell's viability. CFPS based on eukaryotic and prokaryotic lysates was used to synthesize Shiga-toxins originally derived from pathogenic bacteria. Shiga toxins are AB_5 toxins composed of an enzymatically active A-subunit and a pentameric B-subunit ring. We show that the synthesis of the individual subunits as well as the holotoxin was possible. The pentameric ring formation of the B-subunit could even be detected by performing classical SDS-PAGE followed by autoradiography. Further, the activity of the Shiga holotoxin could be shown in cell-based assays on HeLa and U87-MG cells at concentrations of 0.1 nM. The A-subunit as the integral part of the RIP was further characterized in an *in vitro* translation inhibition assay. It could be shown that the A-subunit inhibits the synthesis of model proteins, thus representing an enzymatically active toxin subunit.

In some cases, the mode of action of toxins is difficult to investigate. Hence, the Shiga A-subunit was modified by the introduction of non-canonical amino acids in order to fluorescently label the toxin. This technique can be used to track the toxin within the cell. We show that diverse labeling strategies could be established to fluorescently label the A-subunit. At last, in a biomedical interest, the A-subunit of the Shiga-toxin was fused to an interleukin 13 (IL13) in order to address glioblastoma cells as a targeted toxin. Further analyses of the targeted toxin on HeLa, U87-MG and SH-SY5Y were performed. These data showed that the cells expressing the IL13 receptor were directly addressed by the targeted toxin and the target cells were apoptotic at concentrations of 1 nM. The receptor binding was further confirmed by ELISA binding assays. The functionality of the toxic moiety of the targeted toxin was also investigated in an *in vitro* protein translation assay and confirmed the activity of the toxic moiety within the fusion protein. Taken together these data show the versatile use of CFPS for the analysis and modification of RIPs.

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P01-19

An extended *in vitro* CALUX read-out panel for the assessment of steroidogenesis modulation by endocrine disrupting chemicals on 4 hormonal modalities in the H295R steroidogenesis assay

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Endocrine disruption, as an adverse outcome of exposure to chemicals interfering with the endocrine system, can lead to a range of health

problems. From a regulatory point of view, the endocrine disruption-directed EATS (Estrogen, Androgen, Thyroid and Steroidogenesis) modalities testing has become an important tool for regulatory bodies to ensure safety of chemicals reaching consumer market. EATS testing is in fact required in EU's Biocidal Products Regulation (BPR) and Plant Protection products (PPP) for certain categories of chemicals. One of the assays applied in the context of EATS testing is the H295R steroidogenesis assay [1]. The H295R assay is a well-established *in vitro* assay designed to assess the impact of chemicals on the production of steroid hormones using the determination of 17β-estradiol and testosterone levels modulation as an endpoint, which are then quantified using LC/MS or antibody-based assays. Recently, an alternative cell-based bioassay read-out method has been proposed for the H295R assay, which makes use of the AR and ERα CALUX receptor transactivation assays [2].

However, the effect of chemicals disrupting steroidogenesis may not be limited to androgens and estrogens as the progestogen and glucocorticoid synthesis pathways can also be affected by these chemicals. Therefore, with the aim of capturing the effect of a chemical on multiple endocrine modalities (androgens, estrogens, glucocorticoids and progestogens) in the H295R assay, we propose to extend the H295R-CALUX read-out panel from 2 to 4 hormonal read outs via the addition of the GR and PR CALUX assays [3] for glucocorticoids and progestogens hormonal modalities. In this method, the H295R exposure is conducted as described in the OECD TG456, cytotoxicity in H295R is determined and the medium is harvested to be analysed in the 4 different CALUX assays. Prior to including those two novel read-outs, the glucocorticoid and progestogen hormonal activity in H295R were monitored over time and quantified using the PR and GR CALUX and proper pathway induction/inhibition controls for H295R were selected. Then the 4-assay read-out panel was tested on a set of 12 chemicals from the Conazoles family, of which members are known to have effect on at least one of the 4-tested steroidogenesis modalities. The direct effects of the test compounds on the nuclear receptors in the respective CALUX assays (ERα, AR, GR, and PR agonism and antagonism) as well as cytotoxicity, were also established to exclude false positives in the CALUX read-out. In conclusion, from a single H295R exposure, effects of test compounds on four steroid hormone synthesis pathways could be captured.

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P01-20
Gender-specific toxic effects of methyl mercury and thimerosal on the SH-SY5Y neuroblastoma cell line

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Purpose: Humans are frequently exposed to organic forms of mercury, particularly to Methyl mercury (M) and ethyl mercury (thiomersal, T). Although many physiological processes in the organism occur similarly for both genders, there are also particular differences in some biological processes. This study aimed to investigate the gender-specific neurotoxic and epigenetic effects of thimerosal (T) and/or methyl mercury (M) in SH-SY5Y cells.

Methods: Study groups were control group; T group; M group; high testosterone/low estradiol+T (TT) group; high estradiol /low testosterone+T (ET) group; high testosterone/low estradiol+methylmercury (TM) group; high estradiol/low testosterone+M (EM) group. Cells were exposed to the inhibitory concentrations 20 (IC_{20}s) of M and T for 24 h. For high estradiol/low testosterone medium testosterone and estradiol, doses were 0.1 μM and 7.5 μM, respectively. For high testosterone/low estradiol medium, testosterone and estradiol doses were 1 μM and 0.75 μM, respectively.

Results: Serotonin levels increased in M, TM and EM groups while serotonin transporter (SERT) levels were significantly higher only in T group compared to control group. Dopamine levels dramatically increased in TT and TM groups compared to control group. Dopamine transporter (DAT) levels were only higher in ET group. There were no significant alterations in global DNA methylation levels vs. control. Histone H3 acetylation of M and TM groups were markedly higher in M and TM groups while H3 acetylation was significantly lower in TT group compared to control ($p<0.05$). The results showed that neurotoxic agents might exert different effects in different genders and gender may be an important factor in the occurrence of neurodegenerative diseases in different clinical presentations and with different incidences.

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P01-21
Determination of 9 Bufotoxins by LC-MS/MS

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A method was developed for the detection of 9 bufotoxins (gamabufotalin, resibufagin, desacetylcinobufagin, resibufogenin, cinobufotalin, bufotalin, cinobufagin, bufalin, and cinobufaginol) in human blood by ultra-high performance liquid chromatography-quadrupole/electrostatic field orbital trap high resolution mass spectrometry (LC-Q/Exactive MS). 1.5 mL of acetonitrile was added in 0.5 mL of blood to precipitate proteins, and then was purified with a Hybird solid phase column. A ACQUITY UPLC HSS T3 column (2.1 mm×150 mm×1.8 μm) was selected. The mobile phase A consisted of 0.1% formic acid in water and B consisted of acetonitrile with a gradient elution programfor separation. The mass spectrum was analyzed by electrospray ion source and positive ion mode. The detection limit of 9 bufotoxins in blood and urine can be less than 1 ng/mL, and the lowest can reach 0.1 ng/mL. The calibration curves of 9 bufotoxins were in good linear over the range from 20 ng/mL to 200 ng/mL ($R^2>0.995$) in spiked blood matrix. The extraction recoveries of 9 bufotoxins from blood ranged from 71.1% to 86.7%, the matrix effect ranged from 98.4% to 106.3%, the intraday precision was within 10%, and the interday precision was within 15%. The method was used to analyze the body fluids collected in animal experiments. The results showed that all 9 bufotoxins had been detected. This method has high sensitivity and broad applicability, and can be used for the identification in bufotoxin poisoning cases.

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P01-22

Anticancer effect of arsenic compounds on apoptosis in oral cavity cancer cells

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Arsenic is a well-documented environmental toxicant. Epidemiological survey implicates that exposure to arsenic will induce neurotoxicity and peripheral vascular disease (known as blackfoot disease). However, arsenic trioxide (ATO) has also been used for medicinal purposes, originally to treat acute promyelocytic leukemia (APL), showing ability for anticancer treatment. Oral cancer has been in top 10 common cancers for decades in Taiwan, and the incidence rate still increases year after year. Around 75 percent of oral cancers are linked to modifiable behaviors, such as tobacco use and excessive alcohol consumption. Also, betel chewing in some certain areas, especially in Southeast Asia, is known to be a strong risk factor for developing oral cancers. Due to the high rate of occurrence and mortality, three oral squamous carcinoma cells (Fadu, OEC-M1, and OC3) treated by sodium arsenite (NaAsO2) and dimethylarsenic acid (DMA) were investigated to determine whether the arsenic compounds could be the anti-cancer agents. Results show that cells appeared rounded up and became membrane blebbing after treatments with NaAsO2 (1 μM) and DMA (1 mM) for 24 hr in OEC-M1 and OC3 cell lines, and NaAsO2 (10 μM) and DMA (5 mM) for 24 hr in Fadu cell line, respectively. These morphological changes revealed characteristics of apoptosis. In cell viability test, the surviving percentage of all three cell lines significantly decreased as the dosage of arsenic compounds increased (10 to 100 μM NaAsO2 and 1 to 100 mM DMA). The impact of arsenic compounds on cell cycle regulation was detected by flow cytometry. Results showed that the percentage of subG1 and G2/M phase cells among three cell lines increased in both NaAsO2 and DMA treatments. In addition, activation of the caspases, such as caspase-8, -9, and -3, and cleavage of poly ADP-ribose polymerase (PARP) were examined by western blot, and results showed that NaAsO2 and DMA-activated caspase-8, -9, and -3 cleavages. Moreover, both arsenic compounds could activate JNK, ERK1/2, and p38 phosphorylation among these cell lines. Taken together, NaAsO2 and DMA could induce cell apoptosis through extrinsic and intrinsic apoptotic pathways and cause the activation of MAPK pathway in Fadu, OEC-M1, and OC3 oral cancer cell lines.

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P01-23

Optimization of predictive liver toxicity modeling based on the drug-induced cholestasis index

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Cholestatic drug-induced liver injury (cDILI) is a frequent reason for failure and withdrawal during pre-and postmarketing stages of drug development. Strategies for reliable detection of cDILI in early drug development are therefore urgently needed. Drug-induced cholestasis index (DICI) was previously introduced as a metric for assessing cholestatic potential of drug candidates. DICI is calculated as the ratio between the cytotoxicity inflicted by a drug in the presence and absence of bile acids. DICI cut-off values of 0.78 and 0.8 have been suggested, below which a drug is flagged for increased cDILI probability.

The present study investigated the applicability of DICI in a high-throughput and large sample setting with focus on leveraging predictive liver toxicity modeling. For this purpose, 58 well-documented drugs were selected and categorized into 3 classes, namely drugs inducing (*i*) cDILI, (*ii*) non-cholestatic DILI, and (*iii*) not inducing DILI. 3D liver spheroid cultures of human hepatoma HepaRG cells were exposed to 9 half-log spaced concentrations of each drug based on the Cmax value and solubility limits for 1, 3 and 7 days in the absence or presence of a 50x concentrated mixture of the 5 most common human bile acids. ATP-based cell viability profiling was performed and DICI values were computed for all drugs and time points. In addition, the area under the curve ratio and the occurrence of a trend in cytotoxicity were included as modeling descriptors for categorization. As such, 3 time-related scenarios were considered upon modeling: (*i*) including individual time points, (*ii*) encompassing all 3 time points simultaneously (mixed model), and (*iii*) selecting the smallest value among the 3 time points (aggregated model). Categories were modeled on a nominal or ordinal scale. Combined models for categorization of the drugs were developed using the R software. Performance of the models was investigated using 10 replicates of a 5-fold cross-validation.

Applying DICI with a cut-off value of 0.8 resulted in a sensitivity of 0.85, 0.00 and 0.21 and a specificity of 0.29, 1.00 and 0.74 for the cDILI, DILI and non-DILI classes, respectively. From the 36 predictive models generated, the best performing models originated from using all descriptors and the ordinal scale for either the 7-day time point mixed model or the 3-day time point. The former model displayed a sensitivity of 0.84, 0.00 and 0.74, and a specificity of 0.58, 1.00 and 0.72, and the latter showed a sensitivity of 0.55, 0.00 and 0.94, and a specificity of 0.76, 1.00 and 0.49, for the cDILI, DILI and non-DILI classes, respectively. While these models were unable to identify DILI drugs, the 7-day time point proved to be most sensitive to identify cDILI drugs and the 3-day time point correctly identified 94% of non-DILI compounds. Overall, this study went beyond the standard DICI quantification and provided an optimized approach for *in vitro* predictive liver toxicity modeling purposes.

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P01-24

Investigation of the proliferative and apoptotic effects of Turkish endemic snake venoms on prostate cancer cell lines

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This study aims to examine the proliferative and apoptotic effects of some endemic snake venoms (*Macrovipera lebetina obtusa, Montivipera xanthina, Walterinnesia morgani*) on prostate cancer cell lines. Prostate cancer is one of the most common types of cancer in men. According to US data, it is the second deadliest type of cancer in men and is a new It is important to develop treatment approaches. Therefore, the discovery of potential drug candidates obtained from natural sources is of great importance in cancer treatment.

In the study, the method used was to determine the cytotoxic poison dose obtained by WST-1 and IC50 determination by cultured cancer cell lines (DU145 and PC3). Determination of the proliferative-apoptotic effect with biomarkers (Ki67, caspase-3, caspase-6, caspase-9, Bcl2, Bax, and Annexin V) by immunofluorescence method and biostatistical evaluation of the data will be presented. The poisons to be

used are new in the treatment of prostate cancer, as they will be tried for the first time. They have the potential to offer an effective approach. Snake venoms attract great interest in medical research due to the bioactive compounds they contain.

Our study investigated the cytotoxic and anti-proliferative effects of endemic snake venoms on prostate cancer cell lines and revealed dose-dependent cytotoxicity with IC50 values ranging from 9.7 to 18.3 µg/mL. Treatment with snake venoms resulted in significant reductions in Ki67 expression; this is indicative of inhibition of proliferation. Furthermore, snake venoms induced apoptosis in prostate cancer cells, as evidenced by increased expression of pro-apoptotic markers (caspase-3, caspase-6, caspase-9, and Bax) and decreased expression of anti-apoptotic marker Bcl2. Annexin V staining confirmed the induction of apoptosis, with the percentages of Annexin V-positive cells ranging from around%30. These findings highlight the potential of snake venoms as novel therapeutic agents in the treatment of prostate cancer and warrant further investigation into their mechanisms of action and clinical efficacy.

The results of the study show that the venom of endemic snake species affects the prostate. By elucidating its effects on cancer cell lines, it may reveal the potential to develop new treatment strategies. The data obtained will make a significant contribution to identifying a new target for prostate cancer treatment and evaluating the usability of naturally derived compounds. The study aims to contribute to the literature by filling a gap in the field of prostate cancer treatment and contributing to the development of more effective and low-side-effect treatment options.

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P01-25
Comparative toxicology and pharmacology of covalent and noncovalent NRF2 activators in human macrophages

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Nuclear factor erythroid 2-related factor 2 (NRF2) is a master transcriptional regulator of cellular defense systems that binds to the antioxidant response element (ARE) to drive the expression of more than 200 cytoprotective and antioxidant genes. Deficiencies in the NRF2 pathway increase susceptibility to numerous pulmonary inflammatory diseases. Given the pivotal role NRF2 plays in regulating many cellular defense systems, transcriptionally altering the NRF2-ARE pathway is a promising strategy for the prevention or treatment of many chronic diseases.

Activating NRF2 can occur through covalent or non-covalent modification of its cellular inhibitor; KEAP1. Sulforaphane (SFN) and dimethyl fumarate (DMF) react covalently with the critical cysteine residues of KEAP1, thereby blocking the ubiquitination of NRF2 and stimulating the expression of NRF2-regulated cytoprotective genes. New Keap1 protein-protein interaction (PPI) inhibitors were recently identified through fragment-based drug discovery and these compounds are cell-active noncovalent inhibitors.

This study utilized NRF2 activators at 10 µM including electrophilic compounds such as SFN and DMF, as well as two previously uncharacterized PPI inhibitors (Compound 1 and 2). Both wild-type (WT) and NRF2 knockout (KO) cells were utilized and cellular differences in viability, RNA expression through RNA-seq, nuclear NRF2 levels and post-translational changes of nuclear NRF2 through immunoblotting were quantified at separate time points including 3, 6, 24 and 48 hours.

Our results indicate that 100 µM SFN and DMF were significantly toxic to macrophages after 24 and 48 hours. In addition, Compound 2 was more cytotoxic in human macrophages compared to Compound 1, but also elicited a stronger transcriptional gene expression. Interestingly, DMF, Compound 1 and Compound 2 generated a more specific (on-target) NRF2 gene expression profile compared to SFN. Many of the genes that were transcriptionally activated in all four conditions (152 genes total ($P<0.05$, FC>1.0)) were NRF2 target genes. The on-target effects of Compounds 1 and 2 were validated in NRF2 KO cells because gene expression in NRF2 KO cells significantly changed approximately 150 genes total. SFN induced a significantly different gene expression profile compared to DMF, Compound 1 or Compound 2 that included 1192 upregulated genes and 1826 downregulated genes in WT cells. More than 2000 genes were significantly changed with SFN in NRF2 KO cells indicating that other transcription factors are activated in the presence of SFN.

Our initial results indicate that SFN is a distinct NRF2 activator that elicits strong NRF2-dependent and -independent responses and that certain covalent and noncovalent NRF2 activators can generate similar cellular responses in macrophages. These previously unknown cellular responses will be investigated further and may improve current and future clinical drug design.

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P01-26
Assessment of endocrine pancreas toxicity – identification of mechanisms of toxicity using Min6 mouse beta-cell model

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Purpose: It if of high importance to identify functional *in vitro* tools to be able to mitigate pancreas toxicity when there is a potential target-mediated risk, or when pancreas pathology is observed in preclinical studies. Previously, our main focus has been assessment of glucose-stimulated insulin secretion, the primary function of b-cells. Since β-cells need to produce and secrete insulin and have a high protein production capacity, they are sensitive to dysregulation of protein folding. Therefore, the focus of this study was on ER stress, and the resulting cellular defense mechanism to avoid cell death, the unfolded protein response (UPR). Additional suggested mechanisms of b-cell failure are de-differentiation or cell death by apoptosis. The objective of this study was to identify assays with improved sensitivity that could provide increased understanding of the underlying mechanisms of b-cell toxicity.

Methods: The mouse b-cell line Min6 is widely used and allows high throughput. Insulin-secretion capacity was assessed by glucose-stimulated insulin secretion (GSIS) at 16.7 mM vs 2.8 mM glucose, and quantified by insulin enzyme-linked immunosorbent assay (ELISA). qRT-PCR was applied to measure gene expression of the ER stress genes CHOP and BiP, as well as the de-differentiation genes Pdx1 and Mafa. Cell death by apoptosis was assessed using the Caspase-Glo 3/7 assay. A focused subset of compounds known to interfere with important mechanisms of b-cell functionality, including Glimepiride, GSK2656157 (a Protein kinase R-like ER kinase (PERK)) inhibitor, Thapsigargin and Tunicamycin were included.

Results: Short-term Glimepiride treatment resulted in enhanced of insulin secretion at low glucose (>4-fold), increased CHOP gene expression (1.8-fold) and decreased Mafa gene expression (50%), without change in Caspase activity. Both the PERK inhibitor and Thapsigargin resulted in reduced insulin secretion at high glucose (>80%) and increased CHOP gene expression (>8-fold), followed by enhanced Caspase-3/7 activity (2- and 6-fold, respectively). Thapsigargin also caused increased BiP gene expression (>1.8-fold) and decreased Pdx1 expression (50%). Tunicamycin caused similar effects as Thapsigargin, but also resulted in reduced expression of Mafa gene expression (>90%).

Conclusion: In summary, the data from this study showed that measurement of ER stress and β-cell maturity gene expression together with Caspase activity are sensitive tools to detect β-cell toxicity and give some insight into the mechanism of toxicity as well as the severity. Further work is required to include more compounds and additional mechanisms of toxicity to increase the likelihood of detecting and predicting endocrine pancreas toxicity caused by future drug compounds. Moreover, there may be interspecies differences and investigation on whether these results can be extrapolated to humans is needed.

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P01-27
Possible environmental factors influencing microtubule disruption and binucleation as determined by an *in vitro* cell coculture system and high throughput screening

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Purpose: High rates (56%) of whole genome duplications (WGD) events – doublings of the entire complement of chromosomes are characteristic of metastatic lesions [1], which are a major cause of death and are associated with poor treatment efficacy of cancer [2]. Brown *et al.* found no differences in the genetic drivers of WGDs among patients with different rates of WGDs, suggesting that these events may be influenced by epigenetic or environmental factors [3]. To find possible environmental causes for WGD, we tested whether different environmental and engineered nanomaterials can influence cell proliferation, perturbed microtubules and mitosis, and trigger binucleate cell formation. This study may thus contribute to the understanding of the mechanism of action of particle-induced genotoxicity and cancer.

Methods: We exposed lung epithelial cells in monoculture or coculture with murine lung macrophages to the following different groups of materials: 4 particles produced by fuel combustion: 3 diesel exhaust samples obtained from a diesel engine using different fuel types, and a carbon black sample; 3 engineered carbonaceous nanoparticles: graphene oxide and 2 types of multi-walled carbon nanotubes; 2 nanoclays; and 4 metal oxides. We performed 3–4 biological repeats of the experiment for each material, each in 1–3 technical replicates. From the 24h time-lapse fluorescence and scattering microscopy images, we quantified the following responses: rate of binucleate cell formation, cell proliferation rate, microtubule disruption in epithelial cells (colocalization of nanomaterial and microtubule fibers), perinuclear damage in epithelial cells (colocalization of nanomaterial and dense membrane/protein structures in the perinuclear region), formation of extracellular tubulin debris, and division cycle impediment of epithelial cells.

Results: Among the tested environmental particles, diesel exhaust, carbon black, and one type of nanoclay significantly increased the rate of whole genome duplication (WGD) / binucleate cell formation in a coculture of lung epithelial cells with macrophages. This model successfully mimicked chronic lung inflammation in mice after single nanomaterial exposure [4]. The effect was less pronounced in a lung epithelial cell monoculture. The suggested mode of action involves disrupting microtubules, lipid-containing cellular organelles in the perinuclear region, and decreased cell proliferation rate with impeded mitosis. Each of the studied materials triggers a different combination of subcellular changes. We also observed these effects, although less pronounced, for two types of engineered nanoparticles, TiO_2 nanotubes and multi-walled carbon nanotubes.

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P01-28
The effects of chlorinated paraffins on thyroid hormone metabolism in human HepaRG cells

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Chlorinated paraffins (CPs) are produced at a high volume and are widely used as additives in industrial products, and are ubiquitously present contaminants in the environment and in food. CPs are generally categorised based on their carbon chain length, i.e. short chain CPs ($C_{10–13}$, SCCPs), medium chain CPs ($C_{14–17}$, MCCPs) and long chain CPs ($C_{>17}$, LCCPs) with different chlorination degrees (30%–70% by molecular weight). In animal studies, liver, kidney and thyroid were identified as target organs, and in particular altered thyroid hormone levels and hypertrophy of thyroid follicular cells are considered to be potentially relevant to humans. However, the underlying mechanisms, and therefore their actual relevance to humans remain unclear. To better understand the mechanism of action of CPs, HepaRG cells were exposed to 8 CPs (3 SCCPs, 3 MCCPs and 2 LCCPs), followed by transcriptomic analysis using RNA sequencing. The results revealed, among others, an increased gene expression of uridine diphosphate glucuronosyltransferases (UGT). Increased expression of UGTs can lead to increased glucuronidation and excretion of thyroxine (T4), which can at least partially underly the observed thyroid toxicity. To assess whether the observed increased UGT gene expression changes translate to increased T4 glucuronidation activity, the induction of T4 glucuronidation by SCCPs was assessed in HepaRG cells. In addition, the gene expression of eight selected enzymes associated with T4 glucuronidation was quantified using RT-qPCR. The results show that concentration-dependent increased gene expression of UGTs by CPs also translates to concentration-dependent increased glucuronidation of T4. Altogether, the results of this study using human-relevant hepatocytes suggest that CPs may disrupt thyroid hormone homeostasis, as is observed in animal studies, via inducing UGT enzyme gene expressions, and increased glucuronidation of T4.

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P01-29

Assessment of *in vitro* approaches to identify succinate dehydrogenase inhibitors

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Succinate dehydrogenase inhibitors (SDHis) belong to a class of fungicides that interfere with the capacity of mitochondria to generate energy and are used in agriculture to combat a range of mold and fungal diseases. Concerns raised over the human safety of such chemicals by a group of French scientists triggered the organisation of an expert working group by the French regulatory authority, ANSES, to address the concerns. Specifically, the remit of the working group was to evaluate the scientific literature and to determine whether an update in data requirements and adjustment of existing toxicological reference values (TRVs) for registered SDHis would be necessary.

In anticipation of the outcome of the expert review in terms of potential additional data requests, a series of *in vitro* studies have been conducted to explore the interference of the electron transport chain (ETC) by reference ETC inhibitors and proprietary, non-developed SDHi compounds provided by Bayer and Syngenta. The objectives of this project were to identify the most sensitive and relevant mammalian cells to use in such experiments, to determine if any relevant species differences exist (human, rodent, target fungi) and if interference was observed, to establish effect concentrations. Five reference inhibitors of Complexes I – III of the ETC and 5 proprietary SDHi fungicides were evaluated in rodent and human cryopreserved hepatocytes and in RPTEC cells for effects on mitochondrial membrane potential (MMP) and for ETC specificity using Seahorse technology. Complex II, complex II/III and complex I/III activities were also determined biochemically using mitochondria isolated from *Botrytis cinerea* (mold), *Zymoseptoria tritici* (fungus) and rat heart.

The data indicated that the seahorse assay was able to correctly identify the five reference compounds in each cell model whereas evaluation of MMP failed to detect both reference and proprietary compounds in most of the cell models. Marked species differences were identified when testing the proprietary compounds for biochemical activity of the various complexes with at least a 50-fold difference in activity between mammalian and fungal species in the majority of cases.

Overall, the data indicate that the proprietary SDHis tested were selective for the fungal SDH target enzyme. Furthermore, there was no significant difference in species sensitivity between human and rodent SDH in the *in vitro* assays used, indicating that the rodent is a relevant surrogate model to assess the human safety of SDH inhibitors.

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P01-30

Application of a human *in vitro* reporter assay to gain insight into the toxicological mode-of-action of chemicals

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Purpose: Traditional toxicological hazard approaches often fail to adequately predict the toxicity of chemicals to humans. To improve the *in vitro* safety assessment and to extrapolate the results to *in vivo* exposure, it is important to understand the underlying mechanisms of toxicity.

Methods: We previously developed a human *in vitro* reporter assay, ToxProfiler, that provides a comprehensive understanding of the toxic properties of chemicals by evaluating the activation of seven major cellular stress response pathways. The assay contains reporters for oxidative stress, cell cycle stress, ER stress, ion stress, protein stress, autophagy and inflammation. Here we applied ToxProfiler to identify the toxicological MoA of 10 unannotated test substances by comparing their toxicity fingerprints with a database of reference chemicals with known toxicological effects.

Results: We generated the toxicity profiles for 10 unannotated compounds and performed hierarchical clustering with a large database of reference compounds. Clustering is based on the preferential activation of the seven different stress reporters in ToxProfiler. Amiodarone as one of the unannotated compounds, induced a strong autophagy and ER stress in ToxProfiler and was clustered together with compounds that are known to affect the cellular calcium levels by direct or indirect blocking of calcium channels. This toxicity profile is often observed for chemicals that induce cardiotoxicity (e.g. diltiazem and verapamil). Bicalutamide induced similar toxicity profiles as known mitochondrial toxicants (e.g. troglitazone, griseofulvin, and Rotenone). In ToxProfiler, these compounds induce strong ER and oxidative stress which are linked to pathological mechanism of drug-induced liver injury (DILI).

Conclusions: Overall, we demonstrated that profiling and clustering of chemicals based on their toxicity signatures can be used to identify the toxicological MoA or cellular targets of unknown substances and in some cases their *in vivo* toxicity.

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P01-31

Preliminary study: measurement of urinary unconjugated pteridines after establishment of the HPLC method

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Pteridines, a group of endogenous heterocyclic compounds, serve as useful biomarkers in clinical diagnosis. Elevated pteridines levels have been reported in various pathologies, including viral infection, cancer, renal and autoimmune disorders. Among them, unconjugated pteridines like neopterin and biopterin, oxidized forms of tetrahydrobiopterin, are of particular interest due to their involvement in inflammation, oxidative stress, cellular immune response and neurotransmitter alterations. A High-Performance Liquid Chromatography (HPLC) method has been developed to determine neopterin, biopterin and creatinine levels in urine samples from healthy volunteers using a single sample collection. The high concentration of pteridines in urine samples allows for the dilute-and-shoot method, involving simple microfiltration before chromatographic analysis. Fluorescence detection, with excitation at 353 nm and emission 438 nm, was utilized due to the strong fluorescence signal emitted by oxidized pteridines. UV detection wavelengths were set at 235 nm. Reversed-phase chromatography was conducted on a C18 column with a mobile phase of potassium dihydrogen phosphate containing 2.5% methanol, at a flow rate of 1 mL min^{-1} and a pH of 7 with an injection volume of 25 µl. The column temperature was maintained at 22°C, and the chromatographic separation was achieved in 15 minutes. Under this condition, the retention times for creatinine, neopterin and biopterin were approximately 2, 4 and 10 minutes, respectively. Three replicates of each concentration level were analyzed. The neopterin and biopterin levels were standardized by their ratio to creatine, expressed as µmol neopterin or biopterin/mol creatinine, otherwise flowrate of urine cause alteration in concentration of pteridines. Our method yielded mean concentrations of 273.25 µmol/mol of creatinine for neopterin and 2459 µmol/mol of creatinine for biopterin in four urine samples. Calibration curves were

generated using standard mixtures of neopterin, biopterin and creatinine at seven concentration levels. Stock solution was diluted up to 150, 125, 100, 75, 50, 25 and 12.5 times with ultrapure water. The linearity of the calibration curves (R2>0.99) confirmed the method's reliability. These standardized curves, developed in our lab, serve as a benchmark for future analyses in the quantification of neopterin and biopterin levels in urine samples.

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P01-32
Advancing *in vitro* nephrotoxicity prediction in drug safety with multiparameter imaging and analysis in RPTEC cells

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Drug-induced kidney toxicity remains as a significant concern in clinical practice accounting for 14–26% of cases in Acute kidney injury [1]. It is one of the major factors in the attrition and discontinuation of drug development programs, often being identified in later stages, thereby increasing costs for the pharmaceutical industry. This demands the need of nephrotoxicity assays at earlier stages of drug development to predict, detect and mitigate the risks of kidney toxicity. Previously, we have established an *in vitro* assay using conditionally immortalized proximal tubule epithelial cells (ciPTEC cells) to predict kidney toxicity [2]. We have now expanded the assay by using primary human renal proximal tubule epithelial cells (RPTEC) which are closer to kidney tissue, based on transcript profiling, and advanced the imaging and analysis pipeline. This multi-well plate-based assay is performed by dosing RPTEC cells with different concentrations of known nephrotoxic drugs and non-nephrotoxic controls for 48 hours. The cells are then subjected to multi-colour labelling using specific live cell and fixed cell dyes targeting different organelles, followed by high content imaging (HCI) at high resolution. The analysis involves detection and quantification of individual parameters and overall phenotype indicative of cellular toxicity, employing both manual image analysis and artificial intelligence (AI)-based tools to cluster cells. The assay generates data indicative of finer changes in nuclear morphology, mitochondrial, actin cytoskeleton and cell membrane properties, cell confluency, overall phenotype, and label intensities. The results from the assay enabled concentration dependent detection of toxicity parameters by extracting AC20 (20% activity concentration) values, and by integrating the clinical exposure data in humans, we were able to rank the compounds based on toxicity. The high-resolution imaging across different concentration ranges also enabled mechanistic understanding of initiation of toxicity for specific compounds at sub-cellular level. Interestingly we observed drug sensitivity differences between primary cells and the immortalised cell line, underscoring the importance of selecting appropriate cell types for specific questions about mechanistic understanding of drug-induced nephrotoxicity. This high throughput assay with primary cells is expected to expand our capabilities in precise identification and understanding of drug-induced nephrotoxicity and contribute in devising strategies to mitigate risks at different stages of drug development.

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P01-33
Toxicity induced by cigarette smoke and electronic cigarette aerosols

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Cigarette smoking represents a prominent factor of morbidity and mortality worldwide. Modified risk products (MRPs), such as electronic cigarettes (e-cigs), have been introduced recently to the market with great success, increasing popularity every year. The comparison between MRPs and traditional combustible cigarettes is pivotal for the evaluation of the potential health consequences of their use.

In this study, H292 human bronchial epithelial cells were exposed by air-liquid interface (ALI) to conventional cigarette smoke (1R6F reference cigarette, University of Kentucky) and e-cig aerosol of four different commercial e-liquids in order to compare cytotoxic and metabolic effects. Additionally, Reactive Oxygen Species (ROS) production was evaluated. H292 cells were exposed to 5 puffs of 1R6F cigarette undiluted smoke or 10 puffs of e-cig undiluted aerosols using the Borgwaldt LM1 smoking machine and the Borgwaldt LM4E vaping machine, respectively. The cytotoxic effects of cigarette smoke / e-cig aerosols were evaluated by Neutral Red Uptake (NRU) assay, cytofluorimetric Annexin V apoptosis analysis, and xCELLigence Real-Time Cell Analysis (RTCA) system. The mitochondrial status was evaluated with the use of fluorescent JC1 dye in High Content Screening (HCS) analysis system. The production of ROS in the smoke (from 9 to 45 puffs) / aerosols (from 20 to 80 puffs) was assessed through bubbling in PBS and the use of 2′,7′-Dichlorofluorescin diacetate (DCF-DA) in a "cell-free" assay.

1R6F combustible cigarette smoke exposure resulted in a significant reduction in cell viability in comparison to AIR control (32.02%±1.78 at 24 hours by NRU assay), with more than 70% of cells in advanced apoptosis / necrosis, and inability to grow. In addition, cigarette smoke induced a strong and long-lasting reduction in mitochondrial function in comparison to AIR control, which was noticeable as soon as 12 hours after the exposure. Moreover, it contained a high ROS level rising in a dose-dependent manner, corresponding to 508.85±15.65 μM of H_2O_2 for the 9-puff exposure up to 1885.8±144.24 μM of H_2O_2 for that of 45 puffs. On the contrary, e-liquids showed a significantly reduced cytotoxicity. More specifically, 90–100% of cells were viable at 24 hours by NRU assay after the exposure to e-cig aerosol, while apoptosis analysis and RTCA indicated 64.7–74.7% and 80–100% of viable cells, respectively. Furthermore, cells exposed to e-cig aerosols maintained mitochondria functionality and all vaporized e-liquids tested did not contain ROS.

These results support the reduced potential toxicity of e-cigs compared to combustible tobacco cigarettes in an *in vitro* model of human bronchial epithelial cells resembling real-life smoke/aerosol exposure.

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P01-34

Identification and toxicity assessment of volatile and semi-volatile non-intentionally added substances from polyester-based can coating

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Polyester based coatings are one of the technologies used in can coatings following the suspension of the intentional use of Bisphenol A (BPA) in food packaging in France. The aim of this study was to identify volatile and semi-volatile non-intentionally added substances (NIAS) from a single polyester-based formulation (5 batches) to evaluate their health risks in order to provide consumer's safety regarding consumption of canned food.

Extractions and migration tests of coated metal plates were performed with acetonitrile (ACN) (incubation at 40°C for 1 day) and ethanol (EtOH) 95% (incubation at 60 °C for 10 days) in Calipack migration cells.

Antioxidant degradation product 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionic acid (CAS RN 20170-32-5), or fenozan, was identified as a NIAS and confirmed by reference standard in both ACN and EtOH 95% samples using gas chromatography–high-resolution mass spectrometry.

To evaluate the toxicity of fenozan, several toxicity endpoints were investigated using different *in silico* quantitative structure-activity relationship predictive tools, such as Derek Nexus, Sarah Nexus, VEGA-HUB software and Danish (Q)SAR Database. Positive predictions for carcinogenicity in mice, developmental toxicity, as well as several endpoints of mutagenicity, endocrine disrupting activity, and organ toxicity (hepatotoxicity and thyroid toxicity) resulted from used *in silico* tools.

To confirm positive *in silico* mutagenicity alerts, fenozan was subjected to the *in vitro* Ames test (to test point mutation) in accordance with OECD 471 guideline, using three strains of *Salmonella Typhimurium* (TA100, TA98, and TA1535) and one strain of *Escherichia coli* (WP2) with and without metabolic activation system. A negative response was obtained in the Ames test for the four strains tested with and without metabolic activation system using direct incorporation and pre-incubation methods. Further, bioassays are required to verify the positive predictive outcomes for other toxicological endpoints to ensure the safety of polyester coatings.

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P01-35

Assessment of beta-cell functionality and toxicity – identification of mouse beta-cell line Min6 2D model and primary human islet 3D model as useful *in vitro* tools

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Purpose: Even though endocrine pancreas toxicity is not commonly occurring in drug development, it is of high importance to identify functional *in vitro* tools to mitigate pancreas toxicity when there is a potential target-mediated risk, or when pancreas pathology is observed in preclinical studies. The objectives of this study was to identify *in vitro* assays for b-cell toxicity relevant for mouse and human species, which would allow both short-term and long-term treatment.

Methods: To identify a simple assay with high throughput for assessment of b-cell toxicity, the mouse b-cell line Min6 was investigated. Since insulin secretion in response to a glucose challenge is the primary function of b-cells, insulin-secreting capacity was assessed by glucose-stimulated insulin secretion (GSIS) at 16.7 mM vs 2.8 mM glucose, and quantified by insulin enzyme-linked immunosorbent assay (ELISA). In parallel, cell viability was assessed by the CellTiter-Glo assay, to separate functional changes from cytotoxicity. A focused subset of compounds known to interfere with important mechanisms of b-cell functionality, including Diazoxide, Glimepiride, Thapsigargin, GSK2656157 (a Protein kinase R-like ER kinase inhibitor (PERKi)) and Exendin-4 were tested in either acute (1 h) or chronic (24 h) settings. To assess the mouse-human translation, the same compounds were assessed in primary human pancreatic islet microtissues, a more complex *in vitro* model derived from fresh human endocrine pancreas (InSphero). The human islets were treated with compounds for either 6 days (re-dosing at 3 days) prior to GSIS or 2 h during GSIS.

Results: The stimulation index (insulin secreted at 16.7/2.8 mM glucose) in the Min6 cells was more than 5-fold at baseline. In this cell model, Glimepiride and Diazoxide had acute stimulatory (4.7-fold) and inhibitory (>90% reduction) effects on insulin secretion during the low and high glucose condition, respectively. Thapsigargin and the PERKi showed decreased insulin secretion (>80% reduction) at high glucose after 24 h, with slight effects on cell viability at high concentrations. In the human islets, the stimulation index was more than 20-fold. Acute experiments showed enhanced insulin release by Glimepiride (28-fold) at low glucose and by Exendin-4 (4.5-fold) at high glucose as well as reduced insulin release by Diazoxide (>90%) at high glucose. Treatment with Thapsigargin and PERKi for 6 days resulted in decreased insulin-secretion capacity (>70% respectively >90% decrease) at high glucose. However, reduced viability was observed for both treatments.

Conclusion: This study has shown that the investigated tool compounds can be used to both stimulate and inhibit insulin secretion in presence of low or high glucose in the two cell models utilised. Moreover, a good correlation was demonstrated between the mouse and human models. The data set is currently expanded by testing a larger set of compounds.

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P01-36

Proeryptotic effects of non-functionalized polystyrene nanoparticles with different diameters

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Plastics in the environment are exposed to various factors, such as UV radiation, mechanical abrasion, temperature and biological agents, resulting in the degradation of plastics to microparticles (MPs) smaller than ≤5000 μm, and further to nanoparticles (NPs), whose dimensions are smaller than 1 μm. [1] Since MPs and NPs have been formed in the environment, they are widely distributed and pose a potential threat to living organisms. Microplastics have been detected in feces and human urine, as well as in tissues, like placenta, lung, blood and others.

Nanoparticles, which can be potentially more toxic than MPs, were detected in 2023 in the blood of the entire study group of 196 people, both healthy and sick donors. [2] NPs present in the blood can affect its cells, including erythrocytes. Therefore, in our study, we determined

the effect of polystyrene nanoparticles with different diameters on eryptosis induction in human red blood cells. Different concentrations of PS-NPs, including those, which are likely present in the human blood were tested to account for sublethal results.

Eryptosis is a form of programmed erythrocyte death, and is triggered by oxidative stress and an increase in cytosolic Ca2+ concentration, which leads to phosphatidylserine exposure on the cell surface [3] Eryptosis that is induced by various micro- and nanoparticles may depend on the level of ROS and oxidative stress (increase in caspase-3 activity) and/or on an increase in cytosolic Ca2+ level (activation of Ca2+-sensitive potassium (K+) channels, also called the Gardos channels, and increase in calpain activity).

We determined phosphatidylserine externalization and the levels of ROS, methemoglobin, and intracellular calcium ions in erythrocytes incubated for 24 h with non-functionalized polystyrene nanoparticles (PS NPs) with diameters of (~30 nm, ~45 nm, ~70 nm) in the concentration range of 0.001–100 μg/mL. It was found that only nanoparticles with a diameter of 30 nm induced an increase in phosphatidylserine externalization at a concentration of 100 μg/mL. In turn an increase in intracellular calcium ion level was noted at 50 μg/mL and 100 μg/mL of PS NPs with a diameter of 30 nm, and nanoparticles with a diameter of 44 nm at 100 μg/mL. Nanoparticles did not induce ROS formation and hemoglobin oxidation in the tested concentrations range. The largest PS NPs with a diameter of 70 nm did not cause any statistically significant changes.

The above data indicate that the smallest PS-NPs probably cause PS externalization by calcium ion influx. The increase of Ca2+ ion level leads to activation of Ca2+-sensitive potassium (K+) channels, ultimately resulting in the loss of water as it osmotically follows the loss of potassium chloride from the erythrocyte. Additionally, the increase of Ca2+ ion level activate calpain, which cause degradation of the erythrocyte's cytoskeleton.

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P01-37
The INSPIRE Initiative: using *in vitro* systems to predict respiratory toxicity of surfactants by pipetting and aerosol exposure

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Inhalation is a major route through which substances can cause toxic effects. The INSPIRE Initiative (*In vitro* System to PredIct REspiratory toxicity) aims at building scientific confidence in non-animal methods to assess inhalation toxicity. In this study, a human bronchial epithelial cell line (BEAS-2B) and a reconstructed human tissue model (MucilAir™) both grown at the air-liquid interface were used to predict the toxicity of two classes of chemicals. Building upon recently published data for the testing of silane vapors, a similar testing approach was used to assess surfactants exposed to cells as aerosols or liquids (pipetting).

BEAS-2B cells and MucilAir™ were exposed to five different concentrations of two surfactants (Triton X-100 and oleoyl sarcosine). The surfactants were either directly pipetted (30 μL) onto the apical side of the cells or aerosolized using a collision atomizer paired with a VITROCELL® 6/4 exposure system. Cellular effects were assessed ~24 hours (in BEAS-2B and MucilAir™) or seven days (in MucilAir™) after exposure. For both cell systems, these effects included cytotoxicity (lactate dehydrogenase release), cell viability (resazurin-based assay), inflammatory response (secretion of interleukins 6 and 8), and additionally for MucilAir™, transepithelial electrical resistance (TEER), cilia beat frequency (CBF) and average active area (AAA), and histology.

Results show concentration-dependent increase in cytotoxicity and decrease in cell viability in the two cell systems and for both exposure methods. Benchmark dose modelling revealed relatively homogeneous point of departures irrespective of the exposure method for BEAS-2B and oleoyl sarcosine. Generally, BEAS-2B cells are more sensitive in comparison to MucilAir™. These observations are similar to previous studies testing silane vapor exposures in these cell systems.

These results help to further increase scientific confidence in the use of *in vitro* testing approaches and show how they can be used to meet regulatory requirements. The study also helps to identify what cellular effects, exposure methods, and model systems may be most appropriate for use, depending on the purpose of testing.

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P01-38
Low cytotoxicity of environmentally relevant microplastics in lung cells and a co-culture

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Microplastics (MPs) are formed when plastic material breaks down into ever smaller pieces due to abrasion and ageing from environmental factors. Large scale and increasing plastic use have led to the accumulation and persistence of MPs in the environment with human exposure becoming inevitable. Inhalation is a significant route of exposure to MPs indicated by that MPs have been detected in human lung tissue and bronchoalveolar lavage samples (1, 2). However, the health risks associated with MP inhalation and in particular with environmentally relevant (irregularly shaped and aged) MPs are largely unknown.

On this ground, the aim of this study was to investigate the potential adverse effects of aged and irregularly shaped MPs *in vitro* following short term exposure of human bronchial epithelial cells (HBEC), macrophages (differentiated THP-1 cells), and a co-culture of these. MPs were produced through simulation of weather-induced changes. Polystyrene (PS) and polyethylene terephthalate (PET) MPs were UV-aged and low-density polyethylene (LDPE) was aged by thermo-oxidation. Particle-cell interactions were investigated using Nile Red dye and confocal imaging. The toxicity assessment covered cell viability, generation of reactive oxygen species (ROS) and cytokine release endpoints.

Confocal imaging confirmed particle-cell interaction. Further, quantification of particle-cell interaction indicated that after 3 hours of exposure, 5–10% of applied particles were adhering to cells. Viability of bronchial cells in monoculture or co-cultures with macrophages was generally not affected after MP exposure. In contrast, macrophage monocultures showed increased viability/metabolic activity compared to control after exposure to the highest LDPE dose (500 μg/mL). In general, no increase in intracellular ROS was observed except in macrophages when treated with aged PS at the highest dose (500 μg/mL). The measurement of cytokines released by macrophages revealed no

statistically significant increase in levels of IL-8, IL-6, IL-1β, or TNFα following exposure to either particle. In conclusion, environmentally relevant microplastics showed little or no cytotoxicity and inability to generate ROS or trigger an inflammatory response in cell models of relevance for lung exposure.

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P01-39
Mitochondrial toxicity screening to support the R&D crop protection pipeline

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Toxicity assessment is key in the development of new chemicals to avoid costly late stage attritions. Mitochondrial toxicity is underlying several organ toxicities, such as liver, cardiac, and kidney toxicity as well as neurotoxicity. In pharma, mitochondrial toxicity testing is routinely done to test the pipeline and many publications are available on the subject. We can apply those tests to also evaluate agrochemical safety and support the pipeline.

In Bayer Crop Science, a first screen comprises of biochemical assays to identifying species specificity between pest and rat mitochondrial complexes I and III. Compounds with sufficient differences in binding between both species, can be tested in higher tiered assays. One of the first tests done in investigative toxicology is based on the shift of cytotoxicity of HepG2 cells grown in different medium conditions promoting ATP synthesis by oxidative phosphorylation (OXPHOS) or glycolysis, the Glu-Gal method. A higher tiered assay for the most promising compounds is to assess indirectly OXPHOS and glycolysis, by means of defining oxygen consumption and proton production (XF96 Seahorse, Agilent). In addition, the ToxProfiler (Toxys) assay gives insight on the deregulation of several cell stress pathways, by means of biomarker genes. We are also supporting promising future developments such as cell painting and high throughput transcriptomics show promise to be applied early in screening and gain valuable knowledge around biology-induced effects. Indeed, signature-based clustering can be meaningful only after enough reference compounds have been tested.

Mechanistic approaches are needed besides a mitochondrial toxicity screening. For example, to support the elucidation of Adverse Outcome Pathways (AOPs) for the resubmission of a substance for which a potential mitochondrial function concern was identified. *In vitro* mechanistic studies include using distinct cell types to address cell specific sensitivities, adapting cell medium (Fatty Acids for B-oxidation), performing specific HCA or flow cytometer endpoints.

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P01-40
Contemporary *in vitro* toxicology assessment of a novel Herbal Heated Product (HHP) compared to cigarette smoke

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To support Tobacco Harm Reduction (THR) there have been many new emerging product categories coming to market, with the aim to give adult smokers alternative and potentially reduced harm alternatives to traditional cigarettes. Heated Tobacco Products (HTP) emissions show reductions in the number of chemical analytes and *in vitro* toxicity as compared to traditional cigarettes due primarily to the absence of combustion. Here we present the *in vitro* assessment of a tobacco-free herbal heated product (HHP), assessed against reference cigarette responses.

Composition includes non-tobacco heated substrate, nicotine and flavours. Test products 1R6F reference cigarette and novel herbal heated product (HHP), variants were examined in two test matrices. Firstly, aqueous extracts (AqE) were produced under HCI or HCIm regime as appropriate using Glo™ Hyper X2 device for the variants. Test extracts were assessed in Real time Cell Analyser (RTCA) as a measure of cytotoxicity in H292 lung cells and ToxTracker™ screening assay for genotoxicity. Furthermore, whole aerosol experiments were also performed with undiluted aerosol exposure using the Korber LM4E vaping robot and MucilAir™ organotypic lung cultures (Epithelix), measuring cytotoxicity via MTT, trans epithelial electrical resistance (TEER) and cilia beat frequency and active area.

In all assays conducted, HHP variants showed reductions in cytotoxicity and genotoxicity compared to 1R6F research cigarette, showing their potential as a reduced harm product in comparison to traditional cigarettes.

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P01-41
De-risking new cancer therapeutics with *in vitro* platelet assays: comparing the effects of BH3-mimetics on platelet viability

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Background: Following the successful development of ABT-199 (Veneto-clax) for the treatment of chronic lymphocytic leukaemia (CLL) and acute myeloid leukaemia (AML), other BH3-mimetics that target BCL-XL have been investigated to treat solid tumours. However, new therapeutics have been limited by on-target and dose-limiting thrombocytopenia.

The PROTAC DT2216, designed to avoid on-target platelet toxicity of ABT-263 (Navitoclax), was reported to be platelet sparing in *in vitro* assays published in the literature. However, moderate on target platelet toxicity of DT2216 has been reported *in vivo*.

We present higher throughput *in vitro* platelet assays with improved capability for screening and de-risking such therapeutics in drug discovery and development.

Aim: To develop and validate *in vitro* platelet toxicity assays with improved capability for screening and de-risking BH3-mimetics and other therapeutics in development.

Methods: Washed platelets were isolated from healthy volunteers and incubated for 3 or 24 hr with ABT-199, ABT-737, ABT-263 and DT2216 prior to assessment of platelet viability by MTS and caspase assays.

Results: Both *in vitro* assays showed high reproducibility and there was good correlation between the MTS and caspase activity assays for predicting platelet toxicity. ABT-199 had little to no effect on platelet viability after 3 hr. A slight reduction in platelet viability was observed at high concentrations following 24 hr treatment. The pan-BCL inhibitors ABT-737 and ABT-263 both reduced platelet viability after 3 hr treatment and the potency increased at 24 hr. DT2216 was shown to reduce platelet viability in our *in vitro* assays at 24 hr and matches observations from *in vivo* studies.

Conclusion: Using BH3-mimetics with known effects on platelet viability, we have demonstrated the suitability of sensitive, higher throughput *in vitro* platelet viability assays that could be used for predicting *in vivo* platelet toxicity to de-risk new therapeutics in development.

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P01-42
In vitro test methods and petroleum UVCB substances: critical review of dosing methods

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In multiple regulatory contexts, *in vitro* test methods can be used, i.e. from prioritization and screening, to supporting chemical grouping and read-across of data for human health and the environment. It is anticipated that *in vitro* tests, as part of New approach methodologies (NAMs), will be used to a larger extent moving forward in place of whole organism *in vivo*testing. An important challenge of most *in vitro* testing methods is how to establish, maintain and confirm defined test substance concentrations throughout the test. This is particularly challenging for petroleum UVCB (Unknown or Variable composition, Complex reaction products or Biological materials) substances that typically contain a large number and variety of hydrophobic and (semi)volatile hydrocarbon constituents that are very prone to evaporative and sorptive losses. The ability to deliver and maintain stable exposure of petroleum substances in *in vitro* test systems is challenged by several factors, including: i) high surface area to volume ratios of multi cell well plates, which increases the likelihood of sorption to plate walls; ii) the inability to seal some test vessels (e.g., volatile constituents can escape from open test vessels and may contaminate neighbouring cell wells); iii) poor solubility of hydrophobic constituents in biological media and iv) presence of lipids and proteins in biological media may differentially bind individual constituents. Here we present the initial findings from a critical systematic review on the state of science of *in vitro* dosing methods, their challenges and their applicability in (eco)toxicological assessments of petroleum UVCB substances. In fact, due to the challenges mentioned, *in vitro* testing for these types of substances is generally performed to date using extracts derived from solvent extraction of the neat substance. Two different search strategies were used and combined for the literature search (search by specific terms, and in parallel a citation search based on 6 key papers on Sorption and loss challenges and Exposure control), in order to cover as much of the relevant literature as possible. The outputs will be used to identify relevant *in vitro* tests, dosing methods and adaptations best suited for petroleum substances in future research and regulatory testing activities.

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P01-43
Developmental toxicity assessment using human iPSCs by automated measurement of FGF signaling disruption

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Signal interactions are vital for the regulation of fetal development. We hypothesized that developmental toxicity eventually relates to signal disruption, and have reported an approach (DynaLux/c)[1,2,3] for evaluating developmental toxicity through an FGF-SRF signal reporter assay utilizing human iPS cells.

This approach uses human iPS reporter cells (SRF-Nluc) that luminesce in response to the FGF signaling pathway crucial for morphogenesis. We measured the luminescence intensity of the cells upon the addition of chemical substances to continuously assess signal disruption. Subsequently, we defined the curve area between the luminescence intensity curves of the treated group and the solvent control group as the Area Between Curves (ABC) and calculated the Sum of ABC. In this assay, chemiluminescence associated with FGF signaling activity was continuously monitored, demonstrating that signal disruption occurred at different time points depending on developmental toxicants. This approach demonstrated a notable accuracy of 89%, surpassing existing test methods. By integrating signal disruption over time, it was possible to accurately distinguish known developmental toxicants. However, since chemiluminescence was measured manually in our previous study, it was challenging to capture detailed temporal changes and to measure during the night. Therefore, in this study, we automated luminescence measurements to capture detailed and long-term luminescence alterations.

The continuous monitoring of chemiluminescence reveals that there were two peaks in 72 hours, unlike the single peak in 24 hours in our previous study. Moreover, substances with developmental toxicity causing expanded disruption after 24 hours, such as valproic acid, were more accurately detected.

The automation of luminescence measurements demonstrated the potential for more precise tracking of signal disruption. Future efforts will focus on optimizing measurement conditions and increasing the number of test substances to further elucidate the utility of this method.

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P01-44
Particulate matter constituents trigger the formation of extracellular amyloid β and Tau-containing plaques and neurite shortening *in vitro*

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Background and Purpose: Air pollution is an environmental factor associated with Alzheimer's disease, characterized by decreased cognitive abilities and memory. Despite a strong epidemiological correlation between exposure to airborne particulate matter and the development of the disease, the causal relationship between particulate matter in air pollution and Aβ plaque deposition with neurite degeneration in Alzheimer's disease remains controversial. Environmentally driven models of Alzheimer's disease are thus timely and necessary.

Methods: To address this, we investigated whether various types of particulate matter commonly found in polluted air, including metal oxides and carbonaceous particles, could trigger the formation of Aβ plaques and neurite shortening in an *in vitro* model. We exposed neuron-like cells, differentiated from the human neuroblastoma cell line SH-SY5Y, to different nanomaterials (TiO_2 nanotubes, Fe_2O_3, diesel exhaust particles, and CeO_2). We employed live-cell confocal fluorescent imaging combined with high-resolution stimulated emission depletion (STED) microscopy to follow the morphological changes of cells and the formation of extracellular amyloid β and tau plaques, visualised by fluorescently labelled mouse monoclonal antibodies.

Results: A high dose, single exposure of *in vitro* neuron-like cells to particulate matter constituents reproduces a neurodegenerative phenotype, including extracellular amyloid-β containing plaques and decreased neurite length. After 100 hours of exposure to TiO_2 nanotubes, γ-Fe_2O_3, and diesel exhaust particles, the median length of neurites shortened from 60 µm in control to 40, 32, and 30 µm, respectively (all $P<0.001$, Mann–Whitney U test). Particularly for exposure to γ-Fe_2O_3 and diesel exhaust particles, the relative proportion of short neurites (<10 µm) dramatically increased, while there were almost no very long neurites (>100 µm) left. In contrast, CeO_2 nanoparticles did not cause any reduction in length even after 100 hours, which is consistent with their beneficial effect observed in traumatic brain injury models. In the samples that shorten neurites the most, also more Aβ deposits were observed.

Conclusions: High dose *in vitro* exposure of neuron-like cells to particulate matter constituents, like diesel exhaust and iron oxide nanoparticles, reproduces a neurodegenerative phenotype, including extracellular amyloid-β-containing plaques and reduced neurite length and density. Although the exact mechanism behind this effect remains to be explained, the high dose rate *in vitro* model, comprising wild-type neuron-like cells, could serve as an alternative environmentally driven model of Alzheimer's disease.

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P01-45
Examination of 19 drugs and metabolites in hair samples by UPLC-MS/MS

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Aim: To establish a sensitive detection method for the analysis of 19 drugs and metabolites in the hair samples, including methylamphetamine, amphetamine, 4,5-methylene dioxyamphetamine, 3,4-methylene dimethamphetamine, 3,4-methylene dioxy-n-ethylamphetamine, demethylketamine, ketamine, cathinone, methcathinone, 4-methyl methcathinone, cocaine, benzoylecgonine, heroin, codeine, acetyl codeine, morphine, O^6-monoacetylmorphine, tetrahydrocannabinol and tetrahydrocannabinic acid, to prove the use of these drugs within a period of time.

Methods: After the hair sample was cleaned and dried, then it was grinded to powder by grinding machine. Methanol was used for extraction. The extract was filtered for UPLC-MS/MS analysis. Chromatographic separation was performed in Phenomenex Kinetex Biphenyl(2.1mm×100mm, 1.7µm) column, acetonitrile and 1mmol/L ammonium formate (with 0.1‰ formic acid in it) as mobile phase, and 10 min total run time with the flow rate of 0.4 mL/min and MRM mode of mass scan.

Results: 19 drugs were well separated, with the limits of detection of 0.001–0.05ng/mg and limits of quantitation lower than 0.05ng/mg. showing a good linear relationship within a certain concentration range.

Discussion: The normal rate of hair growth is 1 cm/month in adults. The detection of drug in a certain length of hair can reflect the drug use in the corresponding period. The method of grinding can crush the hair to micron size, greatly increase the specific surface area and increase the recovery rate of drug extraction. The chromatographic column of diphenyl bond phase with hydrophobic selectivity, aromatic selectivity and enhanced polarity selectivity can be used to simultaneously detect a variety of drugs with different polarity and shorten the experiment time.

Conclusions: The method is simple, convenient and sensitive, which is suitable for the detection of 19 drugs and metabolites in hair samples.

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P01-46
Effects of doxorubicin-loaded UCNP@MSN core-shell particles with a thermoresponsive nanovalve in melanoma cells

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Melanoma, one of the most aggressive forms of skin cancer, has an increasingly higher incidence. When detected in advanced stages, tumour eradication is often incomplete, contributing to poor prognosis with conventional treatments. Upconversion nanoparticles (UCNPs) have

unique optical properties that allow their effective use in several biomedical applications. This includes the excitability under near-infrared (NIR) excitation light, which has a relatively high penetration depth in tissue, a multitude of characteristic emission bands in the ultraviolet (UV), visible (Vis), NIR, and short-wave infrared (SWIR), along with long luminescence lifetimes, and high photostability. Mesoporous silica nanoparticles (MSN) with nanovalves or derived coatings have widely been used for triggered and targeted drug delivery in the past. Anticancer drugs can be loaded into the pores of MSN, enabling spatiotemporally controlled drug release. In the last decades, photoactivated drug delivery systems have received much attention. In this work, UCNPs were coated with a mesoporous silica shell yielding UCNP@MSN core-shell nanoparticles which were equipped with thermoresponsive retro-Diels-Alder nanovalves and then loaded with DOX, a chemotherapeutic agent for melanoma treatment. Subsequent DOX release from this drug delivery system was triggered by 980 nm NIR light. *In vitro* DOX release profiles demonstrated successful NIR light-triggered drug release. Melanoma cells exposed to UCNP@MSN-DOX exhibited reduced viability, with further reductions observed upon combined exposure to UCNP@MSN-DOX and subsequent irradiation, correlating with increased DOX release. These findings underscore the potential use of UCNP@MSN drug delivery systems with thermoresponsive caps as effective drug delivery platforms for melanoma therapy.

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P01-47
Considerations for *in vitro* dosimetry to study uptake of inhaled toxicants

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Previously, we have illustrated the role that *in vitro* models can play in considering human-relevant exposure scenarios for hazard identification of inhaled toxicants. *In vitro* exposures provide the opportunity to consider variations in concentration (exposure intensity), exposure duration and exposure frequency [1]. Additionally, the Air-Liquid-Interface (ALI) offers many possibilities for exposure of cells. When choosing an exposure method, it is recommended that (a) it is considered what information really is needed for hazard or risk assessment (*e.g.*, what specific question is to be answered); (b) the exposure system that is most suitable for the chemical to be assessed is chosen; (c) a deposited dose that mimics human deposition in the respiratory tract is used and (d) the post-exposure sampling methodology should be carefully considered and relevant to the testing strategy used [1].

In the application of *in vitro* models to assess uptake of inhaled substances, the above mentioned principles for dosing apply. In addition, the cell model should meet some criteria to generate relevant data for human uptake and systemic availability. It is important that the cell model should form a tight barrier, including tight junctions. The cells should express relevant transporters for compounds that are transported via active processes over the lung epithelium and they should have a relevant clearance capacity. ALI cultured cells that produce mucus are an advantage over submerged systems when testing compounds that may undergo changes when dissolved or volatiles. Still, the use of ALI cultured cells to assess uptake of inhaled toxicants is limited, with most data available on inhaled pharmaceuticals.

Our previous experiments have shown the applicability of ALI-cultured human primary bronchial epithelial cells (PBECs) to asses translocation of nicotine and ethyl maltol [2]. Defining relevant dosimetry and criteria for cell models is the first step needed for assessment of translocation. As a next step, we will illustrate the applicability of ALI-cultured PBECs to study the uptake of inhaled pesticides using a relevant exposure scenario. Although mechanisms of uptake are unknown for inhaled toxicants, we believe that ALI-cultured respiratory models can play an important role in assessment of systemic availability.

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P01-48
Expanding functional neurotoxicity investigations: exploring synchronized calcium-driven oscillatory activity in a human dopaminergic 3D model

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Animal experiments are still widely used to predict drug toxicity on human neuronal systems. Lund human mesencephalic cultures (LUHMES) are of human origin and have a dopaminergic phenotype. These cells display electrical properties typical to neurons and are a strong candidate to provide an alternative method to test drug effects without animal use. Our ongoing work involves the development of assays utilizing LUHMES cells for high-throughput quantification of free intracellular Ca2+-concentrations, which serve as a representative indicator of neuronal activity.

Here we show that LUHMES cultures are capable to establish synchronized slow Ca2+ oscillations at frequencies near 0.2 Hz. This oscillatory behaviour can be triggered by certain neurotransmitters such as dopamine or serotonin or by depolarization with Tetraethylammonium. The network oscillations are stable and highly reproducible in 2D monolayers and 3D spheroids. The activity can be pharmacologically modified to exhibit bursting-like behaviour, complete cessation, or alterations in frequency. We could so far not detect any spontaneous synaptic activity in LUHMES cells in patch-clamp recordings. It is therefore unlikely that the phenomenon is driven by typical electrical signalling mechanisms seen in for example glutamatergic neurons. Although the mechanism isn't fully understood we speculate contributions of dopamine signalling or the electrical coupling and transfer of small molecules via gap junctions.

Once fully characterized, the oscillations could serve as a widely applicable parameter for testing of drug toxicity, such as assessing the overexcitation of the network or the decoupling of the neuronal system. A crucial milestone involves the full characterization of the oscillatory activity to understand what underlying mechanisms are driving the phenomenon. Subsequently, this work aims to establish resilient assays to assess drug toxicity on the network level that can be universally applied in both academic and industrial settings worldwide.

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P01-49

In vitro study of the antioxidant activity of newly synthesized N-Pyrrolyl Hydrazones

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Oxidative stress is associated not only with the normal aging process but may also play an important role in the development of a variety of neurodegenerative diseases, especially Parkinson's and Alzheimer's diseases. Recent perspectives in drug design include multifunctional drugs interacting with several targets for the treatment of neurodegenerative diseases with complex aetiology. Such perspective active compounds could be derived from the class of N-heterocyclic compounds including pyrrole and its derivatives. These compounds possess various pharmacological activities, among which antioxidant, antitumour and anti-inflammatory.

The aim of this study is to investigate the antioxidant activity and safety of a series of newly synthesized N-pyrrolyl hydrazones in *in vitro* model systems.

SH-SY5Y cell line serves as a suitable model for studying neurotoxicity and neuroprotection in various neurodegenerative diseases, including Parkinson's and Alzheimer's diseases. HepG2 cell line is widely used to assess the hepatotoxic potential and safety profile of novel compounds.

In an oxidative stress model (1 mM H_2O_2, 15 min), compounds **7c**, **7d**, and **8e** exhibited a promising antioxidant protection in the neuroblastoma cell line SH-SY5Y. Compounds **7c** and **7d** demonstrated stronger neuroprotective effects compared to the reference compound melatonin. In HepG2 liver cell line, compounds **7c, 7d, 7e, 8d,** and **8e** showed protective effects against H_2O_2-induced oxidative damage (500 µM, 60 min). The highest antioxidant activity was observed with compound **8e**.

In conclusion, the newly synthesized N-pyrrolyl hydrazones exhibit low toxicity and significant antioxidant activity *in vitro*. These findings highlight their potential for further experimental pharmacological and toxicological studies, particularly in exploring their efficacy against induced oxidative stress in neurodegenerative pathological processes.

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P01-50

Mitochondria-specific targeting to overcome imatinib resistance in chronic myeloid leukemia cells

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Chronic myeloid leukemia (CML) is a hematopoietic disorder caused by the BCR-ABL fusion gene which is produced by the reciprocal translocation between chromosomes 9 and 22. Emergence of resistance to Imatinib (IMA), as a first-line treatment of CML, leads to therapy failure.

In order to define the role of mitochondria in IMA-resistance development in CML, we inhibited mitochondria function through Gamitrinib (GA, inhibitor of mitochondrial TRAP-1 activity) treatment. Targeting chaperone-directed proteostasis in mitochondria represents a promising antitumor activity. GA selectively accumulates in mitochondria and induces acute proteotoxic stress, shuts off multiple mitochondria functions, including bioenergetics, and represents anticancer activity in different cancer types. We analyzed the effects of IMA, GA and IMA+GA drug combinations on CML cell lines, K562S (sensitive), K562R (IMA resistant-suspension) and K562R-adh (IMA resistant-adherent), considering that GA treatment might be an alternative to overcome IMA resistance in CML. Cytotoxicity, RT-qPCR (BAX, BAD, BIM, BCL-2, BIM and BCL-XL genes), Annexin V staining, caspase activity and cytochrome C assays were performed to analyze the apoptotic effects of GA in CML cell lines. GA induced apoptosis in all K562S, K562R and K562R-adh cells when applied as a single agent and its apoptotic effects were enhanced when used in combination with IMA.

Since GA is an agent that inhibits oxidative phosphorylation, we carried out ATP assays to analyze the ATP production in GA-treated cell groups. ATP production was found to be decreased only in K562R and K562R-adh GA-treated cells. However, ATP production was dramatically decreased in all cell lines when GA was applied in combination with IMA. Combined GA and IMA treatment also decreased the mRNA expression levels of HK2, PKM1 and LDHA in all three K562S, K562R and K562R-adh cells. These results show that IMA+GA combination therapy may be recommended as an alternative treatment protocol to induce apoptosis in CML.

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P01-51

Prediction of drug-induced hepatotoxicity and neurotoxicity using *in vitro* toxicity screening assays

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Significant numbers of drugs are continuing to be removed from the market, or from late-stage development, because of unanticipated toxicity issues. There is therefore a pressing and continued need for improved predictive power in preclinical pharmaceutical toxicology assessment. *In vitro* predictive toxicity assays present a rapid and cost-effective option to identify potential toxicity concerns during the early lead optimization stage.

The proposed *in vitro* hepatotoxicity panel includes the measurement of 4 standard readouts in primary rat hepatocytes seeded in 96-well plates: Cytolysis (live kinetic fluorescence imaging – Sytox Green), Steatosis (Fluorescence – Nile Red), Oxidative stress (Fluorescence – GSH depletion), and Cholestasis (bile canaliculi network using fluorescence imaging – Cholyl-lysyl-fluorescein). Relevant positive controls

were evaluated in each assay including Acetaminophen (10 to 50 mM), Troglitazone (0.1 to 10 μM), Cyclosporine A (0.1 to 10 μM), Bosentan (10 to 300 μM) or Valproic acid (3 to 30 mM).

Stemonix microBrain® 3D are human iPSC-derived cortical neurons and astrocyte culture (excitatory (glutamatergic) and inhibitory (GABAergic) population at a 80/20 ratio) matured for 7 weeks in 384-well plates. These spheroids exhibit spontaneous calcium oscillation patterns that can be monitored to evaluate neurotoxic adverse effects A screening of 20 known neurotoxic compounds (4 separate concentrations) was performed. Each test item was applied for 24 hours before calcium oscillation measurement using a FLIPR platform. The modulations of calcium oscillation frequency and amplitude were computed.

Our data demonstrate that rat hepatocytes are a valid and cost-effective alternative to human hepatocytes for predictive hepatoxicity screening, despite lacking some CYP450 activity. Primary human hepatocytes can be used in more focused secondary screenings using a similar panel of assays, or even in 3D. Early-stage predictive neurotoxicity, with complex human 3D spheroid cultures, can be successfully used in a high-throughput approach to identify neurotoxic compounds. Functional changes can be monitored through calcium modulation and can identify toxic compounds as well as modulators of neurotransmission that can impair the normal physiology of these cell cultures. These approaches confirm the key value and utility of early predictive toxicity screening as part of a preclinical development program. This approach can also be relevant for predictive toxicity screening in other therapeutic domains, using cell types such as cardiomyocytes and kidney cells.

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P01-52
Screening of the induction of an *in vitro* foreign body reaction in two UHMWPE case studies

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Purpose: The eventual failure of a medical device material can often be attributed to the induction of a foreign body reaction (FBR). FBR refers to the adverse response of host tissue to implanted materials. Following implantation, proteins adhere to the material surface, triggering the recruitment of immune cells and acute inflammation. After the physiological wound healing phase, attached macrophages undergo a process termed “frustrated fusion” to form foreign body giant cells (FBGC). Ultimately, this can lead to chronic inflammation characterized by the development of pathologic fibrotic encapsulation of the medical device. Currently, the *in vitro* assessment of FBR is not part of standard ISO 10993 biocompatibility testing for conformity certification of medical devices. Therefore, it is of interest to establish a protocol for assessing medical device mediated FBR induction to improve patient’s safety and to avoid unnecessary animal testing.

Methods: Since macrophages are key in FBR signaling, primary rat alveolar macrophages and human induced pluripotent stem cell (iPSC) macrophages were chosen as model systems. Cells (2 x10^5/per well of 24-well plates) were seeded onto various implantable ultra-high-molecular-weight polyethylene (UHMWPE) material samples. Supernatants were collected after different culture periods for measuring membrane damage and release of different chemokines/cytokines. Subsequently, metabolic status (WST-1) was assessed. Furthermore, formation of FBGC was analyzed by confocal microscopy after 72 h using DAPI (cell nucleus) and fluorescence-coupled anti-alpha-tubulin (cytoskeleton) antibodies for staining.

Results and Conclusion: In line with its use as implant material, unmodified UHMWPE did induce neither cytotoxicity nor TNF-a/MMP9 release after 24 h of incubation, as compared to untreated cells. In two case studies, different ethylene oxide-sterilized UHMWPE materials and a novel hydrogel-coated UHMWPE were then examined for induction of FBR. Notably, distinct material combinations thereof induced FBGC formation, while mediating an only minimal inflammatory status.

The results indicate that certain types of UHMWPE material combinations can induce an FBR in the absence of cytotoxicity and considerable inflammation. Therefore, these distinct UHMWPE materials would pass ISO 10993-5 biocompatibility testing, even though the induction of an FBR is present. Hence, quantification of FBGC could represent a promising add-on for ISO 10993 *in vitro* biocompatibility testing and might thus enhance patient’s safety. Finally, use of iPSC might help in transition of FBR testing to an animal-free methodology.

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P01-53
Standard versus modified Ames test for mutagenicity evaluation of petroleum UVCB

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Testing for *in vitro* gene mutation in bacteria (Ames test), according to OECD TG 471, is a standard information requirement for substances including petroleum UVCB (unknown or variable composition, complex reaction products or biological materials) being registered under REACH (EC Regulation No 1907/2006). Previously, false negative results have been reported for petroleum substances (PS) when tested using the standard Ames test. This led to the development of the Modified Ames test (ASTM E1687), which enhance the sensitivity of the bacterial reverse mutation assay in identifying mutagenicity potential of PS. The test is optimized to detect mutagenicity mediated by polycyclic aromatic compounds (PAC) present in PS. In order to test non-soluble PS in an *in vitro* system, dimethyl sulfoxide (DMSO) is used, combined with an extraction step that concentrates the potentially hazardous 3- to 7-ring PACs into the DMSO-extract fraction. The result from Modified Ames test is expressed as mutagenicity index (MI), which represents the slope of the mutagenic dose response relationship. A positive result in this test indicates potential for *in vitro* gene mutation and the MI value has been also found to be correlated with the carcinogenic potential of petroleum UVCB. To increase the confidence in the Modified Ames test for REACH regulatory testing of petroleum UVCB, the present study evaluated and compared the mutagenicity potential of six PS using both standard and Modified Ames tests. Of the six PS tested, 5 were lubricating base oils (LBO) and 1 was distillate (unrefined/acid-treated oil). All substances were tested in three different forms i.e., neat material (whole substance), DMSO-extract (according to ASTM E1687), and raffinate (remainder of the DMSO-extracted materials) in both tests. Results obtained show that i) none of the test substances, in any tested forms, showed positive outcome in the standard Ames test, ii) all LBOs, in all tested forms, tested negative in the Modified Ames test. MI values for the neat LBO, the DMSO-extracts LBO, and the raffinate LBO ranged from 0 to 0.13, 0.03 to 0.36, and all 0, respectively, iii) distillate, in all tested forms, tested positive in the Modified Ames test, with MI-neat=2.41, MI-DMSO-extract=3.50, MI-raffinate=1.31. Further analysis demonstrated that the tested distillate contains a substantial level of PACs i.e., >8% total wt.% (measured using the IP346 method). These PACs are predominantly 3- to 5-ring (measured using the PAH-0397 method), e.g., phenanthrene,

benzo(a)dibenzothiophene, pyrene, benzo[a]anthracene, chrysene, benzo[a]pyrene. Altogether, the present experimental work confirms the superiority and robustness of Modified Ames test over the standard Ames test, to adequately identify PS with mutagenic properties and carcinogenic potential. This additionally reinforces the involvement of 3- to 7-ring PACs in mediating the observed mutagenicity associated with PAC-containing petroleum UVCB.

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P01-54

New fluorescent probes for quick assessment of drug induced liver injury effects on bile canaliculi dynamism and albumin expression

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Drug-induced liver injury (DILI) is a major cause of study cessation during drug development and market withdrawal. DILI manifests as hepatocellular toxicity or cholestasis or mixed patterns. Yet new tools need to be set up to improve DILI characterisation. Easy-to-use specific fluorescent probes were designed to assess hepatocyte functioning and performance, and DILI. This study aims to characterise two fluorescent probes: Fluobile™ and Fluoalb™ for bile canaliculi and albumin labelling respectively. For this purpose, various hepatic assay systems were used: primary human and rodent hepatocyte and HepaRG® cell models.

Primary human hepatocytes (PHH) and rodent hepatocytes were used at day 1 (D1) and at D10 for PHH only. Undifferentiated HepaRG® cells were used at D3, D7, D10, D14, and D21 of culture, and differentiated HepaRG® cells at D28, D35, and D42. Cells were exposed to taurocholate, chlorpromazine, fasudil and rifampicin for 2 hours prior to the incubation of cells and cell supernatants separately for one hour with fluorescent probes. Drug effects on bile canaliculi and albumin expression were evaluated by fluorescence microscopy and fluorimetry using respectively Fluobile™ and Fluoalb™ probes.

Fluobile™ probe was shown to be localized within the bile canaliculi in PHH, rodent hepatocytes and differentiated HepaRG® cells allowing the visualization of the bile canaliculi network. Efflux of Fluobile™ probe in the bile canaliculi was partially inhibited by taurocholate suggesting an involvement of the BSEP, a canalicular efflux transporter with a pivotal role in cholestasis. As expected, fasudil and rifampicin induced a strong bile canaliculi dilatation, whereas chlorpromazine induced bile canaliculi constriction, both effects visualized and quantified using Fluobile™ probe. Drug-induced alteration of bile canaliculi morphology and dynamism can therefore be assessed with Fluobile™ probe.

In parallel, Fluoalb™ probe was used to label intracellular albumin, as a marker of hepatocyte differentiation and functioning. Intracellular albumin was labelled in hepatocytes of HepaRG® cell model, PHH and rodent hepatocytes and secreted albumin was quantified in cell culture supernatants. Interestingly, albumin staining was only observed in hepatocyte-like cells of HepaRG® cells but not in biliary-like cells, which shows the high specificity of the probe. Moreover, Fluoalb® staining showed that albumin expression gradually increased in undifferentiated HepaRG® cells over culture time to reach a level similar to that of PHH in differentiated HepaRG® cells. Furthermore, drug-induced alteration of albumin expression can be assessed using the Fluoalb™ probe.

Altogether, our data suggest that hepatocyte characteristics such as bile canaliculi morphology and dynamism as well as albumin expression can be assessed and quantified using easy-to-use fluorescent probes through one step protocol. This represents a step forward in improving DILI characterization.

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P01-55

The effect of *in vitro* distribution of polycyclic aromatic hydrocarbons (PAHs) on *in vitro* derived genotoxic potencies

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When toxicity effects are predicted based on *in vitro* data, the nominal *in vitro* exposure concentration is often used to describe the substances' potency, whereas the distribution of the test substances in the *in vitro* system itself is often neglected. Depending on their physicochemical properties, the substances can behave differently in *in vitro* test systems, e.g., through binding to plastic or culture media constituents, leading to differences in the amount and thus concentration of the substances available to the cells and therefore measured bioactivity. Using nominal concentrations to describe substance potency or to derive relative potency factors (RPFs) can therefore give inaccurate results. In this study, we investigated if differential *in vitro* distribution of polycyclic aromatic hydrocarbons (PAHs) affects their obtained respective *in vitro* relative potencyfor genotoxicity. To this end, the genotoxic potency of eight indicator PAHs was determined with the γ-H2AX assay, using three different cell types (i.e., HepaRG, LS174T and HepG2). The genotoxic potencies differed between the tested cell types, resulting in different RPFs based on the nominal concentrations. Then, distribution of the test compounds in the *in vitro* systems was determined using computational models, and predicted values were verified with analytical measurements of the available concentrations of the PAHs in the respective *in vitro* systems. The RPFs determined using the nominal concentrations were subsequently compared to the RPFs corrected for the *in vitro* distribution of the test chemical. It will be discussed how and to what extent (predictions of) *in vitro* distribution of PAHs can be applied to improve *in vitro* to *in vivo* extrapolations. Overall, this study contributes to the improvement of *in vitro* risk assessment strategies.

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P01-56

Primary human hepatocytes as screening platform for imaging-based assessment of DILI liabilities

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Purpose: Hepatotoxicity, known as drug-induced liver injury (DILI) in drug discovery and development, is a potential risk for novel drug candidates and a reason for approximately 30% of all drug withdrawals. Different cell organelles, such as mitochondria and lysosomes, are affected by liver toxicants and their reactive metabolites leading to diverse clinical appearances of DILI. Mitochondrial dysfunction may cause for example fatty liver and lysosomal impairment may lead to phospholipidosis. The purpose of the work was to develop a screening method for these mechanisms contributing to DILI, using primary human hepatocytes. This assay enables rapid and efficient screening of high volume of compounds early on facilitating the identification of potential DILI liabilities. Early detection of such liabilities is crucial to

prevent issues eventually in human use and to avoid costly setbacks if identified later stages of drug development.

Methods: Primary human hepatocytes, pooled from ten donors, were used in the assays to ensure covering human diversity. After drug exposure, specific fluorescent dyes targeting various toxicity markers were multiplexed to stain the hepatocytes. Multiparametric assay was developed to detect drug-induced changes in intracellular levels of neutral lipids (steatosis) and lysosomal phospholipids (phospholipidosis), as well as potential cytotoxic effects leading to cell loss. Additionally, a lysosomal trapping assay was established to identify lysosomotropism, i.e. lysosomal accumulation of drugs. This assay can be used either independently or to complement the lipidosis assay as lysosomotrophic nature of a drug is tightly linked to a risk to cause phospholipidosis. Another multiparametric assay was developed to detect disturbances in cellular respiration and stress, such as decrease in mitochondrial membrane potential and basic parameters for cell loss, nuclear morphology and cell membrane integrity. All these fluorescent assays were optimized to a 2D screening format on 96-well plate and using automatic imaging system coupled with fast image analysis optimized for each assay.

Results: Multiparametric DILI screening assay was successfully developed applying primary human hepatocytes and using fluorescent probes for different toxicity end points. Compounds with known DILI concern and model toxicants for specific mechanisms were used to validate the assays. We showed an increase in steatosis and phospholipidosis with multiple compounds and detected concentration dependent decrease in lysosomal stain, a sign of lysosomal accumulation, with several known lysosomotropic compounds. Our assessment also included cytotoxic evaluation with multiple model toxicants. In summary, this assay serves as an effective screening tool for identifying DILI liabilities and can also be complemented with other assays for mechanisms such as hepatic efflux transporter inhibition leading to cholestasis, a known mechanism for DILI outcome as well.

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P01-57
Antidepressant induced phospholipidosis and lysosomal dysgenesis during *in vitro* adipogenesis

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Citalopram (CIT) and Sertraline (SER), two Selective Serotonin Reuptake Inhibitors (SSRIs), used as antidepressants, have been associated with significant weight changes in patients after long-term treatment. Although SSRIs are largely considered safe and prescribed extensively, the understanding of their metabolic effects is limited and needs further research.

Here, we investigated whether CIT and SER interfere with the process of adipocyte differentiation. Human mesenchymal stem cells (MSCs) were exposed in a range around reported steady state concentrations in a 2D and 3D model, followed by an assessment of intracellular lipids by Nile Red staining and fluorescence measurements (Ex/Em 485/590 nm). To elucidate possible mechanisms, we performed RNA sequencing at NOEC and LOEC concentrations on 3D MSCs (1–10μM CIT and 0.1–1μM SER), followed by differential gene expression (DEG) and pathway analyses.

CIT and SER increased intracellular lipid accumulation in both 2D and 3D MSCs, an indication of enhanced adipogenesis. This effect was observed in a concentration dependent manner, and using the benchmark dose modeling approach, points of departure were found overlapping with reported SSCs (SER: 0,065–0,65 μM, and CIT: 0,12–0,92 μM). Surprisingly, DEG analysis showed downregulation of genes related to metabolism and adipogenesis by the SSRIs and this trend was observed in all exposures. In contrast, genes involved in phospolipogenesis and of pathways related to lysosomes were found upregulated by SSRI exposures. Functional analyses in 2D MSCs confirmed the lysosome and phospholipid-inducing effects of the SSRIs.

Taking all data together, we concluded that these SSRIs lead to a common adverse effect of a group of chemicals, commonly known as Cationic Amphiphilic Dugs (CADs). CADs are a class of chemicals known to accumulate within lysosomes due to their weak basic properties. This lysosomal trapping results in extensive binding to phospholipid membranes and the inhibition of lysosomal enzymes, leading to excessive accumulation of lysosomal phospholipids, a process known as drug-induced phospholipidosis.

Our findings point toward lysosomal accumulation of SSRIs during adipogenesis, due to their physicochemical properties, inducing phospholipids and lysosome formation. Whereas important adipogenic processes are inhibited, potentially leading to dysfunctional adipocytes, which might have implications in maintenance of a healthy metabolic balance.Selective Serotonin Reuptake Inhibitor, MSC, lysosome, adipogenesis, *in vitro*.

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P01-58
Comparative mitochondrial changes caused by ionizing radiation in healthy and cancerous lung cells

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Since the aim of radiotherapy is to kill cancerous cells and shrink the tumor, the death of cancerous cells in the radiated area is a desired result. However, not only cancerous cells die in the irradiated area, but also healthy cells are damaged or die. This non-selective nature of radiation can lead to undesirable health effects. To date, most of the studies conducted to explain the undesirable cellular effects caused by radiation have focused on the genotoxic effects caused by radiation and other cellular effects have been investigated to a lesser extent. Of these, mitochondria in particular are an organelle worthy of investigation in radiation-induced cellular toxicity due to the important roles they play for the cell. Therefore, in this study, we investigated radiation-induced mitochondrial changes in healthy and cancerous cells exposed to acute single dose ionizing radiation. For this, we exposed the cells to ionizing radiation at a dose of 4 Gy for 2 min and investigated the mitochondrial changes on days 1, 3, and 5 compared to the control group. According to our results, we found a significant increase in both total and mitochondrial ROS levels in both healthy and cancerous cells after radiation treatment, but this increase was more pronounced in cancerous cells. We also found that of the enzymes that make up the cell's enzymatic antioxidant system, only CAT was significantly increased in cancerous cells, whereas the other enzymes such as GPx, Cu,Zn-SOD and Mn-SOD only showed a significant increase in healthy cells. In addition, mitochondrial GSH levels increased in healthy cells but decreased in cancerous cells. While cytosolic GSH levels did not change in healthy cells, it caused a slight increase in cancerous cells. Mitochondrial and cytosolic MDA levels increased in healthy cells, while only cytosolic MDA levels increased in cancerous cells. All these results

show that ionizing radiation causes oxidative stress in both cell types, but healthy cells have a better antioxidant defense mechanism. While ETC complex activities, mRNA and protein levels, among the other mitochondrial parameters we examined, varied for complexes 1 and 4, interestingly, the data for complex 3 varied oppositely between the two cell types. As a result of all these mitochondrial changes, ATP levels, which are a general indicator of mitochondrial functions, increased in both cells. In the last step, MMP and mPTP parameters were measured, no change was observed in healthy cells, but a decrease in MMP and an increase in mPTP were detected in cancerous cells. The data so far has shown us that radiation disrupts mitochondrial functions more in cancerous cells than in healthy cells. Lastly we measured the apoptotic factors AIF, Bax, CytC and Caspase-3 with the WB technique and we showed that radiation triggers apoptosis in cancerous cells.

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P01-59
Effects of toxic metal mixtures on oxidative status in prostate on male Wistar rats

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The risk of developing prostate disease is related to aging, race, family history and lifestyle. Also experimental studies support the hypothesis that environmental chemicals, including toxic metals, may increase the risk of developing such disorders. However, traditional toxicological studies primarily focus on assessing the impact of individual chemicals on disease development This approach, while valuable for understanding the toxicity of specific substances, may not fully capture the complexities of real-world exposure scenarios where organisms are exposed to mixtures of chemicals.

The aim of this study was to examine the effect of a mixture of six toxic metal(oid)s (As, Pb, Hg, Cd, Cr(VI), and Ni) on oxidative status parameters in the prostate of male Wistar rats after exposure to different doses of this mixture. Aquatic solutions of toxic metal mixtures were administered to experimental animals via oral gavage during 28 and 90 days.

Dose levels were determined on the basis of the prior human biomonitoring investigation. The experiment included control and treatment groups that received doses reflecting the lower confidence limit of the Benchmark dose for effects on hormone levels (M1), median concentrations (M2), 95th percentile concentrations (M3) and reference values of each individual metal according to literature sources (M4, only 28 days of exposure). After 28 and 90 days of exposure, the animals were euthanized, their prostates were extracted and the redox status parameters (IMA, MDA, SH groups, GSH and SOD) were determined in the tissue by spectrophotometric method.

In animals that were treated for 28 days, there was a statistically significant increase in IMA in group M1. Additionally, there was a decrease in SH groups and GSH in groups M2 and M4, which indicates the depletion of antioxidant protection reserves in the tissue. After 90 days of exposure, a significant increase in the level of IMA was observed in all treated groups, while in the case of other parameters there were no significant differences compared to the control.In conclusion, the experimental study suggests that exposure to low doses of toxic metals reflecting environmental exposure can disrupt prostate function by altering the redox status, leading to oxidative damage. Despite the observed increase in oxidative damage, the level of antioxidant protection remained stable. The results of this study point to possible toxic effects of real-life metal mixture on the prostate following prolonged exposure. These effects could be mediated through oxidative stress induction.

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P01-60
Integrated testing strategy for evaluation of pulmonary fibrosis based on fibroblast-to-myofibroblast transition (FMT)

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Fibroblasts are tissue mesenchymal cells that play a role in reconstructing a normal and well-structured ECM during the wound healing repair process. Activation of these fibroblasts is involved in the formation of chronic lung diseases such as pulmonary fibrosis. The activation step is the proliferation of fibroblasts and differentiation of fibroblasts into myofibroblasts (fibroblast-to-myofibroblast transition (FMT) process). The FMT process can be a good indicator to evaluate pulmonary fibrosis. However, there are no reports on pulmonary fibrosis focusing on FMT. Therefore, in this study, we established a method to evaluate pulmonary fibrosis based on FMT. The major key events (KE) of pulmonary fibrosis were TGF-β1 activation (KE1), differentiation into myofibroblasts (KE2), ECM deposition (KE3), and enhanced cellular migration and contraction (KE4). Based on the changes in these key events, we applied a weight of evidence (WoE) and integrated testing strategy (ITS) approach to improve the reliability of the evaluation of pulmonary fibrosis (AO). To validate this system, MRC-5 fibroblasts were exposed to PHMG-HCl, which is known to induce pulmonary fibrosis. As a result, all key events for distinct fibrotic responses were significantly increased in MRC-5 exposed to PHMG-HCl. In conclusion, the ITS and WoE system designed in this study suggests that it can be utilized as a key tool to assess FMT-induced pulmonary fibrosis.

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P01-62
Development of an *in vitro* model of dry nose

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When the nasal cavities do not contain enough moisture, dry nose symptoms can manifest such as pain and swelling, nosebleeds, and even airway infections. Nasal dryness can often degrade the quality of life, therefore, novel topical treatments formulated as nasal sprays are needed to treat the severe cases. To test simultaneously efficacy and toxicity effect of novel formulations, we developed an *in vitro* "Dry Nose" model based on a fully differentiated human nasal epithelium cultured at the air-liquid interface.

Methods: Epithelia (MucilAir™-Pool) were reconstituted with a mixture of primary human nasal epithelial cells isolated from 14 different heathy donors. Dry air was applied onto the apical surface of the epithelia at a speed of 6 L/min for several exposure time (from 1 to 10 min). To measure the effect of dry air on nasal epithelial cells, the following End-points were evaluated: (i) tissue integrity (TEER); (ii) cytotoxicity (LDH); (iii) ciliopathic effect (Cilia Beating Frequency, active area and mucociliary clearance); (iv) pro-inflammation (IL-8 release). Proof-of-Conception for treatment as well as prevention of Dry Nose symptoms was provided by application of saline solution (0.9% NaCl).

Results: Data suggest that the optimal condition to simulate Dry Nose were 5 minutes of exposure to dry air (6 L/min), which induced a decrease of cilia beating frequency. Kinetics of CBF and Active Area could be restored after application of saline solution. The saline solution also prevented the cytotoxic effect of dry air measured by LDH assay. As expected, addition of saline solution abrogated the induced IL-8 secretion by dry air.

Conclusions: This set of data suggest that the human nasal epithelia is a versatile and convenient tool for assessing the efficacy and toxicity of moisturizing formulations designed to treat dry nose.

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P01-63
The secretion of interleukin 18 by reconstructed human epidermis is a biomarker for *in vitro* prediction of skin photosensitization

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Background and objectives: The photosensitization is an adverse effect induced by a substance that becomes a sensitizer upon exposure to ultra-violet (UV) light. Although the photosensitization remains a major toxicological endpoint for the safety assessment, there is no validated *in vitro* method available for its evaluation so far. We have previously developed a method (SENSIL-18) using the interleukin 18 (IL- 18) production by reconstructed human epidermis (RHE) as a specific biomarker for *in vitro* sensitization assessment [1,2]. Herein, we discuss our most recent study [3] demonstrating the efficacy of a new *in vitro* assay using IL-18 to predict the photosensitization in the RHE model (PhotoSENSIL-18).

Methods: EpiCS™ RHE were incubated with a set of known sensitizing/phototoxic/photosensitizing substances and exposed to ultra-violet (UV) irradiation. Then, the cell viability was analyzed by MTT assay, while the IL-18 secretion was quantified by ELISA. This protocol was used to test 16 substances and the induction of IL-18 production (UV+/UV- ratio) was calculated.

Results: As a first step in the test development, three preincubation (30, 60, and 120 minutes) and two recovery times (23 and 42 hours) were evaluated, in order to select the most resolutive ones for the purpose of the assay. The scheme following 1 h of preincubation with the positive control (ketoprofen) and a recovery period of 23 h promoted the highest IL-18 elevation, being therefore the most resolutive condition among the tested parameters. Under such conditions, we found that a cut-off of 1.5 induction (UV+/UV- ratio) is the most predictive model, being capable of identifying true positive photosensitizers (8 of 9) with a good prediction (sensitivity of 89%) in comparison with *in vivo* data. This approach provides complementary information regarding the toxicity and sensitization potential of tested substances and it can be integrated with other assays (e.g. hCLAT and DPRA) for an accurate prediction.

Discussion and conclusion: Our data suggests that the PhotoSENSIL-18 is a valuable *in vitro* method for identification of photosensitizing substances, allowing the administration of higher concentrations and test lipophilic compounds. In a nutshell, our studies have highlighted that the SENSIL-18 and PhotoSENSIL-18 are promising approaches to evaluate the skin sensitization induced by raw material and finished products, as well as complex mixtures and medical devices.

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P01-64
Approaches to mitigate known safety liabilities of immunomodulatory (IMiDs) drugs used in heterobifunctional degraders

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Heterobifunctional targeted protein degraders (PROTACs) have the capacity to bind and cause the degradation of any number of proteins, including those deemed undruggable, by hijacking the Ubiquitin-Proteasome System (UPS). Due to their potential, they are one of the fastest area of growth in the biopharmaceutical industry, with the first class of molecules in clinical trials. PROTACs consists of a Protein-of-interest (POI) ligand attached to an E3 ubiquitin ligase binder via a chemical linker. The resulting molecule brings into close proximity the POI and the E3 ligase, leading to the transfer of ubiquitin and the eventual degradation of the POI. To date, most of the molecules in the literature and entering the clinic are based on the E3 ligase binders

lenalidomide and pomalidome, otherwise known as IMiDs. IMiDs bind to the Cul4^{CRBN} E3 ligase and result in the degradation of a number of proteins, so-called neosubstrates, including Ikaros, Aiolos, and SALL4. The degradation of these proteins has been associated with some of the observed clinical toxities of IMiDs.As PROTACs made from IMiDs may retain some of these off-targets, we have developed an *in vitro* screening cascade that includes fast proteomics to monitor a number of known neosubstrates, assessment of toxicity in primary human hematopoietic stem and progentor cells (HSPCs), as well as *in vivo* assessment via a CRBN knock-in transgenic model . We show that a number of commercially-available IMiD-based PROTACs, optimized for their primary target, retain off-target degradation activity. We further charaterize the impact of degradation or genetic deletion of Ikaros in the differentiation of HSPCs. Finally, we demonstrate that an IMiD-alternative CRBN binder is able to mitigate the safety liabilities of IMiDs while retaining efficient degradation of a number of POIs when incorporated into heterobifunctional degraders. These results provide a way forward for the development of safer CRBN-based degraders.

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P01-65
In vitro developmental toxicity testing based on real-time monitoring for signal disruption

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In pharmaceutical development, developmental toxicity tests using animals to assess impacts on embryos and fetuses are essential. These tests require substantial investments of time, effort, and resources due to species differences. Some drugs, like thalidomide, do not affect rodents but cause severe malformations in humans. Moreover, the specificity of new modalities, such as nucleic acid therapeutics, toward human complicates safety assessment through traditional animal testing. Addressing this urgent issue necessitates the development of high-throughput, accurate *in vitro* methods capable of precisely predicting developmental impacts. Our research has developed a developmental toxicity evaluation method using human iPSCs, focusing on the disruption of signal transduction by chemical substances (DynaLux/c) [1,2,3]. This method has demonstrated high accuracy and comprehensive coverage in detecting developmental toxicants, particularly through analyzing dynamic changes in FGF-SRF signal disruption by a 24-hour live-cell luciferase assay. Currently, we have enabled continuous detection of dynamic signal disruptions by incorporating a real-time luminescence measurement device with cell culturing capabilities into DynaLux/c. This integration significantly reduces testing time to one week and allows for detailed analysis. Additionally, we've also established the Wnt-TCF/LEF signal disruption reporter system, like the FGF signal. Consequently, our human iPSC-based assay for signal disruption emerges as a promising tool for preliminary screening of potential developmental toxicants.

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P01-66
A workflow to study cellular reactions to volatile organic compounds exposure *in vitro*

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Volatile organic compounds (VOCs) are chemicals that evaporate easily at ambient air conditions and may originate from both natural and human sources. Some of these substances are known to negatively affect air quality and human respiratory health. While immediate toxic effects of such substances are usually known, their potential long-term effects are far less clear. For certain substances it is assumed that long-term exposure even at very low concentrations leads to cellular changes that trigger health problems. Characterizing the molecular mechanisms of such chronic effects is challenging; however, it is suggested that *in vitro* methods play a crucial role in this aspect.

A major challenge to studying the mechanisms of VOCs with *in vitro* models is to generate a stable and defined gas-concentration of the volatile substances at high humidity, to avoid direct contact of the substances with the medium during the exposure, and to have a responsive cell model that mimics the barrier function of the lung. Expanding upon earlier research [1], we characterized and exposed an *in vitro* model composed of differentiated epithelial cells cultured at the air-liquid interface, along with immune cells, to formaldehyde as a model analyte. We used an in-house developed exposure technique that enables long-term exposure up to several days. To capture the cell response comprehensively, we analyzed various omics layers and applied multi-omics integration. Systems network analysis supported the identification of most relevant signatures and affected biological processes.

In summary, we present a workflow that is suitable to comprehensively investigate the cellular effects of volatile chemicals.

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P01-67
Oxidative changes and cell death pathways induced by chlorpromazine photoproducts in breast normal versus cancer cells – contributions to development of new drugs for cancer therapy

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Chlorpromazine (CPZ) is an antipsychotic drug derived from phenothiazine, showing significant potential also in cancer therapy. Previous research proved that laser-irradiated CPZ exhibits antibacterial and antitumoral effects on breast cancer cells [1,2]. Several photoproducts were identified with promazine being the predominant compound [3].

To deepen our understanding of the toxicity mechanisms underlying the therapeutic efficacy of CPZ photoproducts, this study proposes a comparative analysis of oxidative status and cell death pathways activated following exposure in breast normal vs. cancer cells. CPZ hydrochloride solution was irradiated for 10 and 40 min with a 266 nm pulsed beam emitted by a Nd:YAG laser [1]. Laser-irradiated and non-irradiated CPZ solutions were applied to breast normal (MCF-12A) and cancer (MCF7) cell lines for 24 h at various concentrations of 10, 15, and 20 μM, with unexposed cells serving as the control. Assessment of oxidative potential was performed by measuring the production of reactive oxygen species (ROS) and the level of reduced glutathione (GSH), malondialdehyde (MDA), advanced oxidation protein products (AOPP), and protein expression of Nrf2 transcription factor. Protein expression profiles of Bax, Beclin-1, and LC3 were investigated as markers of cell death.

ROS generation was observed in almost all conditions, with the highest levels detected in cells treated with 40 min irradiated CPZ at a concentration of 20 μM. Notably, the photoproducts showed potential tumor-selective toxicity by inducing a high amount of ROS in cancer cells while non-irradiated CPZ in normal cells. CPZ photoproducts obtained after 40 min of irradiation caused a significant increase in intracellular GSH (by 65%) and MDA (by 82%) levels in MCF7 cells at a concentration of 15 μM, relative to control. No statistically significant changes in these parameters were observed in breast normal cells. However, the excessive ROS produced by non-irradiated CPZ in normal cells correlated with a 33% increase in protein oxidation compared to control. Furthermore, CPZ photoproducts obtained from laser irradiation for 10 and 40 min, as well as non-irradiated CPZ, induced AOPP formation in cancer cells after 24 h more than 50% over control. Decreased protein expression of ubiquitinylated Nrf2, Bax, and Beclin-1 in normal cells exposed to 40 min irradiated CPZ suggests the activation of antioxidant defense against oxidative stress and stimulation of cell survival pathways. On the other hand, CPZ photoproducts released after 10 and 40 min irradiation induced autophagy in MCF7 cells as shown by elevated protein expression of Beclin-1 and LC-3.

We conclude that CPZ photoproducts have enhanced anti-tumoral activity compared to non-irradiated CPZ by triggering different mechanisms in breast normal vs. cancer cells. The data presented herein highlight the potential of laser-generated compounds in pioneering the development of new drugs for cancer treatment.

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P01-68
Quantification of ciliary beating frequency in differentiated bronchial epithelial cells

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The mucociliary system of the airways is composed of cilia, mucus, and mucus and plays an important role in airway defense by removing inhaled foreign harmful particles. On of these, assessing cilia beating is important in toxicology and can be used to predict respiratory disease, as a decrease in cilia beating can lead to respiratory disease. However, existing methods for quantitatively assessing ciliary beats are rather complicated and require the purchase of specific equipment and programs. Therefore, in this study, we developed a simple and accurate ciliary beat frequency (CBF) quantification technique using a power spectral density measurement method based on image autocorrelation. To validate the performance of this technique, we analyzed the changes in ciliary beat frequency after exposure to PHMG-HCl for 3 hours in a 3D model of normal human bronchial epithelium (NHBE). The results showed a significant dose-dependent decrease in ciliary beating of NHBE cells after acute exposure to PHMG-HCl. Therefore, the assay is a simple and accurate way to analyze ciliary beating in pulmonary bronchioles, which can be used for the predictive evaluation of respiratory diseases.

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P01-69
In vitro markers for cytotoxicity and oxidative stress in human lung cell cultures, exposed to aerosols generated in a biofuel model system

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In the light of the principles of the circular economy, the use of residual products from the rural economy as biofuels is increasingly coming into practice. These can be fruit stones, hay, coffee, sunflower flakes, straw, etc. During combustion processes, however, organic substances

such as polycyclic aromatic hydrocarbons (PAHs), which are proven carcinogens, are expected to be released. That is why additional study of the toxicological characteristics (cytotoxicity and degree of oxidative stress) of ultrafine dust particles, which are produced during the combustion of biofuels, is of essential importance in assessing their safety. In order to use alternative approaches in toxicology, the application of model *in vitro* cell systems and genetic markers provide reliable data.

The present study **aims** to evaluate the cytotoxic response and oxidative stress state of lung cell A549 cell culture exposed to the influence of particle meters (PM) ≤1 with an aerodynamic diameter of less than 1 μm.

Samples of two types of biomass: pellets from wheat straw (WS) and cherry stone (CHS), are burned in a horizontal flow tube reactor (HPTR) described in Naydenova *et al.* (2023). The impactor has 14 cascades, to sample particles in a wide range (from 0.015 μm to 10 μm). Quantitative analysis of 16 priority PAHs was performed by gas chromatographic analysis (Environmental Protection Agency analytical method). A549 cell line was obtained from European Collection of Authenticated Cell Cultures. 10 mg of each particle matter sample were dispersed in 10 ml of distilled water upon sonication, followed by rapid freezing at -80^0C and lyophilisation. A series of dispersions with descending concentrations were obtained by dilution with culture media. For cytotoxicity assessment, MTT test was performed. For oxidative stress assessment, RNA was isolated from A549 cell culture treated with PM from WS and CHS and gene expression of HMOX1 was measured with real-time RT-PCR.

In our previous study it is proved that in the samples detect 16 priority PAHs have been identified. Observes the presence of at least 6 unidentified PAHs, which constitute about 23% of the total PAH mass with unclear toxic properties. The total PAH levels of PM 0.25 in the flue gases of the CHS exceed the measured values at PM 0.25 from WS. Cytotoxic impact analysis performed of ultrafine PM indicates PM derived from WS, reduce to a lesser extent vital ability of A549 cells compared to the particles obtained from CHS. A similar trend was observed in the evaluation of the degree of oxidative stress. The use of *in vitro* model systems and genetic methods represent an alternative tool for assessing the toxicological properties of pollutants generated during combustion.

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P01-70
3D liver models in animal-free nanofibrillar cellulose hydrogels

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The demand for well-defined, reproducible, biologically relevant, and cost-effective *in vitro* liver cell models for toxicology testing is increasing. These models should retain liver-specific cell functionality to provide clinically relevant toxicological profiles of drug compounds, and at the same time they should avoid the use of animals in research. Three-dimensional (3D) cell culture using hydrogels has emerged as a promising approach for developing such models. GrowDex® hydrogels, derived from plant-based nanofibrillar cellulose, have been shown to provide an effective and experiment-reproducible 3D matrix for various healthy and cancerous cell types, including liver cells. These hydrogels mimic the extracellular matrix, support cell growth and spheroid formation while enabling cells to remodel the matrix according to their needs. They exhibit desirable properties such as shear thinning and temperature stability, with minimal lot-to-lot variability, making them suitable for scaling up automated 3D cell-based assays for drug discovery, development, and toxicology testing.

Notably, GrowDex hydrogels have been shown to support the growth and spheroid formation of liver cells such as PHH and HepaRG while preserving their organ-specific functionality. Furthermore, these hydrogels facilitate the 3D coculture of hepatocytes with Kupffer cells, enhancing the physiological relevance of the models. We have developed a multiplexing protocol to simultaneously assess viability, cell death, CYP activity, and albumin secretion from the same sample, thus saving time and resources. Efforts are underway to automate and further optimize this protocol for high-throughput screening, paving the way for animal-free toxicology testing and potential integration into quality control processes for primary samples in the future.

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P01-72
EUROTOX Early Career Forum (ECF): The voice for early career scientists in EUROTOX

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The Early Career Forum (ECF) within EUROTOX provides a dedicated platform for early-stage toxicologists. Established in February 2024 by the EUROTOX Executive Committee, it serves to address the professional needs of early-stage researchers (ESRs) and foster connections between ESRs and EUROTOX. Our vision is to inclusively represent all ESRs regardless of their backgrounds, nationalities, or identities, and to promote the Intersectoral, International, and Interdisciplinary (3I's) nature of science across all our activities.

The ECF Core Team comprises eight early career researchers representing diverse geographical locations in Europe as well as diverse affiliation natures (academia, industry, regulatory experience). This team includes a chair who serves as the primary contact for ECF and liaises with the EUROTOX Executive Committee, a co-chair to assist with specific tasks and act as a substitute in the chair's absence, a secretary responsible for organizing and documenting ECF meetings,

and five officers who provide general support to the core team and lead specific member activities.

Our primary objectives are twofold: firstly, to serve as a central point of contact for ESRs with less than ten years since their highest degree, who are interested in toxicology across various domains (*in vivo*, *in vitro*, in silico, etc.); and secondly, to advocate for their interests. We aim to develop new EUROTOX activities tailored for ESRs, propose scientific programs such as workshops and symposia at EUROTOX annual congresses, and promote educational initiatives focusing on advancements in fundamental and applied toxicology. By actively engaging early career researchers, ECF seeks to align these developments with EUROTOX activities to drive progress in *in vivo* toxicology, *in vitro* methods, and *in silico* modeling.

In addition to their core roles, all members will serve as ECF ambassadors, maintaining communication with members and fostering collaborations with other ESR networks. We recognize networking as crucial at all levels to support the next generation of toxicologists.

The establishment of the EUROTOX Early Career Forum signifies our commitment to empowering young (and young of mind) toxicologists, elevating their professional stature, and facilitating significant advancements in safety science.

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P01-73

Development and use of reconstructed respiratory epithelium tissue models from multiple species for translational inhalation studies

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Highly differentiated *in vitro* models of the respiratory tract have been commercially available since 2000s and are widely used for toxicological, respiratory infection, tobacco safety, and inhaled drug delivery studies. Availability of tissue models representing the different segments of the respiratory tract were instrumental in gaining insight into the underlying mechanisms of SARS-CoV-2 and other infections as well as for screening of anti-viral compounds. Despite these useful applications, there are large databases of animal toxicity data which are not directly translatable to data obtained from the human *in vitro* airway tissue models due to the species differences.

Methods: To close this translational gap, cells harvested from both rat and non-human primate (rhesus monkey) tissues were utilized to develop models similar to EpiAirway, the human reconstructed tracheobronchial tissue model offered by MatTek. The tissues were characterized for structure (histology), epithelial cell markers (IHC), barrier integrity (transepithelial electrical resistance, TEER measurement), and functionality (inhalation toxicological studies). To verify the reliability of these models worldwide, quality control (QC) data for tissue lots produced in the US and Europe were compared.

Results: The animal cell-derived 3D tissues exhibited similar characteristics to human tissues including well polarized epithelia with physiological TEER values of >300 Ω*cm^2, cilia formation on the apical surfaces, and mucin production mimicking the airway microenvironment. Acute exposure to 4 chemical toxicants (CT) showed species-specific changes in the tissue viability and membrane integrity as measured by MTT and TEER assays, respectively. The effective dose concentration that reduces tissue viability by 50% (ED-50) for vinyl acetate (VA) and chloroacetaldehyde (CA) were both <2 mg/tissue and the ED-50 for propylene glycol (PG) was >20 mg/tissue for all species. However, the ED-50 values for toluene (T) showed differences between the species: human >20 mg, primate 16.2±1.7 mg, and rat 13.8±0.1mg. Based on the MTT viability and TEER values, the test chemicals were rank ordered from high to minimal toxicity: CA>VA>T>PG and the vehicle controls (water and corn oil). TEER values from standardized QC tests averaged 1094±325 (n=141 lots) in the US vs. 913±238 (n=64 lots) in Europe. TEER values were not statistically different ($p<0.001$).

Conclusions: Although more chemicals need to be tested, the multispecies 3D airway tissue models may become useful translational tools to predict airway inhalation toxicity and to bridge the *in vitro - in vivo* knowledge gap to reliably predict human responses, while providing a worldwide alternative approach to animal experimentation.

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P01-74

Bioinspired polydopamine nanoparticles trigger apoptosis in HCT-116 colorectal carcinoma cells

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Colorectal carcinoma (CRC) stands as one of the leading causes of cancer-related deaths worldwide, becoming a serious public health issue owing to its alarming and continuous increase in incidence. Polydopamine is an artificial melanin-mimetic polymer that imitates the structure and the properties of the natural pigment. Recently, polydopamine nanoparticles (PDA-NPs) gained outstanding popularity especially due to their application as nanoplatforms in cancer diagnosis and treatment. Nonetheless, the efficiency of PDA-NPs in CRC therapy lacks a comprehensive investigation at present. In the light of these data, the current study proposed the *in vitro* exploration of PDA-NPs in terms of therapeutic efficacy and safety as potential nanotherapeutics in CRC management.

PDA-NPs were produced via dopamine polymerization in alkaline conditions. The experiments were conducted using two human cell lines, HCT-116 and CCD 841 CoN, selected as *in vitro* models for CRC and normal colon cells, respectively. The influence of PDA-NPs on cell viability was assessed by applying the MTT test. Cell confluence and morphology, as well as the intracellular accumulation of PDA-NPs were observed through bright-field microscopy. The impact of PDA-NPs on HCT-116 and CCD 841 CoN cells' nuclear aspect and cytoskeletal F-actin and tubulin distribution was evaluated using immunofluorescence techniques. The expression of pro- and anti-apoptotic markers was determined using the RT-qPCR method. The 24 h treatment of HCT-116 and CCD 841 CoN cells with PDA-NPs (10, 25, 50, 75, and 100 μg/mL) lowered their viability in a dose-dependent manner, with higher cytotoxicity being exerted in CRC cells compared to normal colon cells. Bright-field microscopy highlighted the intracellular accumulation of PDA-NPs in both cell lines, evidenced by visible signs of pigmentation. The cell rounding and shrinkage caused by PDA-NPs at high concentrations in HCT-116 cells were accompanied by a massive loss of confluence, as well as condensation of chromatin, and constriction of F-actin and tubulin filaments. PDA-NPs reduced the expression of anti-apoptotic Bcl-2 and Bcl-XL markers in HCT-116

CRC cells, while also increasing the expression of pro-apoptotic Bad, Bax, and Bak markers.

These novel findings unveiled the *in vitro* anti-tumor efficacy of PDA-NPs which caused a significant and selective cytotoxicity in HCT-116 cells by triggering apoptosis.

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P01-75

Between-laboratory reproducibility of the modified STE to identify UN GHS Category 1 surfactants

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The Short Time Exposure (STE) test, a method that measures cytotoxicity in corneal epithelial cells, has been adopted by the Organisation for Economic Co-operation and Development (OECD Test Guideline 491) for identifying chemicals inducing serious eye damage (United Nations (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS) Category 1) and chemicals not requiring classification (UN GHS No Category). However, the method was not optimized for predicting eye irritation of chemicals having surfactant properties. A Defined Approach (DA) for eye hazard identification according to UN GHS has been developed for surfactants (DASF). The DASF is based on the combination of Reconstructed human Cornea-like Epithelium (RhCE) test methods (OECD TG 492) and a modification of the STE test method. The modified STE measures relative cell viability after a 5 minutes exposure to a 0.5% concentration and uses a viability cut-off of 20% to distinguish between UN GHS Cat. 1 and not UN GHS Cat. 1. Between-laboratory reproducibility (BLR) of the OECD adopted STE test method was evaluated during validation studies. The purpose of the current study was to evaluate the transferability and reproducibility of the modified STE.

The BLR of the modified STE was evaluated based on a subset (N=16) of the full set of surfactants (N=47) used to assess the performance of the DASF. The subset includes the 3 UN GHS categories and the full range of viabilities. The surfactants were tested in a European laboratory and the results were compared with those obtained by the test developer (Kao), which is located in Japan. Complete (100%) concordance in predictions (UN GHS Cat. 1 vs not UN GHS Cat. 1.) was obtained between the laboratories. Furthermore, all UN GHS Cat. 1 surfactants (N=7) were predicted Cat. 1, whereas the 4 UN GHS Cat. 2 and 5 UN GHS No Cat. surfactants were all predicted not Cat. 1 in both laboratories. Cell viability values ranged from 0 to 104% for the test developer and from 0 to 110% for the European laboratory with an excellent agreement between the laboratories (Pearson correlation coefficient of 0.96). This demonstrated successful transferability from Japan to Europe.These results indicate that the modified STE, which is part of the DASF, appears to be a reliable method for the identification of UN GHS Cat. 1 surfactants in a first tier of the Top-Down approach or the second tier in a Bottom-Up approach. This provides an important advancement that makes feasible eye irritation hazard assessments in a fully *in vitro* framework.

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P01-76

Human health effects and immunomodulating properties of marine toxins using *in vitro* human cell line models

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Marine toxins are a diverse group of natural toxins produced by marine micro-algae. They occur in high amounts during harmful algal blooms and cause human intoxications by consumption of contaminated shellfish. Moreover, marine toxins also end up in sea spray aerosols, causing an inhalational exposure route associated with several effects including respiratory symptoms. Despite their association with harmful algal blooms and shellfish poisoning, recent studies suggest that some marine toxins may exhibit health-promoting properties at low concentrations, such as anti-alzheimer, anti-inflammatory or anti-cancer effects. We investigated the human health effects of marine biotoxin exposure by *in vitro* (immuno)toxicity testing. A sub selection of marine toxins with high potential were selected, namely yessotoxin, homoyessotoxin and pectenotoxin-2. Two human cell line models, A549 and THP-1, were exposed to different concentrations of the phycotoxins. Various types of colorimetric and fluorescent assays provided insights into toxicity and immunomodulating effects of the phycotoxins. At low doses, both yessotoxin and homoyessotoxin selectively impact lysosomes, preserving metabolic activity and cell membrane integrity. This distinct toxicity mechanism was not observed for pectenotoxin-2. Furthermore, the yessotoxins exhibit a concentration-dependent modulation of cytokine expressions.

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P01-77

Phytochemical characterization and *in vitro* assessment of *Galium mollugo* L. extract on murine melanoma cells

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Galium sp. are recognized as effective and easily accessible traditional natural remedies, with a content rich in phytocompounds, mainly from the class of flavonoids, terpenes, phenolic acids, and phytosterols. This vast composition has led to the use of the genus *Galium* as nutraceuticals, in combating health problems involving the immune system and inflammation [1,2].

Until now, there is no data on the antitumor potential of *Galium mollugo* L., thus, the current study aimed to investigate the cytotoxic effect of the ethanolic extract of *G. mollugo* L. (GmEtOH) on murine melanoma cells (B164A5) and to determine the chemical composition responsible for therapeutic effect.

Through the LC-MS technique, the phytochemical profile of GmEtOH was outlined. The *in vitro* anticancer potential was investigated by determining the viability (MTT method), by microscopic eval-

uation of the cellular morphology, by quantifying the cytotoxicity (LDH test), and more deeply by observing the changes in the nuclei using the Hoechst assay, after stimulation with five concentrations (50–400 µg/mL) of GmEtOH for 24 hours.

The results showed that GmEtOH extract contains polyphenolic compounds, with chlorogenic acid in the highest percentage. Moreover, at the highest concentration, a decrease in viability of B164A5 cells below 50% was observed, together with a strong release of lactate dehydrogenase. At 50 µg/mL, the confluency reduced visibly, while at 400 µg/ml, the elongated cells became round and detached from the plaque. Furthermore, at 400 µg/mL, considerable pro-apoptotic signs were exposed, with nuclear disintegration and chromatin condensation.

Our research completes the lack of data from the literature with new information regarding the bioactivity of *Galium mollugo* L. herba, especially regarding the antitumor activity. The results exhibit that the extract has a dose-dependent cytotoxic action on murine melanoma cells, future studies being necessary to understand the mechanism of action.

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P01-78

Evaluation of immunomodulatory effects of micro/nanoplastics in human induced pluripotent stem cell-derived intestinal epithelial cells and human dendritic cells

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Evidence exists that humans are exposed to micro- and nanoplastics (MNPs) via diet and these MNPs have been described to cross intestinal barriers and enter the bloodstream. Inflammatory effects of MNPs were shown in *in vitro* models, but data on immunomodulatory effects in the intestine and intestinal resident immune cells are still scarce and poorly understood.

In this study, we evaluated the immunomodulatory effects of MNPs in human induced pluripotent stem cell (hiPSC)-derived intestinal epithelial cells (IECs) and human dendritic cells (DCs) derived from human MUTZ-3 cells (CD34+ progenitor cells). IECs, grown as 2D layers, represent a model for the intestinal epithelial barrier and consist of multiple cell types, including enterocytes, goblet cells, stem cells, Paneth cells, and enteroendocrine cells. The immune responsiveness of the IECs was characterized using a challenge with an inflammatory cytokine cocktail. DCs were selected for testing due to their natural presence in the intestine, where they function as key regulators of the immune system and have the potential to sense and sample compounds through the intestinal barrier. A protocol to differentiate MUTZ-3 progenitor cells into immature (iDCs) and mature (mDCs) DCs was optimized and markers were selected to distinguish progenitor cells from immature (iDC) and mature (mDC) cells. To study immunomodulatory effects of MNPs, MNPs with different polymer compositions (i.e. HDPE, PET, PS) and different particle size distributions (ranging from nanometer to micrometer scale) were selected and tested at 25, 50 and 100 µg/ml.

IECs challenged with a pro-inflammatory cocktail showed no indications of cytotoxicity or barrier disruption, but a light decrease in cell viability and barrier integrity was noted. Cytokine expression in the IECs increased (transcriptomics analysis and protein expression), indicating good immune responsiveness. This exposure was further used as a positive control. Exposure to MNPs for 48h showed no cytotoxicity and no effects on pro-inflammatory cytokine expression. Transcriptomics analysis, qPCR, and flow cytometry was used to select the best protocol resulting in mDCs resembling intestinal DCs (e.g. expression of CD1c, CD103, CD11b, and IRF8). These mDCs were further used as a positive control. Next, the potential of MNPs to activate maturation of iDCs into mDCs was evaluated after 48h exposure. MNP exposure did not appear to be cytotoxic compared to the controls. Furthermore, MNPs did not activate maturation of the iDCs into mDCs. However, cytokine expression of some cytokines (i.e. IL-6, IL-8, IL-23) was significantly induced for the PS MNPs (albeit still lower than the positive control). Currently, cellular uptake of the PS particles (that are doped with Europium) in IECs and DCs is being evaluated using single particle-ICPMS.

In summary, exposure of human iDCs and hiPSC-derived IECs to MNPs showed no cytotoxicity and minimal to no immunomodulatory effects.

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P01-79

The applicability of GARDskin for assessing skin sensitization potential of hydrophobic esters during product development

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The field of skin sensitization assessment is rapidly evolving and the recent advancements in New Approach Methodologies (NAMs) has made it possible for the industry to perform *in vitro* skin ssensitization testing with good predictivity across a large chemical space. However, challenges remain for "difficult-to-test" chemicals, those with challenging physical/chemical properties or of Unknown or Variable composition, Complex reaction products or Biological materials (UVCBs), which are often outside the applicability domain of conventional cell-based assays. GARDskin (OECD TG 442E) is a genomic-based assay with demonstrated applicability to "difficult-to-test" substances. The aim of this study is to assess the skin sensitization potential of two ester substances of biological origin, substance A and B, using the GARDskin assay. These substances are very hydrophobic and fall outside of the applicability domain of the conventional *in vitro* assays. Both substances were successfully solubilised in cell media by utilizing a combination of heating, sonication, and selection of appropriate solvent vehicle (ethanol or acetone). No cytotoxicity was observed for either substance, thus 500 µM was chosen as the input concentration for cellular stimulations. GARDskin combines a genomic readout with machine learning to predict skin sensitizing hazard, where values above the threshold (DV=0) is predicted as skin sensitizing and below as non-sensitizing. Both substances resulted in negative mean Decisions Values and thus were classified as non-sensitizers in GARDskin (A: -1.54, B: -0.339).

In conclusion, with the inclusion of GARDskin into the OECD test guidelines, the range to where NAMs are appropriate has been increased. This study demonstrates the applicability of the GARDskin assay to assess skin sensitizing hazard of hydrophobic ethyl esters, which provides an ethical alternative to animal methods for safety assessment during product development.

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P01-80

***In vitro* assesment of the safety profile of caffeine and magnesium ascorbyl phospate incorporated in HPMC on human keratinocytes**

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Introduction: Caffeine (CAF) and magnesium ascorbyl phosphate (MAP – a derivative of ascorbic acid) are two ubiquitous substances in our society and widespread in the pharmaceutical sphere due to their multiple properties in topical formulations. CAF and MAP are photoprotective compounds, help skin care and prevent skin aging, and moreover, have important anti-inflammatory and antioxidant properties. Hydroxypropyl methylcellulose (HPMC) is found in a variety of formulations being a good viscosity agent, bioadhesive and has the ability to increase the stability of compounds. The use of formulations in which CAF and MAP are included in HPMC increases the need for a more in-depth knowledge of the biosafety profile, including at the cellular level, for future directions of use and to avoid adverse effects.

Aim: The aim of the study was to test CAF and MAP included in HPMC on HaCaT – human keratinocytes cell line to determine the safety profile of the compounds at the cellular level. Due to the wide use of these compounds, knowledge of their impact incorporated in HPMC brings improvements in terms of future directions in use.

Materials and Methods: For the *in vitro* evaluation of CAF and MAP in HPMC, cell viability determination (using MTT assay) cell morphology analysis and cytotoxicity potential (using LDH method) were performed after 24 hours of treatment with the compounds of interest in concentrations of 100, 200, 300, 400 and 500 µg/ml.

Results: Cell viability results indicated that the greatest decrease in cell viability was reported only at the highest concentration for both formulations (500 µg/ml), reaching the threshold of approximately 80% viable cells compared to untreated cells. Furthermore, at the lower concentrations, cells appear to be stimulated after the application of the formulations. Cell morphology was not affected, and there were no signs of cellular stress or cytotoxicity at the concentrations tested.

Conclusion: The data showed that the two compounds included in HPMC do not affect keratinocyte cells. There were no considerable changes in cell shape and in addition there was no statistically significant decrease in cell viability after treatment even at the highest concentration, expressing that both formulations have a good safety profile at the cellular level, being promising formulations in cutaneous treatments.

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P01-81

Exploring Gold/PLGA nanoparticles as potential drug-delivery system in a co-culture blood-brain barrier model

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The brain is the epicenter of the human body, protected by the blood-brain barrier. Although this has a protective role, its low permeability represents a disadvantage for the delivery of drugs at the level of the central nervous system, in order to treat various pathologies such as tumors, stroke, multiple sclerosis, Alzheimer's, Parkinson's, etc. Thus, a way to deliver medications to the brain needs to be developed and nanoparticles are a promising candidate due to the physico-chemical properties they present. In this study, the translocation capacity of two types of nanoparticles (NPs), based on Poly (lactic-co-glycolic acid) (PLGA) or gold, was investigated using a blood-brain barrier model represented by a co-culture of hCMEC/D3 endothelial cells and neuroblastoma, SH-SY-5Y grown in 1:1 ratio on Transwell cell-culture inserts. The main aim of this study was to investigate the potential of selected nanoparticles to cross the blood-brain barrier and act as drug delivery systems. PLGA and gold NPs were added in the apical compartment at different concentrations (12.5; 25; 50 and 100 µg/mL) and the inflammation process was checked by measuring the levels of interleukins IL-6 and IL-8 after 24 and 96 hours of incubation. The viability and cytotoxicity were analyzed by the MTT and LDH tests after 24 hours. Also, the quantity of transported and internalized nanoparticles were evaluated by spectrophotometric measurements after 24, 48, and 96 hours. The cell viability, integrity membrane, and nitric oxide revealed no significant changes compared to the control, suggesting that PLGA and gold NPs exerted no cytotoxic effects on blood-brain barrier models. The inflammatory process after 24 hours indicated a growth compared to control in the blood-brain barrier model following incubation with PLGA and gold nanoparticles. The analysis of nanoparticle transport through cellular models showed an increased permeability of the blood-brain barrier for PLGA and Gold nanoparticles, respectively. In addition, the measurement of transepithelial electrical resistance (TEER) showed that the permeability of the co-culture hCMEC/D3: SH-SY-5Y did not change in the presence of PLGA and gold NPs. In conclusion, our results showed that PLGA and gold NPs exerted insignificant toxic effects on the blood-brain barrier and can be used as multifunctional platforms for delivering therapeutics in monitoring their biodistribution and therapeutic efficacy. Nonetheless, ongoing research in this field holds great promise for advancing the treatment of neurological disorders and brain tumors.

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P02 | New Approach Methodologies: 3D models, stem cells, organ-on-a-chip, microfluidics

P02-01

Comparison of nickel toxicity in Air-Liquid Interface models of human and rat bronchial epithelial cells

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Nickel (Ni) compounds are indirect genotoxic carcinogens with threshold mode-of-action. While Ni subsulfide (Ni_3S_2) and Ni sulfate hexahydrate (hereafter referred to as $NiSO_4$) are classified as human carcinogens, the former induces lung tumors in rats while the latter does not. In preparation for studying the carcinogenic mode of action of these Ni compounds, we conducted toxicity studies in Air-Liquid Interface (ALI) models of rat (newly developed model) and human bronchial epithelial cells. Rat (MucilAir-RF) and human (MucilAir-HF) MucilAir were exposed to Ni_3S_2 or $NiSO_4$ via droplet or air exposures.

TEER, LDH release as a measure of cytotoxicity, and IL-6 as a measure of inflammation were assessed following exposure. MucilAir-RF were exposed for 24 hours to 10, 30, and 90 μg/cm^2 Ni_3S_2 or 1.33, 4, and 12 mM $NiSO_4$ in droplet exposures. MucilAir-RF were exposed to 281, 728, and 4767 mg/m^3 $NiSO_4$ or 302, 854, and 2318 mg/m^3 Ni_3S_2, whilst MucilAir-HF were exposed to 280, 934, and 2503 mg/m^3 $NiSO_4$, or 237, 916, and 2353 mg/m^3 Ni_3S_2 , during single 6-hour air exposures.

In the droplet studies in MucilAir-RF, Ni_3S_2 caused ≤15% cytotoxicity, whilst $NiSO_4$ caused up to 25% cytotoxicity. Both compounds decreased the TEER values and increased the IL-6 levels in a concentration-dependent manner.

In the air exposures, both $NiSO_4$ and Ni_3S_2 decreased membrane integrity in a concentration-dependent manner, increased cytotoxicity, and increased IL-6 production in MucilAir-RF and MucilAir-HF. At comparable mg/m^3 levels, $NiSO_4$ caused a greater decrease in TEER and a greater increase in inflammatory IL-6 than Ni_3S_2. These results are consistent with *in vivo* rodent studies, where at equal mg/m^3 or mg Ni/m^3 exposure, $NiSO_4$ triggered a higher increase in the expression of an inflammatory cytokine than Ni_3S_2. Higher intracellular levels of Ni_3S_2 than $NiSO_4$, particularly in the nucleus, were observed; this was in agreement with previous cell uptake studies.

In conclusion, the MucilAir models were able to reproduce the differential pattern of toxicity seen *in vivo* for these two compounds. Although further work is needed on the reproducibility of the Ni air exposures, these models may provide a welcome alternative to animals for mode of action studies.

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P02-02
Pycnogenol® as a safe suplement for treating ADHD

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Purpose: The awareness of ADHD grew throughout the ages and the overall view of this disorder has changed. With new diagnostic criteria and understanding of ADHD types, the generic picture of a child who can't withstand sitting on a chair and paying attention in class drifts away. More adults get diagnosed, more parents take their children for a treatment, more patients search for safer substitutes than methylphenidate or atomoxetine, which side-effects can even lead to addiction. That's when people turn to more natural remedies and one of them is Pycnogenol® (PYC) – an extract of French maritime pine bark.

Methods: The experiments were conducted on SH-SY5Y cell cultures which after differentiation process were turn to dopaminergic neuron model. Based on literature review, 10 and 25μM atomoxetine (ATX) and 10–100 μg/ml PYC concentrations where chosen for experimental setting. To assess the potential benefit in ADHD treatment, we have evaluated the effect of PYC on norepinephrine (NE) and its transporter (NET) as theirs levels are diminished in neuronal tissue. Moreover, we have analysed if PYC could work as an Alpha-2B adrenergic receptor (ADRA2B) and vesicular monoamine transporter 2 (VMAT2) stimulator leading to synaptic release of neurostimulants via exocytosis.

Results: None of the tested PYC concentration caused decrease in dopaminergic neurons' viability which was measured by SRB assay. In NE analysis, the highest concentration in the interneuronal space was observed after 25μM ATX treatment (878 pg) and after 50μg/ml PYC administration (547 pg). In cellular lysates 25μM ATX incubation led to 32% increase in NE concentration (2,33 pg) while after 100μg/ml PYC incubation 1,98 pg of NE was detected on 100μg protein. In NET the highest efflux from the cells was observed after 10μg/ml PYC (1000 pg) and 25μM ATX (969 pg) with around 40% higher levels comparing to the untreated cells. Whereas, in lysates 10μM ATX incubation caused more than 200% higher concentrations (818 pg) comparing to the control cells. The best effect of PYC was measured after 10 μg/ml with almost 30% increase (515 pg). The highest concentration of ADRA2B in supernatants were obtained after 25μM ATX (11886 pg), 50 and 100μg/ml PYC treatment (11022 pg) which is similar to the values obtained for control cells. In lysates 50 and 100μg/ml PYC stimulated the highest values of ADRA2B (around 830 pg) comparing to the untreated cells and ATX. In VMAT2 in both supernatants and lysates the differences in concentration after ATX and PYC administration were not statistically significant.

The results indicate that Pycnogenol® is not only an option with less side-effects than drugs used in ADHD treatment, but could also optimize the neurochemical environment in the patients' brains. It's already used for allergies, asthma, mild hypertension, diabetic retinopathy and improvement of athletic performance. Pycnogenol® can become a great supplement for ADHD treatment.

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P02-03
Comparison of biological effects on mouse tracheal epithelial cells cultured at the Air-Liquid Interface: a study of cigarette smoke and heated tobacco product aerosol

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Heated tobacco products (HTPs) produce fewer harmful and potentially harmful constituents (HPHCs) compared to cigarettes because HTPs heat but not combust tobacco leaves to generate aerosol. Various studies are underway to evaluate whether this reduction in HPHCs is associated with a reduction of toxicity for HTP users.

In vivo inhalation studies are one approach to investigating the toxicological effects of HTP aerosol, and the results of several studies indicate that HTP aerosol has a reduced biological impact on animals compared to cigarette smoke (CS). However, from an ethical standpoint, there is a need to minimize the use of animals in scientific studies. Air-liquid interface (ALI) cultures of mouse tracheal epithelial cells (MTEC) could serve as a useful *in vitro* model for this purpose. ALI-cultured MTEC have a functionally differentiated airway epithelium with basal cells, ciliated cells, and secretory cells.

In this study, we compared the effects of CS and HTP aerosol on ALI-cultured MTEC. MTEC were isolated from 9-week-old female C57BL/6 mice and passaged twice before ALI culture. The differentiation of ALI-cultured MTEC was examined by gene and protein expression of selected markers for basal, ciliated, and secretory cells. The exposure was performed for 24 hours from ALI day 21. The cells were exposed to total particulate matter (TPM) from 1R6F CS or aerosol collected mass (ACM) from our proprietary direct heating tobacco system 3.0a (DT 3.0a) aerosol up to 1000 μg/mL. We focused our investigation on xenobiotic metabolic processes and oxidative stress responses as they are considered initial responses to CS *in vivo*.

The integrity and viability of ALI-cultured MTEC decreased upon exposure to 1R6F TPM at concentrations above 500 μg/mL but did not decrease with exposure to DT3.0a ACM. Exposure to 1R6F TPM also

increased the release of granulocyte macrophage colony stimulating factor (GM-CSF) and Keratinocyte-derived cytokine (KC). The expression of genes related to xenobiotic metabolic processes (*Cyp1a1*, *Cyp1b1*, *Ahrr*, and *Aldh3a1*)and oxidative stress response (*Nqo1*) were upregulated by exposure to 1R6F TPM at concentrations above 12.5 µg/mL. These genes were also upregulated by exposure to DT3.0a ACM, but the concentrations were higher than those for 1R6F TPM: above 250 µg/mL.

The results obtained with ALI-cultured MTEC showed that the biological effects of HTP aerosol were reduced compared to CS, a trend similar to previous *in vivo* studies. Therefore, ALI-cultured MTEC could be a useful tool to reduce the need for *in vivo* inhalation studies of tobacco products. ALI-cultured MTEC may also be valuable in developing methodologies to extrapolate *in vivo* data from *in vitro* data because experimental data obtained *in vitro* and *in vivo* in the same species can be compared.

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P02-04
ASPIS Academy: empowering the next generation of toxicologists

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The ASPIS Academy is a dynamic networking platform where the landscape of toxicology is being reshaped by nurturing and empowering early-stage researchers (ESRs) through a vibrant networking hub and tailored professional development initiatives. Established in 2023 by the ASPIS cluster, a collaborative initiative comprising three European Horizon 2020 projects ONTOX [1], RISK-HUNT3R [2], and PrecisionTox [3], with a mission to accelerate and refine chemical risk assessment practices within the European Union using animal-free approaches. ASPIS is driving innovation through the development and validation of New Approach Methodologies (NAMs), leveraging *in vitro*, *in silico* techniques, and cutting-edge technologies like artificial intelligence and omics. It focuses on moving away from the reliance on traditional animal models, such as rodents, to increase the precision, efficiency and cost-effectiveness of chemical safety assessment.

Recognizing the pivotal role that ESRs play in shaping the future of toxicology, the ASPIS Academy is dedicated to providing a supportive environment where their talents can flourish. With a membership of over 120 ESRs, the ASPIS Academy offers a suite of specialized programs focusing on comprehensive soft and hard skill training, individualized mentorship, opportunities for lab exchanges, and effective communication strategies. Led by ESR representatives and guided by seasoned researchers, these initiatives aim to foster inclusivity by embracing diverse perspectives and backgrounds.

Join the ASPIS Academy in creating an environment where every voice matters and where the ideas and aspirations of emerging scientists can flourish. The ASPIS Academy serves as a beacon of collaboration, support, and growth, representing a pivotal advancement in toxicological discourse and paving the way for a brighter future in chemical risk assessment.

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P02-05
Validating *In finite* prediction of time-evolved inflammation triggered by particulate matter

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Background: One of the most challenging tasks in current toxicology is predicting long-term health hazard ahead of time, so much needed to deliver new advanced materials safely onto the market. Its success does not depend on existence of smarter long-term animal tests or their animal-free alternatives per se but rather on our ability to identify 1) inability of biological system to resolve changes triggered by the particulate matter (PM) via Molecular initiating events (MIE) as well as to identify 2) potential propagation of MIE into the adverse outcome pathway(s) (AOP) faster than it would naturally evolve in an organism.

During the SmartNanoTox H2020 project we had shown that there exists an in-vitro-to-in-silico translation enabling prediction of inflammation stages beyond the time scale of observation [1]. Later on, Infinite has evolved translation into an "*In finite*" automated platform [2], delivering early Mode-of-Action (MoA) directly from up to 100h long monitoring of an PM-exposed *in vitro* mouse lung model together with inflammation prediction up to 12 months into the future. Currently, nanoPASS HEurope project consortium aims to validate the *In finite* translation against the largest publicly available *in vivo* database, for which the recent results are presented here.

Methods: Despite combining the datasets from several past EU projects, the dataset number cannot exceed 100, defining the maximal number of independent/orthogonal MoAs (N_{maxMoA}). For validated prediction to be applicable to any PM, MoA cannot refer to Mechanism-of-Action as the number of degrees of freedom (Ndf) on molecular level easily exceeds 100.000, far beyond N_{maxMoA}. Instead, MoA here refers to Mode-of-Action on functional level, where the Ndf does not exceed N_{maxMoA}. Thus, *in vitro* monitoring translated by the *In finite* technology is focused to monitor all the functional units at subcellular and cellular levels.

Results: Predicted dose- and time-values are validated against the neutrophils influx measured *in vivo* as the earliest observable key event on the systemic level at 3–5 time points (from 3 days to 9 months) after exposure to 2–4 doses of diverse PM such as metal oxides (e.g. TiO, SiO, ZnO, NiO, NiFeO), nano-clays, carbonaceous particles (e.g. carbon nanotubes, graphene oxides) and combustion products (diesel exhausts, carbon blacks). Currently, the validation has reached the

sensitivity of 72% and specificity of 83% for time points between 3rd day and 9th month as well as for surface doses up to 10:1.

Conclusions: Automated *In finite* platform can determine the early MoA on the functional level within 4–5 days funneling PM-specific MIE into material-agnostic KEs of the AOP without employing any MoA assumption and exceedingly expensive experimentation delivering dose- and time- dependence of the PM of interest, taken either from R&D or air-sampled at the working place, thus enabling fast and validated long-term safety assessment.

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P02-06
New Approach Methodologies for hepatotoxic hazard assessment: development of an *in vitro* human liver fibrosis model

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It is estimated that there are around 70.000 chemical substances for which sufficient toxicological information is lacking [1]. Compared to the general population, this is particularly important in occupational settings where workers may be exposed to high and repeated doses of chemicals. Animal testing has been the gold-standard for chemical risk assessment, however, it is resource intensive and can fail to accurately predict toxicity effects in humans [2]. Given the intrinsic limitations of toxicity testing using animal models, the large number of chemical compounds lacking toxicological information as well as the need to reduce testing in animals, New Approach Methodologies (NAMs) are emerging with the ultimate goal to become safer, more accurate and animal-free chemical risk assessment tools as part of the Next Generation Risk Assessment (NGRA) paradigm shift in toxicology [3].

The Partnership for the Assessment of Risks from Chemicals (PARC) aims to develop and implement NGRA methodology in Europe. As part of this we are developing an *in vitro* human liver multicellular model that recapitulates the pathophysiology of liver fibrosis building from the proposed Adverse Outcome Pathway (AOP) #38 (AOP-Wiki): 'Protein Alkylation leading to Liver Fibrosis'. Human hepatic cell lines including hepatocytes (HepG2), hepatic stellate cells (hTERT-HSC), hepatic liver sinusoidal endothelial cells (TRP3) and primary monocyte-derived macrophages (MDMs), will be cultured on a trans-well system. Cell cultures (monocultures and multicultures) will be characterized for the expression of cell-type specific markers using immunofluorescence analysis. TRP3 cells will be structurally characterized by scanning electron microscopy for the detection of fenestrae. Acute exposures with known hepatofibrogenic chemicals – allyl alcohol, monocrotaline and thioacetamide – will be performed to validate the model. All Key Events of AOP #38, including 'cell injury', 'tissue resident cell activation', 'increased pro-inflammatory mediators', 'hepatic stellate cell activation' and 'extracellular matrix alteration' will be measured in the multiculture system and compared with cells grown in monocultures to assess the model. We aim to develop a liver fibrosis NAM that is able to discriminate hepatofibrogenic from non-hepatofibrogenic compounds, contributing to the NGRA strategy. This study was co-funded by the EU Horizon Europe program PARC.

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P02-07
Using microfluidic hepatic spheroid cultures for assessing effects of T2 mycotoxin

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Mycotoxins represent one of the most important categories of natural toxins to human and animal health and economic impact worldwide. The T-2 toxin (T-2) is a type A trichothecene, produced by several species of *Fusarium* fungi, and it is one of the most toxic and distributed around the world. It is found in several cereals, but mainly in oats. Some of the described mechanisms of action of this toxin are inhibition of protein synthesis, activation of mitogen-activated protein kinase (MAPK) and inhibition of DNA and RNA synthesis. However, there is a lack of information on the effects of T-2 in three-dimensional co-cultures of liver cells *in vitro*. In this work, we used microfluidic devices to create 3D co-culture of hepatic cells (HepG2) and stellate cells (LX2), and the objectives were: (i) to evaluate the albumin secretion in monoculture (HepG2) and co-culture (HepG2-LX2) after 24 h of T-2 exposure by ELISA (ii) to compare the relative spheroid growth upon exposure to T-2, and (iii) to characterize the inflammatory markers after T-2 exposure in monoculture and co-culture spheroids. The results demonstrated that albumin secretion and spheroid size were higher in co-culture compared to monoculture at all concentrations of T-2 (15, 30 and 60 nM) after 24 h of exposure. The secretion of IL-1RA, TNFα and IL-8 in co-culture were significantly higher at 60 nM of T-2 compared to 0 nM. This suggests that pro- and anti-inflammatory signaling programs were upregulated in the presence of mycotoxin. In conclusion, we demonstrate that microfluidic 3D co-cultures may represent a valuable model for investigating effects of mycotoxins on the liver.

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P02-08
Using ToxTracker and DNA Repair-Deficient Cell Lines to Determine the Genotoxic Mode of Action of N-Nitrosamines

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N-Nitrosamines (NAs) are considered probable human carcinogens and were recently detected as impurities in pharmaceuticals leading to a concern for human health. NAs require metabolic activation before they become mutagenic. There are many, differently sized NAs and not all NAs are mutagenic. Understanding which NAs are genotoxic and their mode-of-action (MoA) will improve understanding of the mutagenic potential of these drug impurities as it relates to their structure.

While NAs are potent mutagens *in vivo*, *in vitro* metabolization is generally less efficient. We first optimized a hamster S9-based protocol for *in vitro* NA metabolization. Next, we assessed the genotoxic potential of 8 different NAs to which humans are commonly exposed using the ToxTracker assay. ToxTracker is a reporter assay that can provide insight into the MoA of genotoxicity. All tested NAs were classified as genotoxic in ToxTracker in presence of hamster S9 with a clastogenic MoA. The smaller NAs did induce DNA strand breaks but no detectable inhibition of DNA replication, likely related to the size of the induced DNA lesions.

To further investigate the MoA of these NAs, we applied DNA Repair Profiler, a collection of mammalian cell lines with deficiencies in the major DNA repair pathways. For the smaller NAs, there was no increased cytotoxicity in base excision (BER) and nucleotide excision repair (NER)-deficient cells compared to wild type cells. Currently we are exploring the repair of DNA damage from the larger NAs as well as the relevance of DNA mismatch repair to reduce NA-induced gene mutations.

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P02-09
Testing nanomaterials in complex 3D *in vitro* lung models

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The potential health and environmental hazards linked to nanomaterials (NMs) present an obstacle to their widespread adoption and impede public perception of the advantages of nanotechnologies. DIAGONAL aims to advance Safe by Design expertise and resources to a stage where they can be integrated into the development process of industries dealing with MultiComponent NanoMaterials (MCNMs) and High-Aspect Ratio Nanoparticles (HARNs). The project relies in part on *in vitro* research to study specific hazard properties and potential exposure strategies of MCNMs and HARNs. LIST investigates their effects upon inhalation exposure, which is one of the scenarios raising most NM safety-related concerns. To this end, an advanced *in vitro* 3D co-culture alveolar model is used where the basolateral side is in submerged conditions while the apical side allows exposure at the air-liquid interface (ALI), resulting in a close replication of the human physiology. Exposure of 3D models to NMs can be performed with multiple exposure strategies, all with advantages and disadvantages which should be considered to ensure its relevance with a high enough throughput and reasonable labour intensiveness. Here, we share tips and tricks on common exposure systems used for *in vitro* testing of NMs in the 3D alveolar model.

Submerged exposure, where the system is never airlifted during preparation, offers speedy exposure and nominal concentration proximity but lacks human physiology resemblance. Importantly, the sedimentation and diffusion rates of NMs suspended in cell culture media, which is largely dependent upon the effective density and diameter of formed agglomerates in suspension, should be determined. As a compromise, semi-ALI involves airlifting the system but still delivering the NM in limited amounts of medium (65 µL/cm^2). Aerosol-based strategies like Vitrocell Cloud single droplet exposure systems mimic *in vivo* scenario closely but require extensive cleaning between materials to avoid cross contamination, limiting daily usage. Continuous-flow systems allow ALI exposures that closely resemble long-term exposures but obviously, results in a lesser throughput compared to single droplet exposure systems. Albeit being primarily developed for chemicals, high-precision printers like Tecan D300e dispense suspended NMs up to 100 nm at ALI (1 µL/cm^2) with minimal setup time and low risk of cross-contamination, yet size restrictions limit its versatility.

Reaching the nominal concentration in the *in vitro* system is crucial to ensure reliable experimental outcomes. Deviating significantly from the intended concentration can introduce errors and impacts the reproducibility of the results. Equally important is to consider the necessity of mimicking the true physiology and its' possible effect on the study outcome. Moreover, understanding the differences between these exposure systems is key to facilitate comparison and extrapolations between studies using different strategies.

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P02-10
Effects of storage of LabCyte EPI-MODEL24, the reconstructed human epidermis, on the *in vitro* skin sensitization test EpiSensA

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LabCyte EPI-MODEL24 (manufactured by J-TEC) is the three-dimensional reconstructed human epidermis (RHE) with basic epidermal structure (basal layer, spinous layer, granular layer, and stratum corneum) made by stratified culture of normal human epidermal keratinocytes. Epidermal Sensitization Assay (EpiSensA) is an *in vitro* skin sensitization test developed by Kao Corporation that quantifies changes in the expression of four marker genes (*ATF3, GCLM, DNAJB4,* and *IL-8*) associated with the activation of keratinocyte in LabCyte EPI-MODEL24. By utilizing RHEs, EpiSensA enables to evaluate substances with a wider range of physical states and chemical classes compared to the currently available *in vitro* test methods approved by OECD for regulatory testing. The draft OECD test guideline for EpiSensA has been released and currently under deliberation by the committee. Although EpiSensA is getting attention in recent years, the effects of storage of LabCyte EPI-MODEL24 have been concerned since the RHE is a living tissue. To clarify the shelf life of LabCyte EPI-MODEL24 for use in EpiSensA, we evaluated two positive control substances and ten draft proficiency substances using LabCyte EPI-MODEL24 stored at room temperature for up to 7 days after shipment. Following the draft OECD test guideline, we analyzed whether the mean fold induction value exceed the respective cut-off value at the three or four fixed concentration (described at Annex 1 of the draft test guideline) by measuring the relative changes in each marker gene expression with Reverse Transcription-quantitative PCR (RT-qPCR). It was confirmed

that the results for two positive controls and ten draft proficiency substances generally corresponded to the target criteria and the changes of EpiSensA results depending on the storage period were not so significant when we used LabCyte EPI-MODEL stored for 3 days. The results suggest that storage of LabCyte EPI-MODEL for 3 days after shipment is acceptable for EpiSensA. In addition, it is also confirmed that the results for the cell viability and RT-qPCR for two positive controls met the test requirements even when we used LabCyte EPI-MODEL stored for 7 days. We are currently conducting detailed analysis regarding the relationships between changes in LabCyte EPI-MODEL depending on the storage period and its responses to each draft proficiency substance.

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P02-11
Enhancing genotoxicity assessment: advanced 3D cell models for chemical testing

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The continual development of new chemicals and consumer products, including cosmetics, medicines, food and feed additives, and other everyday products, has raised significant concerns regarding their potential adverse effects on human health, particularly due to their genotoxic properties. The genotoxicity data required for the registration and approval of such chemicals and medicines are typically obtained through a series of short-term genotoxicity tests conducted on bacteria and rodent or human cell lines. However, current *in vitro* test systems often produce false-positive results, primarily because the cell lines used for genotoxicity testing do not adequately express enzymes involved in the metabolism of xenobiotic compounds. To address this challenge and to meet the high demand for the development of more physiologically relevant *in vitro* cell-based systems that provide more predictive results for human exposure, an innovative *in vitro* hepatic 3D cell model from HepG2 cells has been developed. This model involves the formation of spheroids by forced floating method and the culturing of the spheroids under controlled static conditions for several days without compromising cell viability. The GENTOX3D and 3D-ToX collaborative projects facilitated the transfer of knowledge and skills related to the development of 3D cell models to the UNSA-INGEB partner, where spheroids were utilized to assess the genotoxic activity of various chemicals. To validate the accuracy and predictability of the 3D HepG2 cell model, the researchers employed benzo(a)pyrene (BaP) as a model genotoxic compound. The study focused on investigating the influence of BaP on cell division, as well as its cytotoxic and genotoxic effects. The results demonstrate that HepG2 spheroids, owing to their enhanced metabolic capacity, represent an advanced cell system capable of providing more accurate data for human health risk assessment.

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P02-12
Development of a hepatic spheroid co-culture model for prediction of cholestatic drug-induced liver injury

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Cholestatic drug-induced liver injury (cDILI) is a major cause of acute liver failure and poses significant concern during drug development due to its low predictability. Complex *in vitro* systems have been developed to tackle this issue; however, they lack fundamental biological and mechanistic features, rendering cDILI prediction challenging. The present study was set up to develop an *in vitro* spheroid co-culture model consisting of the most relevant cell populations in cDILI pathogenesis, including primary human hepatocytes (UHHs), normal human cholangiocytes (NHCs), and primary human monocyte-derived macrophages, for predictive toxicology purposes. In a first step, homotypic spheroids consisting of 2000 UHHs and NHCs cultured in their respective cell culture media were set up and characterized. NHCs maintained stable size and high viability for 14 days. Similar observations were made for UHHs, albeit with decreasing viability over the last 7 days. In order to set up heterotypic spheroids, a new co-culture (CO-C) medium was developed. Suitability of the CO-C medium was first tested in UHH and NHC monolayer cultures. The 2D cultures showed unaltered cell morphology, proliferation and differentiated gene expression profiles. In 3D configuration, homotypic spheroids of 2000 UHHs showed higher diameter and increased viability throughout the 14-days cultivation time in CO-C cell culture medium compared to UHH cell culture medium. Reducing the number of cells to 1000 UHHs in CO-C cell culture medium led to smaller spheroids and substantially improved viability as a function of cultivation time. Spheroid co-cultures of 2000 UHHs:NHCs in a range of ratios from 1:1 to 14:1 maintained similar diameter and viability up to day 14, compared to their homotypic UHH-spheroid counterparts. However, 1000-cell UHH:NHC 1:1 spheroid co-cultures did not show significant improvement in viability. In conclusion, the novel CO-C cell culture medium provides a major improvement in the performance of UHH spheroids for long-term *in vitro* toxicity assessment and allows co-culturing with NHCs in a spheroid configuration.

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P02-13
Considering life stage and sex when assessing disruption of thyroid hormone synthesis

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Identification of thyroid hormone (TH) system disrupting substances by New Approach Methodologies (NAMs) may require knowledge on sensitive life stages and potentially sex differences. In this study, we have scrutinized these parameters in the rat thyroid gland when challenged with TH disrupting substances. We stimulated ex vivo cultured thyroid glands with thyroid stimulating hormone (TSH) and inhibited TH synthesis with the thyroperoxidase (TPO) inhibiting drug propylthiouracil (PTU). Rat thyroid glands were collected from fetuses on ges-

tation day (GD) 21, and pups on postnatal day (PD) 6 and PD16. After 72 h of culture THs were measured in media followed by gene expression and histology assessments of the glands. TSH simulated expression of TH synthesis genes suggesting that the tissue retains functional characteristics over the culture period. PTU exposure markedly affected hormones at PD6 and PD16 compared to GD21, but no differences were observed between the sexes with PTU exposure. In contrast to most published data, the T4 levels increased with PTU exposure, which suggests that other mechanisms than TPO inhibition were affected, such as DIO inhibition. Our data suggests that the ex vivo culture system is more sensitive to PTU exposure during postnatal stages than the late fetal stages. When predicting *in vivo* responses based on results from cellular models this knowledge is important, as the potential sensitive windows of exposure may prove important to account for when extrapolating effects from NAMs to those observed in a full organism.

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P02-14

In vitro 2D and 3D kidney models to study the nephrotoxic response of uranium exposure

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As a heavy metal and alpha emitter, uranium presents chemical and radiological toxicity risks, accumulating preferentially in the kidneys, specifically in proximal convoluted tubules. The biological mechanisms and pathways impacted by uranium exposure can be formulated into an adverse outcome pathway (AOP) (https://aopwiki.org/aops/447), allowing us to identify the different steps and gaps between an initiating event to the kidney toxicity. Using *in vitro* models of renal proximal tubule epithelial cells (hRPTEC TERT1) or induced kidney organoids (iKO), this study aims to reinforce and contribute to the development of the AOP of kidney toxicity. After identifying the U(VI) concentrations that induce adverse outcomes (apoptosis or necrosis), key events linked to oxidative stress, apoptosis, survival, inflammation, and renal damage are studied at the gene and protein levels. Apoptosis (Casp 3/7, Bax/Bcl2, cytochrome C) is significantly induced starting from exposure to 300 µM for hRPTEC and from 500µM for iKO, and necrosis (LDH) from 500 µM for both models. A time-dependent rise in ROS production and an antioxidant response -increased HO-1, NQO-1, GCLC- are observed in hRPTEC cells. Whereas SOD2, NQO-1 are augmented in iKO but GCLC, CYP2E1 and CAT are diminished in iKO. Uranium concentrations >300µM induced a marked inflammatory response in both models, with increased levels of TNFα, IL-6 and IL-18 in hRPTEC cells and only TNFα in iKO. The study of renal damage markers revealed a slight increase in KIM-1 at 500 µM and a decreased of Collagen IV in both models and an increased level of Calbindin in iKO. In conclusion, this work provides an insight into the key events in the AOP of uranium-induced renal failure featuring a human phenotype of proximal tubule epithelial cells and kidney organoid model, while considering the dose-dependent effect.

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P02-15

Human endometrium-on-a-chip model of the secretory phase for substance exposure studies

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Introduction: The endometrium is a highly dynamic tissue where classical cell culture-based test methods fail to correctly reproduce a relevant phenotype and treatment response. Biology-inspired micro-physiological systems, such as those based on TissUse's HUMIMIC Chip platform, might more closely resemble organ complexity and crosstalk to mimic the endometrial secretory phase. Our human 3D endometrial model (EM) is composed of immortalized endometrial stromal cells (THESC), an endometrial epithelial cell line (EM42) and primary human uterine endothelial cells (HUtMECs) and has been optimized to undergo physiological changes upon addition of the steroid hormones oestrogen and progesterone.

Methods: The 3D EM, initially generated in a static culture system, was integrated into the HUMIMIC Chip2 96-well, whose microfluidic channels were seeded with HUtMECs, and cultured for up to 10 days inside the chip under circulatory perfusion conditions. Viability and homeostasis were monitored by LDH release and metabolic profiling throughout the length of the assay. Furthermore, integrity and functionality of the 3D EM were additionally evaluated by immunofluorescence (stromal marker: vimentin; endothelial markers: CD31 and vWF; epithelial marker: cytokeratin), and following the addition of steroid hormones, decidualisation markers such as IGFPB1, PRL and FKBP5 were confirmed by qPCR and ELISA.

Results: Immunohistochemical analysis and ELISA measurements of decidual protein secretion (IGFBP1) showed cellular transformation of the 3D EM together with an increased protein secretion, respectively. This steroid hormone-induced decidualisation process could be partially inhibited by co-treatment with ulipristal acetate, a selective progesterone receptor modulator. Moreover, CalceinAM viability staining together with endothelial markers´ staining demonstrated that HUtMECs were viable and functional throughout the course of the chip culture.

Conclusions: The implementation of the model in the HUMIMIC Chip2 96-well allows to elucidate treatment effects on (patho-)physiological parameters in a complex tissue environment and could be used in future to model the complete menstrual cycle.

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P02-16

Modelling chemotherapy-induced peripheral neuropathy on-a-chip

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The side effects of chemotherapeutic treatments can be severe and occasionally include neurological implications such as chemotherapy-induced peripheral neuropathy (CIPN). We studied CIPN in human

iPSC derived peripheral neurons that were differentiated into motor neurons using a 3D microfluidic axonal outgrowth model. The neurons were grown embedded in extracellular matrix in the OrganoPlate®, a 3D microfluidic culture platform that allows culture of 40 chips in parallel. The neurons extend their axons into an adjacent layer of gel, while the dendrites and soma remain in the somal compartment.

Our model was tested for its ability to recapitulate nerve damage caused by a range of chemicals from different chemical classes that are known to cause CIPN in the clinic. Toxicity was assessed over the course of three different timepoints – 48, 72, and 96 hours – and for a concentration range of 0.1–1–10–100 nM. The toxic effects were assessed by labeling the neuronal networks with green-fluorescent calcein-AM and imaging using a high content confocal microscope. The extent of outgrowth in the axonal compartment was quantified as a measure of viability/toxicity. High concentrations (10 nM and 100 nM) of all tested compounds resulted in approximately 50% disruption of the neurite network already after 48 hours. After 96h exposures, all compounds were found to be severely toxic in concentrations of 10 and 100 nM, but only vincristine and monomethyl auristatin E (MMAE) caused significant damage at 1 nM. Prolonged exposure to 0.1 nM did not show significant toxicity for any of the drugs tested. In summary, vincristine and MMAE exhibited the highest level of damage, followed by vinblastine, combretastatin and hemiasterlin. Overall, the compounds studied exert damage in a dose-dependent and time-dependent manner. Using the 96h timepoint where we observed the strongest toxic effects, we calculated the Z factor for our assay. With a value of 0.746, the model is an excellent candidate for toxicity screenings.

Our platform can be used to study toxic effects of chemotherapeutic drugs on peripheral neurons. The 384-well format of the OrganoPlate® renders the model easily scalable and automation-compatible. As CIPN primary affects sensory neurons rather than motor neurons, ongoing work focuses on optimization of sensory neuron culture in the setup presented here. After optimization, the sensory neuron cultures will be used for toxicity assessment of antibody-drug-conjugates

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P02-17

Human stem cell-Derived osteoblasts as an *in vitro* assay to assess skeletal developmental toxicity

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Background: Bone or skeletal abnormalities are one of the most severe birth defects linked to medications. Several drugs, such as thalidomide and warfarin, have been reported to cause bone malformations including shortening of bone length, radial dysplasia and brachycephaly. We previously developed ReproTracker, a human induced pluripotent stem cell (hiPSCs)-based biomarker assay that predicts teratogenicity of drugs and other chemicals using three different lineage-specific cells, namely hepatocytes, cardiomyocytes, and neural rosettes. In this study, we introduced a new lineage-specific cell type to capture the direct effect of teratogenic agents on the development of bone.

Methods: Here, hiPSCs were directed to differentiate into osteoblasts. During differentiation, expression of the pluripotency marker *OCT4* decreased, while early mesoderm (*BRACHYURY* and *MIXL1*) and the osteoblast progenitor developmental marker *RUNX2,* were significantly induced. Upon further maturation, increases in the expression of the osteoblast-specific markers *COL1A1* and *BGLAP* were observed. Bone differentiation was further confirmed by immunostaining and bone-specific functional assays. The effect of teratogenic compounds was assessed by their ability to decrease the gene expression of different bone biomarkers, as well as to disrupt normal morphology and/or functionality in a dose-dependent manner at different stages of the differentiation.

Results: The performance of the assay was determined using a set of 5 well-known teratogenic agents that affect bone development *in vivo*, as well as non-teratogens at non-cytotoxic concentrations. Teratogenic chemicals, including thalidomide, led to a decrease in the expression of bone markers (*RUNX2*, *COL1A1* and *BGLAP*) and aberrant morphology at the different time points. Non-teratogenic chemicals (including saccharin) had no effect on the morphology of differentiated cells, nor on the expression of the biomarker genes. These results showcase the potential applicability of this system to identify chemicals affecting bone development.

Conclusions: In conclusion, addition of the osteoblast lineage cells to the ReproTracker assay allows identification of the teratogenic potential of chemicals on the development of bone. This has the potential to significantly expand the spectrum of teratogenic agents detectable by ReproTracker, making the assay an invaluable tool for early *in vitro* teratogenicity screening.

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P02-18

Using individual-centric model to detect immune-mediated idiosyncratic drug-induced liver injury at preclinical stage

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Introduction: Idiosyncratic drug-induced liver injury (iDILI) is a major concern in drug development because its occurrence is unpredictable [1]. Presently, iDILI prediction is a challenge and cell toxicity is observed only at concentrations that are much higher than the therapeutic doses, in preclinical models [2]. Applying a proprietary cell educating technology, we developed an individual-centric spheroid model that contains autologous educated immune cells, that can detect iDILI risk at therapeutic concentrations in less than two weeks.

Methods: Individual-centric spheroids were generated with the cell educating technology (patent PCT/EP2024/052109) using processed blood from healthy donors. The spheroids that contain educated -hepatocytes, -stellate cells, -macrophages, and -dendritic cells, both derived from the same donor, are treated with diclofenac with concentrations up to 100x C_{max}. The cell viability was measured using CellTiterGlo. The expression and the activity of drug-metabolizing enzymes were measured by RNAseq, by qPCR, and by luminescence assays.

Results: We showed that the expression of phase I and II drug-metabolizing enzymes are comparable between educated spheroids and healthy liver tissues. We reported that the activity of two major cytochrome P450, CYP3A4 and CYP2C9, was increased in a subject-dependent manner upon diclofenac treatment. We demonstrated that only a 45-year-old male out of a cohort of 24 individuals, displayed diclofenac-mediated iDILI at therapeutic dose while acetylsalicylic acid, its non-iDILI partner compound, did not show any toxicity at therapeutic concentration.

Conclusion: We present here the first easy to handle and rapid to set up individual-centric model that can detect immune-mediated iDILI at preclinical stage. Integrating this unique system into a high throughput screening platform will help pharmaceutical companies to accurately detect iDILI risk of new molecules de-risking drug development. The model could be also exploited as a companion test to identify patients at risk for iDILI prior to initiation of the treatment protocol.

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P02-19
A newly developed microfluidic system designed for the cultivation of 3D epidermis and full-thickness skin, tailored for nanoparticle safety evaluation

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The development of nanomaterials (NMs) has expanded into a broad range of applications including cosmetic products [1]. Currently, the safety assessment of nanocosmetics uses 2D skin cell models cultured in static conditions making them unable to accurately represent skin physiology. Besides that, EU legislation banned the use of animal models for cosmetic ingredients, so there is a strong need to replace animal testing with reliable and reproducible alternative methods [2]. Available skin equivalent models are static; therefore, they cannot recapitulate the cutaneous dynamic environment, overestimate toxicity, limit the predictive ability, and have differences in physiology together with high experimental costs [3]. Here, we describe the development of a skin-on-chip model with dynamic perfusion and a modular architecture that is suitable for both epidermis and full-thickness skin growth and allows the safety assessment of NMs. The barrier integrity, cellular viability, and skin permeability were assessed using TEER, PrestoBlue™, and Lucifer Yellow assays, respectively. Our microfluidic device allows the growth of a robust and viable epidermis and dermis with histological and immunocytochemistry examinations unveiling characteristics like those found in healthy human skin, showcasing typical keratin expressions such as K10 and K14. Despite exposure to rutile nanoparticles, both models maintained their viability and permeability, albeit a compromise in barrier integrity was observed. Monitoring and sensing ion gradients within living cell models is essential for comprehending cellular functions and both physiological and pathological processes. To advance in this field, we advocate merging the skin-on-chip model with a graphene field-effect transistor array. This pioneering strategy will facilitate instantaneous analysis of ionic fluctuations linked with cellular metabolic changes triggered by exposure to NMs. Such an approach offers significant potential as an improved surrogate model and platform for toxicological, biomedical, and pharmaceutical endeavors in the future.

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P02-20
Development of an evaluation system for drug-induced cardiotoxicity via hepatic metabolism using co-culture of human cryopreserved hepatocytes and engineered heart tissue

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Purpose: Some drugs have been withdrawn from the market due to cardiotoxicity associated with hepatic drug metabolism and drug-drug interactions. Drug-induced cardiotoxicity via hepatic metabolism cannot be detected by a cardiomyocyte-only evaluation system. In addition, such cardiotoxicity cannot be accurately predicted in animal studies due to significant species differences in drug metabolism. Therefore, we aim to establish an evaluation system for drug-induced cardiotoxicity via hepatic metabolism by co-culturing human cryopreserved hepatocytes (cryoheps) and human iPS cardiomyocyte-derived engineered heart tissue (hiPSC-EHT) on a kinetic pump integrated microfluidic plate (KIM-Plate), a microphysiological system (MPS). This plate has a microstirrer installed in the microchannel between two wells to allow medium perfusion. Previously, we found the co-culture medium, that makes cryoheps and hiPSC-EHT fully functional. In this study, cryoheps and hiPSC-EHT were co-cultured on the KIM-Plate, and drug-induced cardiotoxicity via hepatic metabolism was evaluated. Moreover, drug adsorption by the PDMS rack used to create a hiPSC-EHT was examined, because PDMS easily adsorbs low-molecular compounds.

Methods: hiPSC-EHT were created using iCell cardiomyocytes[2] (Fujifilmwako) and cryoheps were obtained from XenoTech. Cryoheps were seeded on the KIM-plate and cultured for 2 days. Then, mature hiPSC-EHT were placed in a well connected to the well where cryoheps were cultured, and co-cultured in hepatocyte maintenance medium without dexamethasone (HM Dex (-) medium). hiPSC-EHT were cultured without cryoheps as control. Terfenadine, whose cardiotoxicity was attenuated by drug metabolism, was added to the well where cryoheps were cultured. Thereafter, the contraction of hiPSC-EHT was recorded over time. In addition, drugs were added to HM Dex (-) culture medium in the well where the PDMS rack was placed and incubated without cells, to evaluate drug adsorption by the PDMS rack. The supernatant was collected over time and measured using LC-MS/MS.

Results: The contraction of hiPSC-EHT cultured alone was attenuated at 1 hour and almost stopped at 24 hours by adding 1μM terfenadine. However, the contraction of hiPSC-EHT co-cultured with cryoheps was maintained for 48 hours. These results suggest that cardiotoxicity of terfenadine was attenuated by metabolism to fexofenadine in cryoheps. Therefore, we believe that the co-culture system with cryoheps and hiPSC-EHT using the KIM-Plate is useful for evaluating drug-induced cardiotoxicity via hepatic metabolism. In addition, we found drugs that are strongly adsorbed to the MPS rack. This result suggests that dose correction is necessary for cardiotoxicity evaluation of these drugs. We expect these studies to reduce drug withdrawals, animal testing, development costs, and development time.

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P02-21
In vitro personalised model based on induced pluripotent stem cells for studying idiosyncratic hepatotoxicity

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Drug-induced liver injury (DILI) is an adverse reaction to drugs and other xenobiotics which is a leading cause of acute liver failure and is

one of the main reasons for drug withdrawal in the drug development process and in pharmacovigilance phases [1]. Idiosyncratic DILI (iDILI) appears in a small number of susceptible patients and its diagnostic and prediction is challenging since it is dose-independent and relies on different patient-specific factors such as age, sex, genetic factors, obesity or underlying chronic liver disease [1,2]. Common cell-based models used in liver toxicity studies such as hepatoma cell lines are not suitable for predicting iDILI *in vitro* due to the difficulty for recreating patient-specific factors which contribute to trigger a clinical episode of iDILI. Thus, new cell-based models that are able to reproduce the specific characteristics of individual patients and are sufficiently adaptable to be of practical application, which is particularly important for the differential diagnosis and prediction of iDILI, have been proposed [3–5]. We have explored the use of induced-pluripotent stem cells (iPSC)-derived hepatocyte-like cells (HLC) from different donors as a cell-based model for DILI studies. The results showed that HLC obtained from the differentiation of different patient-derived iPSC are sensitive to hepatotoxic drugs (i.e. amiodarone) with a comparable response to other hepatic cell models and that they reproduce the mechanisms implicated in drug-induced hepatotoxicity. In addition, the incubation of HLC from an iDILI patient with the suspicious drug (amoxicillin/clavulanate) revealed changes at a transcriptional level that could be compatible with the cholestatic pattern observed in this patient, evidencing the potential of HLC as a personalized cell-based model for *in vitro* studies for specific iDILI cases.

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P02-22
Establishment of a microfluidic neurotoxicity screening model using human iPSCs-differentiated motor neurons within the New Approach Methodologies for chemical safety developed for Precision Toxicology project

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The EU-funded project PrecisionTox pursues to establish a new, 3Rs-compliant, cost-effective testing paradigm for chemical safety assessment through the application of New Approach Methodologies (NAMs). Based on three core concepts (PhyloToxicology, Quantitative Susceptibility and Embedded Translation), the goal is to identify molecular key event biomarkers to predict chemically induced adverse health effects for humans and to promote their acceptance by industry and regulatory agencies. Based on the transcriptomics and metabolomics profiles obtained after exposure to a library of 250 chemical compounds, comparative toxic responses are assessed across five non-sentient species and human cell lines. Highly conserved mechanisms among species later lead to create translational models for predicting population toxicological effects.

For human responses, advanced *in vitro* cell models were developed for testing neurotoxicity, cardiotoxicity, and hepatotoxicity as the most interesting pathways. Commercially available human motor neurons (MN) differentiated from induced pluripotent stem cells (iPSCs) were used to establish a microfluidic chamber *in vitro* model for testing the selected compounds. First, cell plating (cell number, matrix coating) and culture conditions were optimized. Preliminary assays based on cellular morphology and neuronal markers immunostaining were performed for MN characterization both in normal plates and in microfluidic devices. Then, effective concentration range for the tested chemicals was determined through cytotoxicity assessment using the Lactate Dehydrogenase (LDH) Activity assay. Further functional effects were determined in terms of neurite outgrowth, oxidative stress, and mitochondrial activity. Finally, RNA samples for transcriptomics were generated after exposure to the tested compounds at two concentrations (EC_{10} and EC_{25}) and two time points (4 and 24h).

The obtained results settled the experimental conditions for establishing a high-throughput screening platform to study the potential neurotoxic effects of chemicals using relevant morphological, functional and omics read-outs.

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P02-23
A high-throughput gut-on-chip platform to study the epithelial responses to enterotoxins

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Enterotoxins are a type of toxins that primarily affect the intestines. Understanding their harmful effects is essential for food safety and medical research. Current methods lack high-throughput, robust, and translatable models capable of characterizing toxin-specific epithelial damage. Pressing concerns regarding enterotoxin contamination of foods and emerging interest in clinical applications of enterotoxins emphasize the need for new platforms. Here, we demonstrate how Caco-2 tubules can be used to study the effect of enterotoxins on the human intestinal epithelium, reflecting toxins' distinct pathogenic mechanisms. After exposure of the model to toxins nigericin, ochratoxin A, patulin and melittin, we observed dose-dependent reductions in barrier permeability as measured by TEER, which were detected with higher sensitivity than previous studies using conventional models. Combination of LDH release assays and DRAQ7 staining allowed comprehensive evaluation of toxin cytotoxicity, which was only observed after exposure to melittin and ochratoxin A. Furthermore, the study of actin cytoskeleton allowed to assess toxin-induced changes in cell morphology, which were only caused by nigericin. Altogether, our study highlights the potential of our Caco-2 tubular model in becoming a multi-parametric and high-throughput tool to bridge the gap between current enterotoxin research and translatable *in vivo* models of the human intestinal epithelium.

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P02-24
Biologic impact of green solid-lipid nanoparticles on bronchial 3D human reconstructed tissue

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Solid lipid nanoparticles (SLNs) have garnered significant attention as promising candidates in drug delivery systems due to their potential to enhance the bioavailability and efficacy of various compounds as well as to their unique properties, such as high drug loading capacity, targeted delivery, and sustained drug release [1–4]. In the context of bronchial tissue biosafety, the biological impact of SLNs is a topic of interest, as the bronchial tissue plays a crucial role in protecting the lungs from foreign particles and pathogens, as well as in gas exchange. Therefore, any possible harmful effects developed on this tissue must be carefully studied to ensure both the safety and the effectiveness of SLNs.

The current research reports the safety dosage of the green-SLNs to complete their biosafety profile, previously evaluated from a biological point of view on the three-dimensional (3D) bronchial microtissues (EpiAirway™ model, MatTek Corporation) [5]. The green-SLNs were synthesized through a facile, and cost-effective method, consisting of green iron oxide nanoparticles (IONPs) developed from ethanolic extract of *Camellia sinensis* (Cs) plant material [6], followed by the loading with oleanolic acid (OA). Since the synthesis temperature of green IONPs influences their physical characteristics (size, shape, and phase composition), two temperatures were employed (25°C and 80°C). Thus, were obtained two samples, labeled as SLN_Cs 25@OA and SLN_Cs 80@OA. A preliminary report showed good results regarding the acute biosecurity screening of the naked green IONPs at 500 µg/mL, the bronchial 3D human reconstructed tissues manifesting viabilities above 80% with no significant histopathological changes [6]. As regards the preliminary report of green-SLNs, it seems that when applied at a concentration of 500 µg/mL, those induce a significant viability decrease of the EpiAirway™ microtissues, after 24 h exposure interval [5].

In the present research, at the tested concentrations of 150, 200, and 250 µg/mL, after 24 h exposure, the green-SLNs samples caused no impairment in the viability of the treated inserts compared to the control. Moreover, a stimulatory effect was observed (all viability percentages exceeded 100%), although statistical significance was reached only for SLN_Cs 25@OA at all concentrations and SLN_Cs 80@OA at 150 µg/mL.In conclusion, the biological impact of SLNs on bronchial 3D human reconstructed tissues is a multifaceted area of research encompassing drug delivery, therapeutic efficacy, and safety considerations. Understanding the behavior of SLNs in biological environments is crucial for evaluating their safety and efficacy, particularly in applications involving respiratory tissues. The establishment of the interactions of SLNs with biological systems, their potential for targeted drug delivery, and their role in enhancing the bioavailability of therapeutic agents are critical aspects for advancing the field of nanomedicine.

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P02-25
Use of organotypic gingival cultures for nicotine pouch assessment

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Nicotine pouches (NP) are an emerging class of tobacco-free products for oral use. As NP do not contain tobacco and are not combusted, they may pose a lower risk to health than combusted tobacco products and oral smokeless tobacco products. However, there are limited data on the effects of NP on oral health. In this study, three-dimensional human organotypic gingival cultures were apically exposed to phosphate-buffered saline (PBS) extracts from five types of NP with different flavors and nicotine contents, a snus product, and cigarette smoke fractions (total particulate matter combined with the gas vapor phase [TPM-GVP]) for 96 h. Exposure effects were assessed by evaluating histology and inflammatory mediator secretion. At user-relevant concentrations, the test products induced different degrees of morphological changes. Cigarette smoke extract induced the most pronounced alterations, followed by the extract from the NP with a high menthol level and the highest nicotine level, three other NP, the snus, and finally, the extract from the NP with the lowest nicotine content. A similar product-dependent response was observed for inflammatory mediator profiles, with the strongest inflammatory response to TPM-GVP exposure, even at nicotine concentrations 40 times lower than the oral product extracts. Collectively, these results demonstrate that the organotypic gingival model is appropriate for evaluating biological responses to NP, snus, and TPM-GVP extracts and enables differentiation of toxicological responses across various product formulations. Moreover, oral products exerted a lower impact on organotypic gingival cultures than cigarette smoke fractions.

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P02-26
Optimized iPSC-derived hepatocytes offer a novel liver cell model for *in vitro* predictive toxicity and drug efficacy studies

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Background and Purpose: Liver disease is a rising cause of mortality worldwide. Although investments in drug discovery have increased over the last decade, most therapeutics still fail in clinical stages due to the lack of translatability between pre-clinical models and the clinic. Primary human hepatocytes (PHH) and liver cancer cell lines (e.g., HepG2) are currently used, however, they come with limitations, including limited availability, rapid loss of function, and malignant origin. We have hypothesized that optimized induced pluripotent stem cell (iPSC)-derived hepatocytes can overcome these limitations, offering a novel *in vitro* platform for predictive toxicity and drug efficacy studies.

Method: Wild-type iPSCs were differentiated to hepatocytes (Opti-HEP) for six weeks. Characterisation of Opti-HEP function was assessed and compared to PHH and HepG2 cells, including liver maturity marker expression and secretion by immunocytochemistry (ICC) and ELISA, urea synthesis by western blotting and urea assays, CYP450 expression and activity by qPCR and luciferase assays, and efflux transporter expression and function by qPCR and ICC. Suitability of Opti-HEP to predict drug-induced liver injury (DILI) was evaluated by cell viability and albumin secretion assays following 48h of treatment with 27 drugs of known DILI concern.

Results: Opti-HEP, HepG2, and PHH expressed similar levels of albumin, alpha-1-antitrypsin, and hepatocyte nuclear factor 1A as measured by ICC; however, Opti-HEP showed higher levels of albumin secretion compared to PHH over a period of 3 weeks. Opti-HEP demonstrated functional protein levels of all five urea cycle enzymes (OTC, ASS1, ASL, CPS1, ARG) and higher to those seen in HepG2 cells, consistent with higher urea secretion. In addition, Opti-HEP revealed functional mRNA levels and protein localization of the efflux transporters ABCB11, ABCG2, and MRP2, in line with bile acid secretion from the apical side of the cells. mRNA levels of the CYP450 genes *CYP3A4*, *CYP2B6*, *CYP2C9*, *CYP2C19*, and *CYP2A6* as well as basal CYP3A4 activity levels were higher in Opti-HEP compared to HepG2 cells and similar to those seen in PHH. Crucially, Opti-HEP treatment with the CYP3A4 inducers rifampicin and 1α,25-hydroxy-vitamin D3 significantly increased (>5 fold) CYP3A4 mRNA expression and activity, confirming successful CYP3A4 induction. Finally, cell viability and albumin secretion assessments following treatment with drugs of known DILI concern accurately predicted the high-DILI-concern drugs as hepatotoxic and the no-DILI-concern drugs as non-hepatotoxic, validating the relevance of Opti-HEP as a useful tool in hepatotoxicity assessment.

Conclusion: Opti-HEP are metabolically superior to HepG2 cells with liver function comparable to PHH. This data alongside the expansion capacity and amenability of Opti-HEP showcase the spectrum of opportunities these cells can offer in the fields of predictive toxicity, disease modelling, and drug safety.

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P02-27
$PM_{2.5}$ organic extract induced toxicity varies between 2D and 3D cultured bronchial epithelial cells

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Fine particulate matter ($PM_{2.5}$) is one of the most prevalent and dangerous air pollutants. When present in the environment, it can be inhaled and penetrate deeply into the lungs where it can settle and irritate the airways. To study the interaction of inhaled $PM_{2.5}$ with the cells of the lung, there is the need for good cellular models. Recently, great strides have been taken in the development of complex three-dimensional (3D) cell cultures, which are believed to better recapitulate the tissue-like physiological and morphological features, compared to the conventional monolayer cultures. In line with these developments, we previously established a spheroid model, consisting of human bronchial epithelial cells. and explored the use of the spheroid model for respiratory toxicity testing. The model was optimized to allow for the assessment of cytotoxicity, genotoxicity, apoptosis/necrosis, oxidative stress, and more. In the present work, we studied the toxicity of $PM_{2.5}$ organic extracts and compared the results of 16HBE14o- cells cultured as a monolayer with cells cultured as 3D spheroids. Atmospheric samples were collected on filters in Sao Paulo, Brazil, from which $PM_{2.5}$ organic components were extracted. The extracts were characterized for polycyclic aromatic hydrocarbons, polar organic compounds, anhydro sugars, sugar alcohols, major and trace elements, and water-soluble ions at the University of Aveiro. Previously, we published protocols of the generation, collection and seeding of the spheroids [1]. Spheroids were generated by culturing 16HBE14o- cells as a hanging drop. Afterwards, spheroids were transferred to 1% agarose coated wells, at 250 spheroids/well. For monolayer experiments, cells were seeded in 96-well plates at a density of 20–40 x 10^3 cells/well, for 48 and 24 hours exposure respectively. Spheroids and monolayers were exposed to $PM_{2.5}$ organic extracts corresponding to different collection months, for 24 and 48 hours. The effect on cell viability (WST-1 assay), oxidative stress (CellROX) and cell death (YO-PRO®-1/PI) was assessed following previously established protocols [1]. Additionally, the concentration of the pro-inflammatory cytokines IL-6 and IL-8, in the medium supernatant of both exposed spheroids and monolayers, was quantified. Results obtained in monolayers often varied from those obtained in the spheroids and were depended on the assessed endpoint. For instance, while the cell death assay showed comparable results between spheroids and monolayers, the effect of the $PM_{2.5}$ organic extracts on cell viability and oxidative stress was more pronounced in monolayers compared to spheroids. On the other hand, changes in the levels of the pro-inflammatory cytokines, IL-6 and IL-8, appeared to be more prominent in the spheroids. Together, these results stress the importance of the inclusion of different cellular models when examining the toxic effects of environmental exposures.

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P02-28
Microfluidics technology as a tool to improve maturation and drug metabolism capacity of stem cell-derived hepatocytes *in vitro*

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Human stem cell-derived hepatocyte-like cells (HLCs) represent an alternative tool for efficacy and safety evaluation of novel drugs. However, differentiating HLCs in traditional 2D systems results in immature hepatic phenotype, hampering its application in drug development. Microphysiological systems (MPS) have been reported as to mimic more closely the liver microenvironment, namely hepatocyte organization, fluid flow and cell-cell and cell-matrix interactions, improving cell functionality [1]. The aim of this work was to create a hepatic MPS resorting to microfluidic and stem cell technologies. Yet, moving to microfluidic devices (MD) or cell-chips requires adaptations in cell culturing protocols and in microfabrication procedures. As such, a MD suitable for HLC differentiation, maintenance and maturation, which includes the optimisation of the MD design, cell inoculation procedure and coating was firstly developed. The chip was fabricated by photolithography and soft lithography techniques, based on polydimethylsiloxane (PDMS) molding. 3 MD designs were tested: 2 square-shaped MDs, with 3 or 7 inlets, and a rectangular MD with 2 triangular segments and 3 inlets. The first step of stem cell hepatic differentiation was performed in 2D cultures [2]. At day 17 of differentiation, cells were transferred to a chip (1×10^6 cells/mL) sealed against polystyrene dishes, operated under perfusion (0.2 µl/min) or maintained in 2D culture (2×10^4 cells/cm^2), as control [2]. A homogeneous cell dispersion and confluency was achieved in the square-shaped chip with 7 inlets. The optimised cell inoculation procedure consisted on a three-step approach, firstly using the central inlet at a flow rate of 5 µL/min for 2 min, which was then increased to 10 µL/min for 4 min. Lastly, cells were inserted into the outer inlets at a flow rate of 10 µL/min for 4 min. Collagen coating polymerization at a physiological pH enabled HLCs maintenance up to 10 days under perfusion in the MD, with a higher fluorescence intensity of the hepatic markers CK-18, HNF-4a, OATP-C and MRP2, as well as increased ammonia detoxification ability ($p<0.001$). Finally, for the MPS validation cells were exposed to a non-cytotoxic concentration of diclofenac (0.1 mM for 1h). Enhanced biotransformation competence of HLCs was observed, as demonstrated by the 11.96-fold increase of glucuronidation products in the MPS, and 6.85-fold increase in 2D cultures, upon analysis using GC-MS [3]. These findings support the efficacy of MPS for promoting the maturation of HLCs and its suitability for drug metabolism studies.

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P02-29
Predicting drug-induced cardiac contractility alterations in a 3D beating heart-on-chip platform

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Detecting cardiotoxicity during early stages of drug development process still represents a critical step. Hence, the development of more representative *in vitro* models able to generate human-relevant data holds great potential. Here we present a human functional 3D cardiac model, named uHeart, developed within a beating Organ-on-Chip platform for the quantitative assessment of drug-induced changes in cardiac microtissues contractility.

uHeart platform features uBeat® patented technology, an actuating mechanism providing a physiological uniaxial cyclic strain (i.e., 10% stretching, 1 Hz) [1]. Human induced pluripotent stem cell derived cardiomyocytes (h-iPSC-CMs, iCell²) and human cardiac fibroblasts (h-CFs), 75%-25% ratio, were embedded in fibrin hydrogel ($125*10^6$ cells/ml) and cultured for up to 7 days. Evaluation of 20 compounds affecting different variables (e.g., calcium homeostasis, sodium channel inhibition, contraction) were selected to qualify the model. Drug-induced alterations were evaluated at incremental doses by analysing contractility parameters form video analysis by means of MuscleMotion (e.g., beating period-BP, contraction time-CT, relaxation time-RT, contraction amplitude-CA, contraction velocity-CV) [2]. DMSO was used as vehicle and as negative control.

Human cardiac microtissues showed synchronous beating after 6 days of culture. Drug screening campaign evidenced that ORM 10-962 (3 µM, NCX inhibitor) and Levosimendan (3, 30 µM, enhancer of myocytic calcium sensitivity) increased the CA, showing a positive inotropic effect, while Ryanodine (0.3, 3, 30 µM, RyR inhibitor) and Mavacamten (0.3 µM, cardiac myosin inhibitor) reduced the CA, thus eliciting a negative inotropic effect. Ivabradine (0.03, 0.3, 3 µM, I_f inhibitor) and Mexiletine (10, 100 µM, Na^+ channel inhibitor) prolonged the BP, showing a negative chronotropic effect. DMSO (1 µM) did not statistically alter myocardial contraction.

uHeart human 3D cardiac in-vitro model correctly predicted the inotropic, chronotropic, and lusitropic effects of 12 compounds. Two compounds were partially predicted, four compounds had a trend but not significant, and only two compounds were not found with the expected effects. Despite the complexity of action and the multi-target effects of some of the products used, the uHeart appears as a valuable tool for preclinical cardiac safety prediction.

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P02-30

Generation and evaluation of a historical control database for the creation of assay acceptance criteria for a comparative *in vitro* hepatic enzyme assay

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This work is being conducted in collaboration with KaLy-Cell and CropLife Europe.

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In Europe, chemicals demonstrating endocrine disrupting (ED) properties via a human-relevant mode(s) of action (MoA) may be restricted or, in the case of pesticide active substances (ASs), registration may not be possible. To demonstrate species-specific MoA(s) and non-human relevance, robust *in vitro* assays with clear acceptance and interpretation criteria must be developed. The comparative *in vitro* hepatic enzyme assay has been used to compare the enzymatic response of relevant test species and humans. This is in line with Appendix A of the established ECHA-EFSA ED guidance (2018) for assessing non-human relevance of liver-mediated effects on thyroid hormone clearance. Unlike most other *in vitro* ED assays, with published OECD/OCSPP guidelines containing clear guidance and acceptability criteria, this assay lacks regulatory guidance for assay design, acceptability criteria, and data interpretation. This creates challenges when evaluating this assay, both for applicants and regulators, in the context of regulatory ED assessments. In this project, we statistically evaluated the data generated from 29 comparative *in vitro* hepatic enzyme studies conducted at KaLy-Cell to generate a historical control database (HCD) of vehicle and reference enzyme inducer responses, and to develop assay acceptance criteria. The initial HCD evaluation included primary hepatocytes from two rat strains (Sprague Dawley and Wistar) and humans treated for up to 7 days with a vehicle control (dimethyl sulfoxide) and reference CYP inducers (β-naphthoflavone, PCN, phenobarbital, and rifampicin). The endpoints assessed were mRNA gene expression and enzyme activity for both CYPs and UGTs, including UGT-T4 activity. A statistical mixture model with fixed and random effect components (variance components model) is used to examine the contribution of variability from each experimental design source including species, donor/lot, experiment, sex, strain, and vehicle concentration. Recommendations are made based on the analysis using linear models and statistical control charts of the HCD regarding statistical approaches for data evaluation. All statistical analyses were carried out with appropriate transformations necessary to meet statistical assumptions, and outlier detection techniques are employed, accompanied by summary statistics and graphical exploration. Experimental design considerations are proposed to improve assay repeatability and reproducibility. The HCD and proposed acceptability criteria generated, based on background biological variability can be used not only to assess current and future studies conducted at KaLy-Cell to confirm individual assay validity but also to contextualise previously conducted studies, with the HCD evolving as more studies are incorporated. Additionally, it will aid in establishing an agreed MoA framework across sectors to assess species-specific MoAs and non-human relevance and the development of data interpretation criteria.

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P02-32

Development and application of an alternative inhalation toxicity model using an *in vitro* 3D culture system through multiple analysis

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Polyhexamethylene guanidine hydrochloride (PHMG-HCl), a guanidine derivative, is well-known to induce pulmonary fibrosis. Pulmonary fibrosis is characterized by excessive accumulation of fibrous connective tissue in lung tissues, leading to severe respiratory distress. The interaction between differentiated epithelial cells and subepithelial fibroblasts plays a crucial role in maintaining the structure and regulating the function of the respiratory system. Particularly, the damaged epithelial cell has been reported to play a significant role in wound healing by fibroblasts. Therefore, a co-culture system of epithelial cells and fibroblasts could serve as an important model for studying and understanding the pathological mechanisms associated with pulmonary fibrosis. In this study, we designed an *in vitro* co-culture model using multiplex analysis as a high-throughput screening method to increase the predictability and efficiency of toxicity assessment. First of all, to confirm this co-culture model, normal human epithelial cells (NHBE) were cultured under air-liquid interface (ALI) system conditions and validated by detecting differentiation-related epithelial markers using immunocytochemistry (ICC) staining. Then, to confirm the potency of this model for lung fibrosis, the co-culture cells were acutely exposed to PHMG-HCl. As a results, the co-culture cells exposed to PHMG-HCl showed a significant increase of cytotoxicity, cell migration, and gel contration. Whereas cilia beating frequency (CBF) in epithelial cells was significantly decreased. In conclusion, these results confirmed that PHMG-HCl stimulated a significant fibrotic response in the co-culture model. Therefore, the multi-analysis model using this co-culture system is expected to be useful in the predictive evaluation of pulmonary fibrosis as a simple and efficient high-throughput sreening method.

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P02-33

Bridging the gap: human and preclinical animal microphysiological systems for assessing drug-induced liver injury during drug discovery

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Drug-induced liver injury (DILI) remains the most common cause for acute liver failure in the world and is a leading cause of compound attrition in drug discovery. Although sufficient at capturing most intrinsic events, current models used in drug discovery have limitations as they are not effective at predicting or understanding more complex DILI events in humans. Furthermore, for testing new human-specific modalities, cell lines/animal models are less suitable due to gene sequence or immunological response differences.

Using the PhysioMimix® OOC Single-organ System, human and preclinical animal MPS models have been developed to bridge these gaps presented in current preclinical pipelines. Due to its large tissue size and sample volume, the Multi-chip Liver-12 plate, containing 12 individually perfused chips, is well adapted to mechanistic studies to better understand pathways of toxicity and key events before reaching preclinical safety studies. Primary human and animal liver cells

(hepatocytes and Kupffer cells) were cultured as 3D microtissues on scaffolds under perfusion for two weeks in Liver-12 plates. Liver function for all models were assessed for a broad spectrum of liver-specific endpoints on the cellular structures and culture medium, including clinically relevant biomarkers. Tool compounds recommended by the IQ MPS Consortium for DILI validation were used to challenge the models and toxicity outcomes measured against clinical data.

Using these tool compounds, superior sensitivity, and specificity in detecting DILI over classic 2D primary hepatocytes cultures and non-MPS 3D models was detected in the human Liver MPS models (sensitivity 100%, accuracy 85%, and precision 100%). Measured ALT levels in the cell culture media at 48 hours of exposure matched the severity grading of DILI set out by the DILI Network based on clinical data, highlighting the clinical translatability of our human liver MPS model.

Preclinical animal liver MPS bridges the gap between current *in vivo* animal data and clinical outcomes. The preclinical animal liver MPS were compared to *in vivo* data generated from tool compounds, and accurately mimicked the *in vivo* predictivity for both true/false positive/negative results from preclinical testing. Together, using human and animal MPS early to assess DILI can reduce the number of molecules passing through into preclinical testing to support a reduction in the number of animals required, thus contributing to 3Rs efforts, and cutting costs. Collectively, these tools enable adopters to refine preclinical *in vivo* design through deeper and more human-relevant mechanistic insights for the development of safer and more cost-effective drugs thus enabling their fast-track delivery to patients.

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P02-34
iPSC-derived hepatocytes from multiple donors capture the effects of population diversity in drug metabolism and response

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Background and Purpose: The occurrence of population differences in drug efficacy and toxicity is a common phenomenon in drug discovery. These differences are associated with a complex set of variants, including genetic, environmental, physiological, and behavioural factors, downstream affecting drug metabolism, activity, and clearance. However, the current models used in drug development fail to capture this variability, since animal models can assess efficacy and safety within that species, only, whilst *in vitro* liver models (e.g., primary human hepatocytes, hepatocellular carcinoma cell lines) have either limited availability or are derived from a single source. iPSC-derived cell models encompassing individuals of diverse ages, genders, and ethnicities can overcome the above limitations and serve as suitable models to understand how population diversity influences drug response early in drug development. We hypothesized that iPSC-derived hepatocytes from different wild-type donors can recapitulate this population diversity *in vitro*.

Method: iPSCs from three different healthy individuals (gender: 1 male, 2 female; age range: 50–60 years old; ethnicity: Caucasian) were differentiated to hepatocytes (Opti-HEP) for six weeks. Characterisation of Opti-HEP metabolic function was assessed including liver maturity marker expression and secretion by qPCR and ELISA, urea synthesis by urea secretion assays, and CYP450 expression, induction, and activity by qPCR and luciferase assays.

Results: All three wild-type iPSC lines were successfully differentiatedtoOpti-HEP, as evident by comparable mRNA levels of the hepatocyte maturity markers albumin, alpha-1-antitrypsin, and hepatocyte nuclear factor 1A to those seen in primary human hepatocytes. However, significant differences in albumin secretion between the three Opti-HEP lines were observed alongside differences in urea secretion. The basal mRNA levels of the CYP450 genes *CYP3A4*, *CYP2B6*, *CYP2C9*, *CYP2C19*, and *CYP2A6* were also different between the three Opti-HEP lines, in line with variability in basal CYP3A4 activity, as measured by luciferase assays. Consistent with this, 72h of treatment with the CYP3A4 inducer 1α,25-hydroxy-vitamin D3 increased *CYP3A4* mRNA expression in all three Opti-HEP lines, confirming successful CYP3A4 induction, but with distinct differences in the induction levels between the three lines.

Conclusion: We have successfully generated three Opti-HEP lines derived from healthy individuals, revealing significant variation in metabolic function and Phase I enzyme activity. These data highlight the inter-individual differences observed in human population, allowing to capture variability in drug efficacy and metabolism early in drug development. Expansion of the Opti-HEP library using iPSCs of different ethnicities (e.g., African, Native American etc.) is now required to identify adverse drug responses early, de-risk drug development, and ensure successful clinical outcomes.

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P02-35
3D primary human kidney tissue model for nephrotoxicity

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Approximately 20% of drug failures in human clinical trials are associated with kidney damage. The proximal tubular (PT) region is the most common site for compound reabsorption and is highly susceptible to drug and toxin damage. The goal of this study is to develop a novel, physiologically relevant and functional three-dimensional (3D) organotypic tissue model that can accurately predict drug-induced nephrotoxicity. Human primary proximal tubular epithelial cells (PTEC) were isolated and expanded in a monolayer culture prior to seeding onto microporous membrane inserts. The reconstructed PTEC 3D tissues were analyzed by histology, TEER measurements, immunostaining, and qPCR. Also, receptor mediated FITC-albumin uptake and transpeptidase hydrolytic activity of glutamyl transpeptidase (GGT1) and leucine aminopeptidase (LAP) were assayed. The organotypic PTEC tissues organize into characteristic tubular structures, develop a barrier with mean TEER values of $169 \pm 33.4\ \Omega \times cm^2$ and stain positive for the tight junction proteins, ZO-1 and Claudin-1. Gene expression analysis shows brush border proteins together with water channel AQP1 and GGT1 on the apical side and sodium-potassium ATPase pump on the basolateral side. Real-time qPCR confirmed expression of PTEC-specific markers necessary for renal clearance, secretion, and reabsorption including aminopeptidase CD13, p-glycoprotein (PgP; MDR1), multidrug resistance proteins MRP1, 3, 4, and 5, CYP450 enzymes, glucose transporters SGLT1/2, multidrug and toxin extrusion transporter MATE1, organic cation and anion transporters OCT1/2, OCTN1/2, and OAT-P4C1, urate transporter URAT1, and sodium phosphate co-transporter NP2. Concentration and time dependent receptor mediated uptake of FITC-albumin was observed by fluorescent microscopy. Albumin uptake was inhibited by the addition of BSA (competitive binding utilizing a common receptor for albumin) or the drug chlorpromazine, an inhibitor of calthrin-dependent endocytosis. Hydrolytic activity was monitored by the conversion of γ-Glutamyl-p-nitroanilide (GPNA) and L-leucine-p-nitroanilide (LLNA), substrates for GGT1 and LAP, using spectro-

photometric assays of p-nitroaniline (PNA). Specific transpeptidase hydrolytic activity was inhibited by 88.8% (GPNA) and 35.0% (LLNA) in the presence of an irreversible inhibitor acivicin (1.2mM). Glucose uptake was enhanced by the addition of sodium chloride. Treatment of the 3D kidney model with Cisplatin, a known nephrotoxin, causes reduced TEER and reduced viability in a time and concentration dependent manner. Thus, the reconstructed 3D PTEC organotypic tissue is physiological in terms of structure, barrier properties, gene expression, and functionality mimicking the *in vivo* human PT region. This model will help establish confidence in modeling drug-induced kidney damage/injury and reduce animal use for experimentation.

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P02-36

In vitro monogenic liver disease models using iPSC-derived hepatocytes demonstrate an altered CYP450 gene expression profile compared to wild-type cells

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Background and Purpose: The hepatic cytochrome P450 (CYP450) enzymes are the major players in drug metabolism. However, numerous studies have recently shown that their drug-metabolising activities are dysregulated in liver disease states, and this dysregulation is associated with oxidative stress, inflammatory stimuli, and other cellular tensions, downstream altering drug efficacy and toxicity. Despite the importance of these effects in the development of safe and efficacious new drugs, the disease-induced dysregulation of CYP450 enzymes in monogenic liver diseases is poorly described. We have developed 4 different monogenic liver disease models using iPSC-derived hepatocytes to investigate the CYP450 transcriptional profiles in diseased liver.

Method: Using CRISPR-mediated gene editing in healthy iPSCs, four monogenic liver disease models were generated: alpha-1-antitrypsin deficiency (A1ATD; E342K mutation), Wilson's disease (WD; H1069Q mutation), progressive familiar intrahepatic cholestasis type 2 (PFIC2; D482G mutation), and ornithine transcarbamylase deficiency (OTCD; D175V mutation). Genotype confirmation was performed by Sanger sequencing, whilst iPSC pluripotency and differentiation of iPSCs to hepatocytes (Opti-HEP) by qPCR and immunocytochemistry. Disease phenotypes were determined by different functional activity readouts, including intracellular polymeric A1AT accumulation by immunocytochemistry (ICC), copper-induced oxidative stress by ICC, bile acid export by mass-spectrometry, and urea secretion by colorimetric assays. CYP450 expression profiles were determined by qPCR.

Results: Both wild-type and CRISPR-derived iPSC lines were successfully differentiated to Opti-HEP, as shown by comparable levels of the hepatocyte maturity markers albumin, alpha-1-antitrypsin, and hepatocyte nuclear factor 4A to those seen in primary human hepatocytes. All four disease Opti-HEP lines demonstrated the desired phenotypes *in vitro*: increased polymeric A1AT accumulation (A1ATD), increased oxidative cell stress (WD), reduced bile acid export (PFIC2), and decreased urea secretion (OTCD) compared to their isogenic controls. mRNA levels of the CYP450 genes *CYP3A4*, *CYP2B6*, *CYP2C9*, *CYP2C19*, and *CYP2A6* were determined by qPCR, revealing an altered CYP450 expression profile in all four disease models (with either decreased or increased expression of CYPs) compared to wild-type cells.

Conclusion: We have developed four iPSC-derived hepatocyte models that recapitulate monogenic liver diseases *in vitro*. All disease models demonstrated an altered CYP450 expression profile compared to wild-type cells. These data highlight the necessity for functional *in vitro* human liver disease models that can be used at the early stages of drug discovery, and their superiority over wild-type cells in the generation of physiologically-relevant data during toxicity screening studies.

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P02-37

Enhancing performance: positive controls in a 3D _in vitro_ alveolar test system for the prediction of chemical respiratory sensitizers

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Respiratory sensitizers, designated as substances of very high concern (SVHC) under EU REACH legislation, pose hazards that require early evaluation during the development stages of new molecules across various industries. Tissue inflammation, highlighted as the second key event in the proposed adverse outcome pathway (AOP:39), is closely linked to respiratory sensitization. Assessing pertinent biomarkers for respiratory sensitization using physiologically relevant *in vitro* models is essential for predicting the sensitizing potential of chemicals. Additionally, ensuring the reliability of the *in vitro* method's responsiveness alongside the tested articles is crucial. Including positive controls is imperative as it improves the detection of both false-positive and -negative outcomes.

Lipopolysaccharide (LPS), an endotoxin eliciting pro-inflammatory reactions in lung cell models, and thymic stromal-lymphopoietin cytokine (TSLP), an epithelial cells mediator responsible for allergic reactions by activation of antigen-presenting cells, are commonly used positive controls to induce pro-inflammatory responses in *in vitro* test systems that mimic the respiratory tract.

The aim of the study was to assess the responsiveness of an *in vitro* test system, ALIsens®, developed to predict the respiratory sensitization potential of chemicals. The test system relies on a three-dimensional (3D) configuration incorporating three human cell lines: alveolar type II epithelial (A549), endothelial (EA.hy926) and monocytes (THP-1).

ALIsens® was exposed to a mixture of LPS and TSLP rendering a final concentration of 10 µg/mL and 20 ng/mL, respectively. The expression of CD54, CD86 and TSLPr markers was measured on the surface of dendritic-like cells by flow cytometry. Samples of the medium from the basolateral compartment were collected, and the levels of cytokines and chemokines were quantified on a Bio-Plex 3D Array System using the Bio-Plex Pro Human Cytokine Screening Panel, 48-plex.

Flow cytometry analysis demonstrated consistent responsiveness of the test system, shown by the increased relative mean fluorescence intensity (rMFI) values of all the three evaluated cell surface markers, CD54, CD86 and TSLPr.

A significant secretion was measured for pro-inflammatory cytokines and chemokines representative for the mechanisms underlying respiratory sensitization: interferon gamma (IFN-γ), interleukin IL-2, IL-4, IL-6, IL-16, IL-18, granulocyte colony-stimulating factor (G-CSF), growth-regulated oncogene-α (GRO-α), macrophage inflammatory protein 1β (MIP-1β), regulated on activation, normal T cell expressed and secreted (RANTES), tumor necrosis factor alpha and beta (TNF)-α and β.

These results indicate that LPS and TSLP serve as relevant positive controls for inducing pro-inflammatory effects and activating dendritic cells. LPS and TSLP prove to be effective in evaluating the responsiveness of *in vitro* models designed to study respiratory inflammation and sensitization.

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P02-38

Folic acid-targeted cisplatin nano-drug delivery system – approaches for triple-negative breast cancer treatment and hepatic metabolization

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The overexpression of folate receptors on cancerous cell membranes appears to be a promising target in cancer therapy, including triple-negative breast cancer, which is defined by limited therapeutic options. Carbon nanotubes can be functionalized with folic acid (FA) and act as carriers of anticancer molecules to increase the effectiveness of therapies. Besides the high efficiency against cancerous sites, the effects triggered in healthy cells and metabolization sites in normal conditions are incompletely elucidated. This study aimed to investigate the efficiency of single-walled carbon nanotubes functionalized with carboxyl groups, FA, and cisplatin (SWCNT-COOH-FA-CDDP) on breast cancer spheroids and the possible cytotoxicity on breast normal and hepatic 3D-spheroid models. A method for functionalizing SWCNT-COOH with FA and CDDP was developed by dissolving SWCNT-COOH in DMF, adding FA, sonication, incubation, centrifugation with supernatant removal, and resuspension, followed by CDDP addition, sonication, and washing. Spheroids were generated by liquid-overlay technique from breast cancer (MDA-MB-231) and normal (MCF-12A) cell lines and a hepatic (HepG2) cell line in complete medium with 2.5% Matrigel. Five doses of CDDP (5, 6, 7, 8, and 10 μM) and SWCNT-COOH-FA (2.2, 2.8, 3.2, 3.7, and 4.5 μg/mL) were tested for 24 and 48 h. The same doses of the complex were tested. Cell viability was detected by Live/Dead staining. The membrane integrity was evaluated by lactate dehydrogenase (LDH) release in the culture medium and nanoparticles' internalization was highlighted by fluorescent labeling of lysosomes. The functionalization was confirmed through atomic absorption spectroscopy, showing the attachment of the therapeutic agent to the SWCNT-COOH. The results revealed a dose- and time-dependent cytotoxicity of SWCNT-COOH-FA-CDDP for all tested cell lines. SWCNT-COOH-FA induced cell death in MCF-12A spheroids starting with a dose of 3.7 μg/mL after 24 and 48 h, and disruption of the proliferative layer of MDA-MB-231 spheroids after the treatment with doses higher than 3.2 μg/mL after 48 h. In HepG2 spheroids, SWCNT-COOH-FA induced no changes compared to control. CDDP had no cytotoxic effect, except for the highest dose on MDA-MB-231 spheroids, when the loss of proliferative layer integrity was observed after 24 and 48 h. The complex caused the release of LDH from HepG2 and MCF-12A spheroids after 48 h of incubation with doses of 7 μM and 6 μM, respectively, more pronounced for HepG2 spheroids. Free components induced no significant changes in LDH levels. An increase in lysosome number was noticed for MDA-MB-231 and HepG2 spheroids after 24 h of treatment with nanoparticles. In conclusion, the results showed the internalization of the complex correlated with strong cytotoxic effects on hepatic and breast spheroids. Overall, our study may represent a step forward for the improvement of breast-targeted therapy with limited side effects on healthy cells.

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P02-39

Doxorubicin induced change in filtration permeability in podocytes and glomerular microvascular endothelial cells in co-culture at physiological oxygen tensions

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Purpose: Conventional cell culture demonstrated that it cannot recapitulate the cells physiological environment *in vivo,* thereby severely restricting early prediction of nephrotoxicity. Indeed, atmospheric O_2 (21%), used for nearly all routine *in vitro* experiments, exceeds physiological O_2 tensions (10% O_2) in the human renal glomeruli *in vivo* by two-fold. We showed that O_2 tensions modulated drug induced changes in monolayer permeability of a podocyte cell line. In addition, current *in vitro* testing routinely employs one cell type only, overlooking the interdependency of different cell types, including their paracrine influence, required to allow functionality of a tissue unit, e.g. filtration in the glomerulus. Accordingly, we immortalized primary glomerular microvascular endothelial cells (HGMECs) to recreate the *in vivo* filtration unit, aiming to improve prediction of glomerulotoxic events in humans.

Methods: Doxorubicin (DOX) induced change in filtration permeability was tested on cultured PODO/TERT256 cells that formed a stable, contact inhibited monolayer with typical podocyte morphology and developed a size-selective filtration barrier. Cells were adapted to 20% O_2 (AtmOx) and 10% O_2 (PhysOx) for multiple weeks prior to toxin exposure. Permeability was tested with two types of fluorescent dextrans (20 kDa+70 kDa), impedance was measured with a xCELLigence RTCA machine (Agilent) and cytotoxicity was assessed via LDH leakage. The impact of DOX and O_2 tension on cellular metabolism was determined by assessing glucose consumption and lactate production enzymatically. HGMECs were immortalized by introducing hTERT and an additional conditional SV40-LT expression (Tet-On system), allowing for co-culturing with PODO/TERT256.

Results: DOX exposure induced a concentration dependent change in filtration permeability, already at the lowest DOX concentrations tested (0.01 μM) under PhysOx, whereas at AtmOx overt changes were observed at concentrations ≥0.16 μM. The latter changes were not a resultant of cytotoxicity, as LDH leakage assays (significant for PhysOx and AtmOx >0,5 μM), showed no difference in concentration-response at PhysOx and AtmOx. Impedance decreased with increasing DOX concentrations and reached significance at ≥0,5 μM. Increased lactate production to glucose consumption ratios confirmed that PODO/TERT256 cells shifted to anaerobic glycolysis at PhysOx, while DOX decreased glycolytic ATP production in general. PODO/TERT256 cells and immortalized HGMECs were successfully co-cultured for 12 days and are currently investigated for paracrine effects.

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P02-40

Novel *in vitro* protocol for evaluation of safety of intraoral medical devices

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Medical devices (MDs) represent a crucial aspect of modern healthcare, facilitating a wide range of medical interventions and treatments. MDs also play a crucial role in addressing various oral cavity health needs, where they can have both therapeutic and preventive roles. Within the oral cavity, these devices are utilized for a diverse range of applications, including dental restoration and prosthetics, orthodontic treatment, periodontal care, and oral health and hygiene maintenance. The oral cavity presents a dynamic and complex environment characterized by exposure to saliva, food particles, varying pH levels, and microbial flora [1]. Additionally, due to chronic disorders, or prosthetics, patients may rely on these devices for prolonged periods, necessitating thorough safety assessments to evaluate long-term risks. Hence, comprehensive evaluation protocols are essential to verify the safety of medical devices intended for oral cavity use, safeguarding both patient well-being and public health. To ensure the safety of MDs, they must undergo strict biological evaluation defined by national regulations and their respective guidelines. One of the broadly implemented approaches is ISO 10993: *Biological evaluation of medical devices*. This standard is progressively implementing novel *in vitro* methods that are used for screening MDs for potential health hazards. One of the most recent additions was the acceptance of an *in vitro* protocol for sub-cutaneous irritation testing of MDs, which led to the creation of ISO 10993-23 [2–4].

Building on this successful methodology development, we developed an *in vitro* protocol for testing intraoral MDs. To mimic the soft tissues of the oral cavity, we have evaluated the use of two 3D reconstructed human tissue models. In pilot studies, we utilized EpiOcular tissue model as a universal non-keratinized model. Since this model is constructed of primary human keratinocytes it closely mimics soft tissues found in the oral cavity. Divided into 3 categories, 11 products were tested- I. Materials intended for surgical application, II. Sore-throat pills, and III. Products for care of oral cavity. The observed tissue responses were consistent with our predictions. Subsequently, we integrated the keratinized EpiOral model, with enhanced barrier properties, into testing to better mimic the response of target tissue. Testing with EpiOcular and EpiOral yielded nearly identical responses, with minor deviations. In some cases, viability decreased and elevated IL1a response was observed for neat formulations of products for oral cavity care.

The next phase involves the validation of the protocol in a ring trial to allow for implementation into the ISO standards.

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P02-41
Toxicological aspects of *in vitro* oral mucosa wound healing models

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Wounds in the oral cavity tissues can result from surgical procedures, accidents, or canker sores. Oral wounds present a site for potential infection and increased sensitivity to oral irritants. Untreated wounds can lead to pathogen invasion, chronic pain, poor cosmetic outcomes, and in some cases, extended hospitalization. Thus, damaged oral mucosal tissue needs to be treated and healed as soon as possible. The objective of this project is to develop new *in vitro* oral wound healing models and determine their toxicological profile using common dentifrice materials.

Two tissue models were used in this study (both produced by MatTek Corporation and MatTek Europe): a) EpiOral (ORL-200), which consists of normal, human-derived oral epithelial cells cultured to form a highly differentiated model of human (non-cornified) buccal tissue, and b) EpiGingival (GIN-100), a cornified model of the gingival mucosa. On Day 0, tissues were wounded using a 3 mm biopsy punch (representing 11.6% of total tissue area) and wound closure was monitored using brightfield microscopy, histological cross sections, and transepithelial electrical resistance (TEER) measurements. The toxicological profile of the tissues was probed using the common dentifrice additive, sodium dodecyl sulfate (SDS), at 1%. The exposure time which decreased the tissue viability to 50% (ET-50) of wounded tissues (WT) and non-wounded tissues (NWT) was determined.

Histological cross sections and brightfield microscopy of the WT showed that the wounds extend down to the underlying inert microporous culture membrane. Within 2 days post-wounding (PW) for the ORL-200 tissue and 4 days PW for the GIN-100 tissue basal cells migrate into the wound to reestablish a continuous monolayer. Immediately following wounding, TEER for WT decreases to $<20\%$ of the NWT controls, but TEER increases as wound healing proceeds. Wounding also changes the toxicological profile of the tissues. For the ORL-200 model, the ET-50s for the WT and NWT were 27.8 and 49.1 mins, respectively, which represents a 76% increase in ET-50 for the NWT vs WT. By contrast, in the cornified GIN-100 model, the ET-50s were 54.5 and 124.5 mins, respectively, which is 128% increase in ET-50 for NWT vs WT.

These results demonstrate successful development of wound models for tissues of the oral cavity. Wound healing can be monitored with brightfield microscopy, TEER, histology, and sensitivity to a common dentifrice additive. These models will be useful for testing new therapeutic compounds designed to hasten wound closure in the oral cavity and for determining toxicity profiles in barrier-compromised oral cavity tissues. Additionally, wounding has been shown to decrease the ET-50 of ORL-200 and GIN-100 tissues indicating that it could be a useful tool when a more sensitive tissue model is required, such as for testing many in-use tobacco and oral care products that typically give low responses in NWT.

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P02-43
Assessment of acute oral toxicity of a UVCB by integration of New Approach Methodologies

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Acute oral toxicity is requested by regulators around the world to inform about hazard classification, labeling, and risk management of substances. To assess acute oral systemic toxicity, animal testing is required using recently refined OECD guidelines (OECD 420, 423 or 425). Based on the outcome (LD_{50} cut-off values, mg/kg bw), these *in vivo* methods allow to classify the substance into one of the four acute oral toxicity categories according to CLP Regulation (EC) 1272/2008.

New Approach Methodologies (NAMs) have been made to replace, reduce and refine the use of animals ("3Rs" principle). Different approaches such as chemical grouping or read-across, *in silico* predictions ((Q)SAR) and *in vitro* studies are available for this purpose.

In this context, the main objective of this work was to propose a strategy for assessing the acute oral toxicity of a UVCB (Unknown or Variable composition, Complex reaction products or biological materials) substance, with a non-animal testing approach.

First, an *in vitro* cytotoxicity study according to OECD guidance document 129 was performed with the substance and concluded on an estimated $LD_{50} \leq 2000$ mg/kg bw.

According to the gel permeation chromatography, the most representative constituents of the UVCB substance have a molecular weight in the range of 500 and 2500. These constituents represent more than 90% of the substance. Based on our best knowledge on the possible esterified/transesterified structures of constituents, we identified that the substance contains five families of structurally similar constituents. For each family, one representative constituent was selected in the molecular weight (500–2500). Then, the i*n silico* predictions of acute oral toxicity for each constituent were obtained using tools recommended by some authorities: CATMoS and OECD QSAR Toolbox, showing a $LD_{50} > 2000$ mg/kg bw.

In addition, a read-across approach was proposed using a UVCB analogue containing >80% of constituents similar to the studied chemical. For this analogue, an acute oral toxicity study according to OECD guideline 423 was already available and showed a $LD_{50} > 2000$ mg/kg bw. However, three constituents bearing alcohol and acid functional groups were absent from this analogue. Therefore, the acute oral toxicity *in silico* predictions of those constituents, also well-known to have no acute systemic toxicity, were considered.In conclusion, a weight of evidence approach using *in vitro* cytotoxicity testing, *in silico* predictions on representative constituents of the UVCB and a read-across with an analogue substance, enabled to assess the absence of acute oral toxicity, and therefore absence of classification according to CLP criteria, for a UVCB substance, without the use of additional animal testing.

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P02-44

Robotic device for fully automated high-content screening on *C. elegans* as a novel NAMs platform for DART assessment

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Pharmaceutical, chemical, and biomedical research heavily depend on traditional *in vivo* experiments involving vertebrate models. Although animal testing is becoming more resource-intensive and facing ethical and legal concerns, alternative *in vitro* models fall short in providing comprehensive phenotypic data at the organism level. This limitation is particularly crucial for investigating intricate human processes, diseases, developmental and reproductive toxicity effects.

In response, we present a robotic device based on the Organism-on-Chip technology as an alternative high-content screening method that bridges the gap between *in vitro* data and vertebrate testing in early pipeline stages. The robotic platform SydLab One can autonomously test 64 independent conditions on 1000 organisms (*C. elegans* nematodes) in one run. The nematode *Caenorhabditis elegans* constitutes a valuable alternative model for multiple applications and gained popularity for its ideal small size, short life cycle, ease of cultivation and propagation, and powerful genetic toolkit. While *C. elegans* has the potential to complement *in vitro* models to better predict toxic outcomes in mammals and humans, the current experimentation methods lack automation and standardization, limiting their wider use in screenings.

Hence, the aim of SydLab One is to standardize and automatize the whole *C. elegans* experimentation process to enable the use of this powerful 3Rs model in safety and efficacy screenings. The platform combines advanced microfluidic technology, robotics, biology, and AI-based algorithms to automate the entire process of *C. elegans* culture, treatment, high-content imaging, data extraction, and phenotypic analysis. SydLab One is able to execute multiple toxicity assays, including the possibility of using the existing wide collection of reporter strains thanks to the fluorescent imaging capability.

As an illustration, we present here one of the multiple validated assays on SydLab One in the scope of DART. We blindly assessed the reproductive and developmental effects of 20 benchmark chemicals using the proposed platform. SydLab One handled the worms' culture, treatment (5 doses/compound), hourly data extraction and analysis of time-resolved phenotypic readouts, including growth dynamics, sexual maturity, fertility, embryonic viability, progeny accumulation and survival rate. After unblinding, a balanced accuracy of 87.5% was reached (sensitivity: 75%, specificity: 100%) according to ECHA database.

Overall, SydLab One proposes an innovative solution for rapid identification of toxic compounds and their mechanism of toxicity, effectively bridging the gap between *in vitro* and *in vivo* assays. The platform allows not only endpoint measurements' collection, but also the monitoring of biological responses' dynamics. All in all, enabling the automated generation of whole-organism readouts in high-throughput, without the barrier of *in vivo* studies (feasibility, scaling, ethics).

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P02-46

Cultivation conditions in microfluidic environments: insights from 2D and 3D cell culture models

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Microfluidics, a technology enabling precise control over fluid dynamics, is gaining momentum, particularly in tandem with advanced *in vitro* 2D cell cultures and 3D reconstructed human tissue models. Dynamic conditions facilitated by microfluidics offer a pivotal advantage over static cultures by promoting a more faithful replication of natural physiological conditions and providing critical insights into the intricacies of cellular responses and interactions.

The aim of this work was to determine suitable dynamic cultivation conditions for 2D cell culture of kidney cells (VERO E6) from the American Type Culture Collection (ATCC) and the 3D reconstructed human tissue model of the small intestine – the EpiIntestinal model manufactured by MatTek Life Sciences, a BICO company. The microfluidic device MIVO®, i.e., Multi *In vitro* Organ, by React4life, was utilized in the current study to simulate flow conditions.

In the first phase of the experiment, a standard cell growth curve was established for static conditions in cell culture dishes and used to calculate the average doubling time. Subsequently, experiments were conducted with cells grown in cell culture inserts to compare the viability and morphology of VERO E6 cells in static and dynamic conditions over five days. Under dynamic conditions, three different flow rates were evaluated: 0.5 mL/min, 1.0 mL/min, and 1.5 mL/min.

The doubling time ranged from 19–30 hours under standard cell culture conditions, consistent with ATCC's reported population doubling time of approximately 22 hours. When seeded on inserts and inserted into MIVO chambers, the doubling time was slightly prolonged, possibly due to specific membrane characteristics. VERO cells in inserts subjected to a flow rate of 1 mL/min exhibited optimal growth characteristics, as measured by viability (MTT assay) and microscopy. Increasing flow speed led to sub-optimal cell growth, likely due to insufficient substrate for cell adhesion.

Subsequently, the EpiIntestinal model was exposed to flow conditions, and changes in viability, histology, and transepithelial electrical resistance (TEER) values were monitored over five days. Experiments were conducted in the presence and absence of a promising drug for treating diabetic complications. Our initial experiments suggest subtle changes in the response of 2D and 3D models grown in dynamic conditions. Further tests are necessary to confirm the significance of the contributions of dynamic conditions to observed changes.

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P02-47

A rat *in vitro* 3D airway model for translational toxicology

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Background and Purpose: During the last 50 years, the inhalation toxicology has been performed mainly on rodents such as rats. Huge amount of data has been accumulated all over the world during this period. With the paradigm shift initiated by the Tox21 Consortium at 2007, the ultimate goal is to phase out completely the toxicological tests on animals. It would be a great loss if the results obtained on rats *in vivo* could not be used anymore. To bridge the gap between the rats and human, we developed an *in vitro* rat airway epithelial model which allow the side-to-side comparison between rats and human *in vitro*. As such, it would be possible to translate the *in vivo* rat data to predict the effects of toxicants on human health.

Methods: The rat *in vitro* 3D airway epithelial model was made of primary cells isolated from the tracheal and bronchi, cultured at air-liquid interface. Once differentiated, the epithelium (MucilAir-Rat) was fully ciliated with measurable Cilia Beating Frequency. With histological analysis, the epithelium was pseudostratified, composed of ciliated cells, goblet cells, Club cells and basal cells. The mucociliary clearance was also measured. The activity of main ion channels such as ENac, CFTR were detected in Ussing Chambers.

Results: As proof-of-concept for toxicological tests, we assessed the effects of several toxicants including SDS, Formaldehyde, and CdCl2 after 14 days repeated daily 6 hours exposure. The toxicity of these compounds was assessed by several different endpoints, such as Cilia Beating Frequency (CBF), Trans Epithelial Electrical Resistance (TEER), LDH release, and IL-8 secretion. The rat airway epithelia could tolerate up to 1 mM SDS, 10 mM Formaldehyde, 250 µM CdCl2.

Conclusions: These results demonstrated that this *in vitro* rat model is a useful tool for toxicity testing, which could be used for translational toxicology.

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P02-48

Beneficial effect of differently-coated Selenium Nanoparticles in 3D cell culture models mimicking the respiratory tract and intestinal epithelium

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Selenium (Se) is an essential trace element that plays a crucial role in various physiological processes, including enhanced immunity (Avery and Hoffmann, 2018) and antioxidant defense *(Bjørklund et al., 2022)*. Ingested as well as inhaled Se nanoparticles (SeNP) can be absorbed by epithelial and resident immune cells and being incorporated into selenoproteins that scavenge reactive oxygen species (ROS) and protect against oxidative stress-induced damage. Moreover, Se has been shown to modulate the release of inflammatory cytokines, and Se deficiency has been associated with impaired intestinal barrier function and increased susceptibility to intestinal infections and inflammation. Therefore, Se serves as a valuable micronutrient in protecting against oxidative stress and inflammation. Understanding the mechanisms of Se metabolism and its effects on intestinal and lung physiology is crucial for developing therapeutic strategies to combat gastrointestinal and respiratory disorders. However, the direct administration of Se as an antioxidant is not advised due to its narrow therapeutic window *(Hosnedlova et al., 2018)*.

The objective of this study was to evaluate the toxicity as well as the antioxidant and anti-inflammatory capacity of SeNPs in 3D cell culture models. The respiratory tract can be resembled by an alveolar *in vitro* test system called ALIsens® *(Chary et al., 2019)* built on a microporous membrane of hanging inserts by seeding human alveolar type II epithelial cells (A549) and endothelial cells (EA.hy926), as well as macrophage-like (Mφ-THP1) and dendritic-like cells (DC-THP1). The physiologically relevant architecture of the system favors the development of a tissue-like microenvironment and facilitates exposures at the air-liquid-interface (ALI). The *in vitro* intestinal epithelium is based on a tri-culture model consisting of human intestinal epithelial cells (Caco-2) and mucus-secreting HT29-MTX cells, as well as hematopoietic cells (Raji B) able to promote Caco-2 conversion in specialized microfold cells (M-cell) *(Araujo et al., 2013; Schimpel et al., 2014)*.

The treatment shows that the SeNPs are well tolerated in both cell culture systems without inducing neither a strong basal cytokine release nor increasing oxidative stress measured by ROS formation.

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P03 | *In vitro* to *in vivo* extrapolation (QIVIVE)

P03-01

Novel melatonin/donepezil-based hybrids prevents amyloid-induced neurotoxicity and scopolamine-induced cognitive deficits

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by neuron loss and the formation of neurofibrillary tangles and senile plaques, primarily due to amyloid beta-protein (Aβ). Our previous research on thurty melatonin/donepezil-based hybrids demonstrated their potential to inhibit acetylcholinesterase (AChE),

possess antioxidant properties, and exhibit lower cytotoxicity compared to donepezil alone[1]. In this study, we evaluated the neuroprotective effects of two lead compounds, **3a** and **3c**, against Aβ-induced neurotoxicity and memory deficits in mice.

Methods: The anti-fibrillogenic properties of benzylpiperadine derivatives of melatonin **3a** and **3c** were assessed using ELISA-based analysis to quantify changes in Aβ42 formation in normal and malignant cell lines of different origins. The cholinesterase-inhibiting effects in scopolamine-induced neurotoxicity in mice, along with histological analysis of the impact of the tested compounds on the mice brain were also investigated. Additionally, acute toxicity following oral and intraperitoneal administration was evaluated.

Results: The hybrids **3a** and **3c** showed protective effects against Aβ-induced neurotoxicity reducing Aβ42 levels from 3 to 6 fold in the human neuronal cell line SH-SY5Y, widely used as an *in vitro* model to study neurodegenerative processes. The newly synthesized compounds, as well as donepezil administered intraperitoneally for 14 days, were not hepatotoxic or nephrotoxic. The compounds **3a** and **3c** significantly decreased brain MDA levels while increasing glutathione (GSH) levels compared to controls, suggesting potent antioxidant activity. Further, the new compounds exhibited antioxidant activity against scopolamine-induced increased oxidative stress in comparison to the donepezil by reducing lipid peroxidation and increasing GSH levels in mouse brain homogenates. The histology showed that the administration of scopolamine caused neuronal damage in the dorsal hippocampus of the vehicle-treated mice. In contrast to the reference drug donepezil, the two hybrids **3a** and **3c** showed neuroprotection in most of the hippocampal regions.

Conclusion: The present results suggest a possible chemopreventive role of melatonin/donepezil-based hybrids **3a** and **3c** in Alzheimer's disease making them promising for further experimental research in the field of neurodegenerative diseases.

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P03-02
In vitro to *in vivo* extrapolation (QIVIVE) for predicting drug-induced liver toxicity

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Purpose: The objective of this study was to create a model for predicting drug-induced liver toxicity by identifying crucial genes linked to cell toxicity in *in vitro* settings and assessing their ability to predict toxicity in *in vivo*. The objective was to establish a connection between *in vitro* and *in vivo* data by employing quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) techniques, which have the potential to reduce animal sacrifice in *in vivo* studies.

Methods: The TG-Gates dataset is used to analyze gene expression profiles in primary rat hepatocytes that were exposed to 145 compounds at different doses. This dataset also includes a control group for an *in vitro* rat investigation. We performed gene expression preprocessing using the Robust Multi-array Average (RMA) approach and carried out differential gene expression (DEG) analysis. In addition, we analyzed variations in DNA content within the samples. The study evaluated the degree of cellular damage caused by each chemical at various dosage levels. The alterations in DNA content were subsequently categorized into two levels of cytotoxicity (low and high). A t-test revealed substantial statistical differences among the clustered groups. By employing statistical analysis and utilizing a machine learning feature selection method, we have successfully discovered 12 genes that exhibit a correlation with cytotoxicity. Furthermore, our label prediction model has achieved an auc score of 80%. Gene analysis, such as using tools like DAVID and Gene Ontology analysis, is performed to determine the significance of the genes. Subsequently, the process of combining *in vivo* single and repeated dose study data with pathological data was carried out to obtain pathological toxicity endpoints for the corresponding samples. As a result, the *in vivo* dataset consists of 137 compounds with comprehensive toxicity endpoints. Separate predictive models were developed for each toxicity endpoint, such as Hypertrophy, Microgranuloma and Necrosis, by filtering the data.

Results: The *in vitro* analysis discovered 12 genes that exhibited a strong correlation and statistical significance in relation to cytotoxicity. These genes were later utilized to create machine learning models for predicting different *in vivo* toxicity outcomes. The algorithms demonstrated an auc score of nearly 80% in predicting outcomes at various dosage levels for the rodent liver.

Conclusion: This study demonstrated the capacity of the identified genes and the QIVIVE technique to accurately predict toxicity in *in vivo* simply based on data obtained from *in vitro* data.

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P03-03
Quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) for liver-induced thyroid toxicity risk assessment

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New Approach Methodologies (NAMs) are being increasingly developed to explore toxicities without the use of or with a significant reduction in animal use. The use of NAMs is also intrinsic to the development of exposure-driven Next Generation Risk Assessment (NGRA). However, to ensure a robust NGRA, quantitative knowledge concerning the Adverse Outcome Pathway (AOP) for the toxicity in question is required. The objective of this work is to develop a robust framework for *in vitro* risk assessment for liver-induced thyroid toxicity using a QIVIVE approach and to develop non-animal methods for the biological characterization of hepatic UGT induction and T4 clearance. For this, the accuracy of a QIVIVE approach was assessed using data from short-term *in vivo* toxicodynamic studies (mRNA/enzyme activity/thyroid hormone measurements).

In vitro data consisting of CYP and UGT mRNA expression and activity and T4 clearance, were obtained from male rat and human hepatocytes, exposed to two reference hepatic enzyme inducers, phenobarbital (PB) and pregnenolone-16α-carbonitrile (PCN). The implementation of the QIVIVE approach for rats and humans involves extrapolating the Benchmark Concentration (BMC) and its lower confidence bound (BMCL) from *in vitro* data to obtain the administered equivalent dose (AED) in the *in vivo* scenario, taking into consideration the *in vitro* free concentration of the chemicals. This is followed by a comparison with the Benchmark Dose (BMD) and its lower confidence bound (BMDL)

derived from *in vivo* experiments. The BMC(L) and BMD(L) were computed using the EPA and EFSA approaches, leveraging BMDExpress3 and the EFSA Bayesian BMD tool, respectively. Furthermore, the extrapolation was achieved through reverse dosimetry, using two distinct tools for physiologically based kinetic (PBK) modeling: httk and PK-Sim. The results from the PBK models were compared with pharmacokinetic data of PB and PCN from experiments with rats, and with pharmacokinetic clinical data of PB obtained from the literature for humans.Different pharmacokinetic parameters were used to obtain scaling factors for the extrapolation. For rats, the AUC(0–24h) of plasma concentration was found to give, on average, the most accurate oral AED, with average fold-changes of 1.6±3.7 for BMD and 5.3±16.3 for BMDL compared to the *in vivo* BMD(L). The QIVIVE was also performed with *in vitro* data from human hepatocytes. In conclusion, extrapolating the BMC(L) from *in vitro* experiments and comparing to the BMD(L) from *in vivo* experiments with different biomarkers can shed some light on how to accurately extrapolate a relevant point of departure (POD) from *in vitro* experiments to the *in vivo* scenario for risk assessment.

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P03-04

Integrating *in vitro* data and physiologically based kinetic modeling-facilitated reverse dosimetry to predict diethylhexyl phthalate-induced hepatic lipid metabolic disorder in humans

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Diethylhexyl phthalate (DEHP) has been widely used as a plasticizer in food packaging materials and its toxicity remains a major public health concern. The current point of departure (PoD) used to derive tolerable daily intake (TDI) for DEHP is based on reproductive effects observed in animal studies. However, recent research demonstrates that DEHP can induce metabolic disorder at lower doses than the PoD, indicating that the critical effect of DEHP in humans remains debatable. One reason could be that hazard assessments of DEHP have relied on animal studies, which show poor correlations with human effects due to inter-species differences in toxicodynamics and toxicokinetics. The aim of the present study was to develop an alternative animal testing method to predict DEHP-induced lipid metabolism disorder in humans. The physiologically based kinetic (PBK) model of DEHP and its metabolite MEHP was developed using parameters derived from in silico simulations, *in vitro* kinetic assays and the literature. HepG2 cells combined with a high-content imaging technique were used to determine the *in vitro* concentration-response curve based on lipid droplet accumulation. Using PBK modeling-based reverse dosimetry, the *in vitro* concentration-dependent data were converted to *in vivo* dose-dependent hepatic lipid metabolism disorder in humans, taking into account the toxicity of MEHP and protein binding in the *in vitro* and *in vivo* situation. Benchmark dose (BMD) analysis of predicted *in vivo* data was used to derive PoD. Our results showed that MEHP was 13-fold more potent than DEHP in inducing lipid droplet accumulation and its unbound *in vitro* effective concentrations were within the range of its unbound blood concentration upon the daily exposure to DEHP, implying that MEHP might play a substantial role in DEHP-induced metabolic disorder in humans. The predicted $BMDL_{10}$ was 0.5 mg/kg bw, which was 10-fold lower than the PoD based on reproductive toxicity, suggesting that hepatic lipid metabolism could be a more sensitive endpoint for DEHP. The *in vitro*-in silico derived TDI (0.005 mg/kg bw) was compared to human dietary DEHP exposure levels, suggesting that the potential risk of DEHP on metabolic system in human populations warrants close attention. The present study provides a proof-of-principle of using an *in vitro*-in silico approach to predict hepatic lipid metabolic disorder induced by DEHP in humans, further facilitating the development of risk assessment using New Approach Methodologies for phthalate-induced metabolic disorders in public health.

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P03-05

qIVIVE modelling of liver steatosis

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Introduction: Due to increasing societal and regulatory demands as well as high costs- and ethical considerations there is an urgent need for improved animal-free strategies for chemical testing. Besides ever improving organ mimicking testing systems an important and promising development in this context is the increased application of *in silico* tools. This includes quantitative *in vitro* to *in vivo* Extrapolation (qIVIVE) and the transition from Adverse Outcome Pathways (AOPs) to quantitative AOPs (qAOPs) for the improved prediction of adversity in humans. This project aims for the integration of qIVIVE with the AOP for liver steatosis.

Materials and Methods: Focus is set on the late key event (KE) relationship of the AOP for liver steatosis, which includes the main KE of triglyceride accumulation. This work was performed based on data produced for a fungicide plant protection product. Physiologically based toxicokinetic modelling (PBTK) was conducted to predict active substance (AS) concentrations in plasma and liver using the *httkR* package. The PBTK model was evaluated by comparison with *in vivo* measurements in rat. Further, intracellular concentrations and concentrations of the AS unbound in the medium were predicted for the hepatocyte *in vitro* exposure system using the *in vitro* distribution model implemented in *httk*. Based on the predicted concentrations qIVIVE was performed by application of reverse dosimetry and predicted dose-response relationships were compared to results from two mouse *in vivo* experiments. Finally, predicted and observed data were compared by Benchmark Dose (BMD) analysis.

Results: Values predicted by the PBTK modelling fit well with the experimentally determined *in vivo* plasma and liver concentrations of the fungicide. The *in vitro* concentration-response data for triglyceride accumulation were translated to equivalent oral doses and showed good correlation to the mouse *in vivo* data on liver fat vacuolation after exposure to the substance of interest. qIVIVE-derived BMDs and associated credible intervals were highly similar to the values obtained from the *in vivo* experiments.

Conclusion: The PBTK and *in vitro* distribution models proved fit for the purpose of qIVIVE. The results confirm the usefulness of integrating AOPs and qIVIVE for human adversity prediction particularly with regard to the “replacement” aspect of the 3R principle.

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P03-06

Distribution kinetics of azole fungicides in an *in vitro* liver steatosis model

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Azole fungicides are a class of synthetic chemicals commonly employed in agriculture to protect crops from fungal diseases and in medicine to treat fungal infections in humans. However, exposure to some of these fungicides is associated with hepatotoxicity, including liver steatosis or fatty liver disease. The fungicides are thought to disrupt lipid metabolism in the liver by interfering with the activity of cytochrome P450 enzymes. Within the EU Horizon 2020 project ONTOX, *in vitro* models are being developed using human hepatoma cell line, HepaRG, to test for the steatotic potency of chemicals, including azole fungicides. The aim of this project is to analytically assess the concentration of six triazole fungicides, namely triadimefon, cyproconazole, propiconazole, tebuconazole, ketaconazole and itraconazole, in exposure medium, well plate plastic, collagen-I and HepaRG cells over the exposure time in the ONTOX *in vitro* steatosis assay. Normally, potency is expressed as nominal concentrations *in vitro*. Nominal concentrations refer to the concentration of a chemical that is added to the exposure medium in an *in vitro* toxicity assay. However, the binding of chemicals to microtiter plate plastic, extracellular matrix and medium constituents like serum protein may reduce the amount of the chemical that reaches the cells to cause toxicity. The extent to which non-specific binding occurs is likely dependent on the chemical and the assay setup, explaining in part the variability in *in vitro* potency between chemicals and *in vitro* assay setup. Indeed, results from this study indicate that the intrinsic clearance and binding affinity to well plate plastic, collagen-I and serum differ between azole fungicides, and therefore, the concentration of the chemical associated with HepaRG cells differ even if nominal concentrations are the same. A compartmental model was developed to estimate cell-associated concentrations of these azole fungicides in time in the assay. The model was used to compare potency ranking of the azole fungicides based on different *in vitro* dose metrics and illustrates how the maximum concentration of azole fungicides associated with HepaRG cells is a more robust dose metric for quantitative *in vitro* to *in vivo* extrapolation than the traditional *in vitro* nominal effect concentration is.

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P03-08
Metabolism of Enniatin B in primary mouse, rat and human hepatocytes

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Enniatins produced by *Fusarium* species belong to emerging mycotoxins commonly found in cereals and raising concern for food and feed. They are potentially toxic to humans and therefore represent a public health issue. However, due to the lack of toxicokinetic and toxicodynamic data that could be used to derive a Health-Based Guidance Value, enniatins are not regulated at the European level [1]. Enniatin B (ENNB), a cyclic hexadepsipeptide with the molecular weight 639.83 g/mol, is the most prevalent among the 28 identified homologues [2,3]. Previous work [4] has investigated the metabolism of ENNB using liver microsomal fractions of different species (rat, dog and human). In order to establish a sound data basis for quantitative *in vitro*-to-*in vivo* predictions (qIVIVE), additional studies in *in vitro* cell models have to be performed. The present study has investigated (i) ENNB cytotoxicity in primary human (PHH), mouse (PMH) and rat hepatocytes (PRH), (ii) kinetic depletion in PHH, PMH and PRH, and (iii) respective ENNB biotransformation products that were analysed using liquid chromatography-high resolution mass spectrometry (LC-HRMS).The cytotoxicity of ENNB was determined by measuring metabolic activity in the MTT assay. PHH, PMH and PRH in 48-wells plates were exposed to ENNB in the range of 0.16 to 10 µM. The kinetic experiments were performed with hepatocytes in suspension exposed to the sub-toxic concentration of 1 µM ENNB. The enzymatic reactions were stopped by adding acetonitrile at incubation time points up to 4h. The samples were analysed with LC-HRMS, detecting ENNB and its biotransformation products by using an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a heated electrospray ion source (HESI-II) and coupled to an Ultimate 3000 UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA). Matrix-assisted standard curves were used for calibration ; the limit of quantification was established at 39 nM. First-order kinetics of ENNB depletion in PHH, PMH and PRH were determined and used for qIVIVE. The characterisation of the ENNB metabolites in the different species is under way.

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P03-09
Predictive performance of physiologically based kinetic (PBK) models based on *in-silico/in vitro-in vivo* extrapolation (IS/IVIVE)

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Recent advancements in chemical risk assessment have emphasized the importance of New Approach Methodologies (NAMs), adhering to the 3R principles, increasingly utilizing *in vitro* assays for non-animal toxicity testing. These methods inform predictions of *in vivo* chemical levels in humans from *in vitro* data, supported by physiologically based kinetic (PBK) modeling, which integrates absorption, metabolism, and protein binding processes. The combination of non-testing (QSAR) and testing methods (*in vitro* assays) are pivotal in predicting *in vivo* exposure levels.

This study investigates 24 compounds from the ToxCast database with reported *in vitro* hepatocyte clearance (CL_{int}) and plasma protein binding (f_{up}) data. PBK models were implemented in GastroPlus® version 9.9 (Simulations Plus Inc., Lancaster, CA) to predict the plasma exposure of the chemicals following oral administration. Machine learning models from ADMET Predictor® version 11(Simulations Plus Inc., Lancaster, CA) were used to *predict* all physicochemical and biopharmaceutical properties, except for plasma protein binding (f_{up}) and *in vivo* clearance, for all chemicals. Experimentally measured f_{up} reported in the ToxCast database was used in all simulations and three different approaches were evaluated for prediction of *in vivo* clearance: Approach 1: predicted human liver microsomal clearance (CYP_HLM_Clint model) from ADMET Predictor; Approach 2: predicted hepatocyte clearance (HEP_hCLint model) from ADMET Predictor; Approach 3.

QIVIVE using reported *in vitro* CL_{int} from the ToxCast database with a focus on comparing the performance of restrictive vs non-restrictive clearance of the chemicals. Prediction accuracy was evaluated by comparing the predicted total chemical exposure over time (AUC) and maximum plasma concentration (Cmax) with the observed values. Approach 1 with restrictive clearance predicted the AUC of 14 chemicals and the Cmax of 16 chemicals within 5-fold of the observed data. Approach 2 with restrictive clearance predicted the AUC and Cmax of 15 chemicals within 5-fold of the observed data. Approach 3 with restrictive clearance predicted the AUC of 15 chemicals and the C_{max} of 16 chemicals within 5-fold of the observed data and Approach 3 with non-restrictive clearance predicted the AUC of 12 chemicals and the C_{max} of 14 chemicals within 5-fold of the observed data. Overall, modelling restrictive clearance performed better than non-restrictive clearance for this set of chemicals. The higher prediction errors were investigated more closely, identifying contributions of non-hepatic clearances (e.g., active urine excretion, intestinal first pass) and elimination via bile as main reasons for the higher fold differences between predicted and measured AUC and C_{max}. Future work will focus on defining applicability domain for categorizing the compounds to minimize the variability in prediction accuracy to establish animal free risk assessment framework.

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P03-10
Synthetic cannabinoids & neuronal senescence: distinctive responses of *in vitro* models to AMB-FUBINACA

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Among the array of new psychoactive substances, synthetic cannabinoids (SC) stand out as highly popular among consumers. These substances closely resemble, in terms of their pharmacology, Δ^9-tetrahydrocannabinol (THC), cannabis' main active principle, albeit exhibiting full agonism at the cannabinoid receptors 1 and 2. In light of recent scientific findings suggesting that cannabis use can exacerbate ageing-related parameters, the present work was designed to explore whether SC share similar potential effects. For this purpose, we employed two distinct *in vitro* models. The first model involved primary hippocampal cultures (PHC), isolated from Wistar rat embryos at embryonic day 18–19; after seeding, cells were kept in culture and exposed to the popular SC AMB-FUBINACA (AMB-FUB) at 1 pM, 1 nM and 1 μM, starting at day-in-vitro (DIV) 3 or DIV7, and perpetuated until DIV21. DMSO at 0.02% was used as the solvent control. At the end of the exposure, ß-galactosidase activity (a common first-line cell senescence biomarker) was assessed using a commercially-available kit. Our findings under these experimental conditions, revealed that PHC exposed to all AMB-FUB concentrations had less ß-galactosidase activity than the control condition ($p<0.01$, 1 pM; $p<0.001$, 1 nM and 1 μM). The other *in vitro* model used herein was the human neuroblastoma cell line SH-SY5Y. Beginning at passage 24, cells were seeded and exposed to 1 nM and 1 μM AMB-FUB. At passages 24 (48h after drug exposure) and 28, samples were collected for the analysis of several senescence-related endpoints, namely ß-galactosidase activity, cell cycle analysis (via flow cytometry, following DNA staining with propidium iodide) and relative telomere length measurement (using qPCR). Surprisingly, no discernible effect of AMB-FUBINACA was observed for any of the endpoints examined. The apparent observed "anti-ageing" effect of AMB-FUB on PHC warrants further investigation. Moreover, the differential reponse observed between the two *in vitro* models also requires scrutiny, particularly in light of recent published findings suggesting that THC has the potential to increase ß-galactosidase activity. Further experiments will ensue to hopefully shed some light on these interesting results.

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P03-11
Assessing kinetic properties in food and feed: tebuconazole case study

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As part of the ADME4NGRA project, a tiered testing strategy is under investigation to better characterize interspecies differences for ADME properties. The *in vitro* ADME parameters will be used as input for physiologically based kinetic modelling approaches to estimate the bioavailability of substances found in feed and food.

Nine case studies evaluate *in vitro* approaches to measure transport and metabolism of the liver, intestine and kidney. They also take into account metabolism by the gut microbiome using data rich model compounds.

One case study is presented here, which investigates the degradation of the pesticide tebuconazole in different life stages. Metabolism in the adult women is compared to pregnancy, also including the situation of the foetus in the first, second and third trimester.

Tebuconazole is degraded to hydroxy-tebuconazole by CYP3A4 and CYP2C9. Both enzymes are increasingly expressed during pregnancy, while CYP2C9 in particular is significantly less present in the foetus. In the case study, the rate of degradation of tebuconazole by different CYPs is determined experimentally. PBK modelling is then used to model and compare the bioavailable concentrations of tebuconazole in the different phases of life.

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P03-12
Physiologically based kinetic modelling (PBK) using QIVIVE to predict the toxicokinetic profiles of triazoles via oral route of administration

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A PBK model comprises of a set of mathematical equations to estimate the bioavailable concentration of a dosed compounds in the blood or tissues of an organism. Quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) is therefore a central aspect in the process called Next Generation Risk Assessment (NGRA), which base the hazard assessment of the unknown compounds on New Approach Methodologies (NAMs).

The ZeroPM (an EU funded project under horizon 2020) project develops an assessment strategy to reduce manufacture, environmental emissions and use of persistent and mobile compounds based on several criteria including their toxicological properties. Toxicological properties are screened using New Approach Methodologies, which provide *in vitro* benchmark concentrations, for example EC50 values. Starting from there, the work presented here demonstrate the *in vitro* to *in vivo* extrapolation to estimate the corresponding human equivalent concentrations, which can be used as the point of departure for human risk assessment.

The VIVD model [1] has been reconstructed as it was a comprehensive *in vitro* virtual cell-based assay mass balance model. The model considers the cell system with respect to partitioning to air, cells, proteins and to the plastic surface area in contact with the medium. Most importantly when considering the persistent and mobile substances, the model also considers ionization. The redeveloped VIVD model [1] was used to obtain the free medium EC50 values available in the medium and the possible concentrations within the cells from the administered nominal EC50 values. This *in vitro* biokinetic correction is given as an input to the PBK model to perform QIVIVE.

Using the *in vitro* measured ADME values such as *of* apparent permeability (Papp) value, plasma protein binding, blood – plasma ratio and the intrinsic hepatic clearance and with the *in vitro* biokinetic modelling, we parameterize an oral route bottom-up PBK model to model 'tebuconazole' a data rich compound from the triazole family. A rat based oral route PBK model with rat physiological parameters is constructed. The PBK model predicted plasma concentration for tebuconazole is within the 2-fold range when compared with the *in vivo* plasma concentration data for rat, before being extrapolated to the human oral route PBK model.

The *in vitro* ADME measured valuesof tebuconazole is extrapolated using read-across approach with respect to the molecular weight and other physiochemical properties to fill the data gaps for other triazoles in selected compounds, such as difenoconazole, paclobutrazol, fenbuconazole and bitertanol. The main goal here is to perform the hazard characterization to understand how triazoles affect the human body and the environment due to their long-term presence and slow decomposition rate.

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P03-13
Identifying potential developmental toxicants in an IVIVE workflow: lessons learned

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Purpose: The purpose of this abstract is to document lessons learned and opportunities for improvement in a recently developed *in silico* workflow for *in vitro* to *in vivo* extrapolation (IVIVE) to identify substances with potential developmental toxicity (DevTox).

Methods: Multiple DevTox-relevant public databases were used to identify 26 chemicals with various levels of known or suspected DevTox. The US Environmental Protection Agency (USEPA) CompTox database was then used to obtain *in vitro* and *in vivo* information for the 26 chemicals. High throughput (HT) IVIVE using the USEPA httk package was used to determine an administered equivalent dose (AED) from all *in vitro* data points using a method similar to that described in Paul Friedman *et al.* (2020). Several assays and *in vivo* endpoints were identified as being "DevTox-relevant" and used to compare points of departure (PODs) and AEDs from all available data to those assay endpoints related to DevTox. A bespoke physiologically based pharmacokinetic (PBPK) model was also generated based on a number of published pregnancy PBPK models and used to calculate AEDs for 8 subset chemicals with published PBPK models. Endpoints were compared to evaluate the utility of current IVIVE approaches for estimating DevTox points of departure (POD), and areas for improvement in the methodologies were identified, including: 1) assay/endpoint categorization, 2) *in vitro* distribution (IVD) model adjustment, 3) PBPK modeling and IVIVE.

Results: We identified several assays relevant to DevTox that were not included in the CompTox DevTox classification, which increased the number of chemicals for which DevTox assays could be evaluated. Comparison of 50% activity concentration (AC_{50}) values for DevTox relevant vs. all *in vitro* assays resulted in 57% of the chemicals being designated as high priority follow-up as potential Dev toxicants and 30% for medium prioritization. Among the 3 chemicals not identified as DevTox using the *in vitro* data was sulfasalazine, consistent with its categorization as safe during pregnancy. HT-IVIVE was generally more conservative than bespoke PBPK, and in half the models, provided an AED that was closer to the *in vivo* POD. This is likely due to the highly conservative assumptions built into the httk model. The ability to predict IVD with current models was limited by lack of information on *in vitro* assay parameters. However, in one case (Stemina® DevTox assay), the IVD model predicted that free concentrations for some chemicals were <0.1% of the nominal concentration, highlighting the importance of accounting for *in vitro* distribution of chemical in these assays. Based on these studies, we identified the most needed improvements in documentation of assay conditions for IVD modeling and improved range of assays related to DevTox. Overall, updating the IVIVE process would improve accuracy of the IVIVE, but would likely make AED estimates less conservative.

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P04 | Adverse outcome pathways

P04-01
An adverse outcome pathway (AOP)-based approach of assessing the potential for neurotoxicity – a literature review with fenazaquin

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Fenazaquin is a synthetic acaricide used in agriculture to control pests such as mites on various crops and is an approved active substance in the EU. It belongs to the chemical class of quinazolines and acts by disrupting the energy production process in the target pests, i. e. mitochondrial complex I inhibition. The Adverse Outcome Pathway (AOP)

No. 3 [1] links binding to the complex I of the mitochondrial respiratory chain (the molecular initiating event, MIE) to motor deficits found in parkinsonian disorders (the adverse outcome). Binding to complex I leading to its inhibition has been shown to trigger downstream key events (KE) such as mitochondrial dysfunction and impaired proteostasis, which can cause degeneration of dopaminergic (DA) neurons of the nigrostriatal pathway.

The presented literature analysis indicates that fenazaquin binds to and inhibits complex I *in vitro*, affecting tau protein, synaptic density, inducing neuronal cell death and decreasing mitochondrial ATP levels. Although the reliability of the publications is noted as limited, the identified data provide evidence for the MIE (binding of complex I), KE1 (complex I inhibition), KE2 (mitochondrial dysfunction) and KE4 (degeneration of DA neurons) of AOP 3.

While actual effects always require a sufficiently high dose-response in situ, available data also indicate that fenazaquin potentially reaches the brain of mice and rats. However, the data do not allow to determine the concentrations of fenazaquin in the substantia nigra under realistic exposure conditions in humans and in animal studies. Thus, physiologically based kinetic (PBK) modelling would be desirable to support a robust *in vitro - in vivo* extrapolation (IVIVE) of the effect data. In addition, acute or repeated neurotoxicity (e. g. OECD Test Guideline 424) could be studied in rodents with special focus on the histopathological investigation of the brain. Routine sampling of coronal sections of the brain in standard general toxicity studies, including a detailed histopathological investigation of the midbrain with the substantia nigra as one of the specific areas of interest, would be recommended, but in order to truly capture the hallmarks of PD, specific procedures (e. g., tyrosine hydroxylase/TH staining) could be necessary [2].

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P04-02
Adverse outcome pathways to support assessment of food nanoparticles toxicity on gut microbiota and intestinal barrier integrity

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Background: Nanoparticles (NPs) are present in our plates as the food industry takes advantage of their physicochemical properties to coat, color and protect the packaging or the food itself. But the use of food NPs as potent bactericidal agents rises concerns about their potential impact on our gut microbiota. Mice studies showed evidence of gut dysbiosis following NPs ingestion[1]. However, the microbiota being commonly studied at the compositional level, we lack a comprehensive understanding of the functional changes and the long-term health significance remains unexplored. Rodent and *in vitro* studies also pointed that food grade NPs could disrupt the intestinal barrier[2], a phenomenon implicated in many diseases. However, the mechanisms behind this effect are unclear, and evidence missing due a lack of appropriate methods.

Aims: We aim to exploit Adverse Outcome Pathways (AOPs) to support mechanistic-based assessment of food NPs toxicity[3] on (i) gut microbiota and long-term effect on liver and on (ii) intestinal barrier disruption.

Methods: The AOPs were developed (i) based on previous results obtained in the lab[4] and (ii) by using the recently developed AOP helpFinder tool[5] to identify studies investigating food NPs and intestinal barrier disruption. Assays associated to the main perturbed biological events were identified. Evidence was collected from literature or produced in experimental conditions.

Results: An AOP was formulated linking gut dysbiosis to the modification of intestinal bile acid metabolism resulting in reduced bile acid-mediated anti-inflammatory effect and subsequent escalation of inflammation in the liver.

Regarding intestinal barrier, two AOPs were developed to depict the mechanisms from NP cellular uptake in enterocytes and goblet cells to disruption of the epithelial and mucosal intestinal layers respectively.

Evidence from both literature and experimental data supported these pathophysiological pathways upon NPs exposure while also underscored notable gaps in knowledge emphasizing the need for future research.

Conclusion: Humans are exposed to NPs via their food yet their toxicity on gut microbiota and gut barrier is not well understood. Producing evidence from assays anchored in an AOP-aligned mechanistic understanding will be instrumental in facilitating the assessment of food NPs toxicity on gut.

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P04-03
Adverse outcome pathway-based approach to reveal the mechanisms of skin sensitization and long-term aging effects of chlorothalonil

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Chlorothalonil (CHT) is a widely used antifungal agent and is reported as a sensitizer that can cause allergic contact dermatitis (ACD). ACD initiation is associated with various innate immune cell contributions

and is usually accompanied by persistent inflammation, which is a potential contributing factor to skin damage. However, detailed information on the mechanisms which CHT induces skin sensitization and damage is still insufficient. This study focused on the possible sensitization process and mechanism of CHT and the adverse effects of repeated CHT exposure. CHT activates dendritic cells and promotes the proliferation of lymph cells in the skin sensitization phase, causing severe inflammation in ACD patients. Keratinocytes activate the NLRP3 inflammasome pathway to cause inflammation during CHT treatment, and macrophages also secrete inflammatory cytokines. In addition, CHT-induced inflammation triggered skin wrinkles, decreased epidermal thickness and decreased collagen. Cell experiments also showed cell proliferation and senescence were inhibited under repeated CHT exposure, and autophagy dysfunction was a cause of aging. This study defined the possible process through which CHT is involved in the skin sensitization phase and elucidated the mechanism of CHT-induced inflammation in innate immune responses. We also determined that repeated CHT exposure caused persistent inflammation, ultimately leading to skin aging.

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P04-04

Development of a Putative Adverse Outcome Pathway (AOP) for the identification of substances with a potential link to Alzheimer's Disease (AD)

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In recent years, there has been an increasing need to define appropriate models to support risk assessment to improve understanding of the mechanisms by which chemicals may cause adverse effects in the population and to identify new approaches to replace animal models. Adverse Outcome Pathways (AOPs) serve a dual purpose: to provide a mechanistic basis for animal and epidemiological studies and to facilitate the development of Integrated Approaches to Testing and Assessment (IATA). AOPs are analytical models that describe causally connected Key Events (KEs), originating from a Molecular Initiating Event (MIE), occurring across various biological levels, leading to an Adverse Outcome (AO) allowing for the integration of different types of data. Systematic review and meta-analyses suggest a positive association between exposure to Organochlorines (OC), Organophosphorus (OP) pesticides and Alzheimer's Disease (AD) [1]. The study aimed to develop a strategy for creating an AD network based on the biological plausibility of KE Relationships (KERs) connecting the pathological signs of the disease. The objective was to investigate a potential correlation between the dysregulation of clinically recognised biological AD features and the exposure to stressors such as OC and OP compounds.

An *a priori* protocol has been drafted and a putative AD network was developed based on several criteria:

- Mapping and selection of AOPs involving adult neurotoxicity (ANT) found in AOP-Wiki, a platform collecting developed AOPs.
- The refinement of the network based on expert knowledge and an initial analysis of the consolidated literature. AOPs addressing ANT in AOP-Wiki were selected based on predefined eligibility criteria. The resulting network was curated by merging KEs from different AOPs that address the same or similar biological concepts. The curated ANT network was used to identify KEs and KERs that address AD already developed and data gaps. While the ANT network contained the elements such as tau hyperphosphorylation and synaptic dysfunction, other core events of AD, including β-amyloid accumulation and neurofibrillary tangles, were absent. These features were added to complete the network and prioritised for their relevance in AD progression. MIEs were selected based on the biological events involved in amyloidogenic processing.

  The development of prioritised MIEs, KEs and KERs will be conducted through a systematic retrieval and relevance screening of the information, data extraction and evidence appraisal according to the protocol. To support the assessment of empirical evidence, a stressor-based approach will be adopted using OC and OP pesticides, including those used in the EU, considering both parent compounds and active metabolites.

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P04-05

Artificial intelligence for the development of Adverse Outcome Pathway (AOP) to assess neurodegenerative disorders following ionizing radiation exposure

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Recent research on non-cancer effects of low to moderate doses of ionizing radiation (IR) has shown interest in and growing concern about cognitive and neurodegenerative effects. Adults exposed to doses from 1 to 2 Gy demonstrated cognitive decline, but research is limited and the effects of lower doses remain poorly studied. Associations between IR dose, dementia, Alzheimer's disease and Parkinson's disease were positively and significantly correlated in cohorts of nuclear workers [1,2,3,4]. Development of adverse outcome pathways (AOPs) has recently gained wide interest as a framework with a strong potential to improve the understanding of putative biological modes of action of IR leading to diseases, including neurological disorders. AOPs are a sequential structured representation of knowledge, starting

from a molecular initiating event (MIE) that can be triggered by IR and ending with a specific adverse outcome (AO) via a series of key events (KE). To construct an AOP, the first step is to identify and extract existing information from various sources such as databases and the scientific literature. AOP-helpFinder, a tool based on artificial intelligence (AI), was recently designed to help researchers in creating AOPs by systematically identifying stressor (e.g., IR) – event (e.g., MIE, KE, AO) and event-event associations through text mining of available scientific literature from the PubMed database. Additionally, this tool includes a scoring system that assigns a Confidence score (Cs) to each link, categorized from 'Low' to 'Very High', that evaluates the strength of association supporting the Weight of Evidence for AOPs.[5–6]

To build an AOP for neurodegenerative disorders associated with post-natal exposure to IR, we applied AOP-helpFinder on a list of selected events pre-defined by experts. We identified 6356 publications for IR and gamma ray's impact on KE linked to mitochondrial dysfunction and neurodegenerative disorders, such as Alzheimer's disease and memory impairment, involving studies on species including humans, rodents, and zebrafish. Based on the extracted publications and the calculated Cs assigned to each link (IR-event and event-event), we drafted a new AOP containing a total of 7 KEs, that is complementary to the existing ones from the AOP-Wiki database.

In summary, our proposed AOP provides an organization of multi-biological level information starting from IR and leading to neurodegenerative disorders. The present approach using AI allows to build AOP in an automatic and quick manner by screening the literature to identify relevant dispersed knowledge. It is complementary to other existing toxicological tools, that allows for more robust risk assessment, identification of knowledge gaps and prioritization of strategic future directions based on the needs of the regulators.

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P04-06
The unspoken challenges of the use of polystyrene as food contact material

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Polystyrene is a material with very wide applications, including packaging for food products. It is made by polymerization of styrene, which is currently listed in the EU Reg 10/2011 with a Specific Migration Limit (SML) of 60 mg/kg food. During polymerization, shorter oligomers are formed, especially dimers and trimers, that could potentially migrate to the food as NIAS (Non Intentionally Added Substances). Literature indicates those oligomers as non mutagens, however it does not provide proper characterization of their toxicological profile, that is needed for the risk assessment. The present poster is a critical review of the available data, explains the application and the limits of some tools (i.e., Matrix calculator and FACET) in the risk evaluation, and suggests applicable solutions for a robust evaluation.

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P04-07
Assessment of organophosphate-induced toxicity and evaluation of antidotal efficacy on cellular level

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The widespread use of organophosphorus compounds (OPs), primarily as pesticides but also as nerve agents, continues to pose significant health risks globally, underlined by cholinesterase inhibition, pivotal enzymes in hydrolysis of neurotransmitter acetylcholine. While the neurotoxic effects of OPs are partly documented, their impact on other organs systems remains poorly understood. Motivated by this knowledge gap, we aimed to provide insight into the broad impact of OP exposure. In this study human neural and hepatic cell lines were exposed to OP pesticides and nerve agents, including methamidophos, fenamiphos, and cyclosarin, sarin, across varying concentrations and durations. Our investigation extended to assessing the effects of these compounds on pathways related to oxidative stress, cell homeostasis, and cell death. The findings revealed significant toxicity induced by OPs in both hepatic and neural cell lines, while exposure with nerve agents also induced high level of oxidative stress and led to change of mitochondrial potential. Furthermore, our objective included the evaluation of an antidote designed to provide protection against the detrimental effects of these toxic compounds. We explored the potential of antidote, a bioscavenging complex comprising of butyrylcholinesterase (BChE) and pyridinium oxime, that we have previously shown to be effective in reactivating OP inhibited cholinesterase in *ex vivo* conditions (80% recovery in less than 2 minutes). The bioscavenging complex significantly improved cell viability, ranging from 50% to 100%, after exposure to OPs. Notably, the efficacy varied depending on the timing of antidote administration. Additionally, we evaluated the complex's antioxidant capacity and its ability to protect mitochondrial function. Our results suggest that the combined application of BChE and oxime as a bioscavenging complex may act synergistically to preserve cell homeostasis. These results bring us closer to our aim of achieving comprehensive protection against OP toxicity, by addressing both the immediate and long-term health risks associated with OP exposure.

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P04-08
Insights into the mechanistic pathways underlying the neurotoxic effects of 2C-I and 25I-NBOMe drugs

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The emergence of New Psychoactive Substances (NPS) presents a tough challenge to global public health and legal systems. Among these NPS, phenethylamine derivatives, such as 2C and NBOMe drugs stand out for their psychedelic properties, yet their toxicological profiles remain poorly understood [1,2]. Addressing this gap in knowledge, our study focuses on the synthesis and neurotoxicity evaluation of 2C-I (2-(4-iodo-2,5-dimethoxyphenyl)ethanamine) and its corresponding NBOMe derivative (2-(4-Iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine), with particular emphasis on elucidating the mechanisms underlying drug-induced cytotoxicity.

2C-I and 25I-NBOMe were synthesized, followed by structural characterization using nuclear magnetic resonance and mass spectrometry techniques. For all the *in vitro* experiments, SH-SY5Y cells differentiated into a dopaminergic phenotype were used, which were exposed for 24 hours to the drugs under study.

The drugs (0–800 μM) neurotoxicity was assessed through the neutral red uptake assay, with a concentration-dependent cytotoxic effect being observed for both drugs. 25I-NBOMe was significantly more neurotoxic than 2C-I (EC_{50} values of 121.2 μM and 38.5 μM for 2C-I and 25I-NBOMe, respectively), in accordance with the drugs' lipophilicity data (chromatographic hydrophobicity index assessed by Fast-Gradient RP-HPLC). Significant depolarization of mitochondrial membrane (JC-1 probe assay) along with a significant intracellular ATP depletion (bioluminescence assay) were detected. However, no significant alterations in ROS intracellular levels (DCFH-DA probe assay) were noted for both drugs. Additionally, a significant increase in early apoptotic cells was also observed, being this effect more evident for 2C-I and correlated with a significantly increased caspase-3 activity. Accordingly, the co-incubation with an irreversible pan-caspase inhibitor (Z-VAD-FMK) significantly attenuated the previously observed drug-induced cell death.

Overall, the obtained data corroborate the reported clinical toxic effects of these drugs, with 25I-NBOMe presenting an increased neurotoxicity compared to 2C-I. Our study demonstrates that both NPS induce mitochondrial dysfunction, subsequently leading to apoptotic phenomena

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P04-09
Development of a microphysiological system to quantify the liver fibrosis AOP *in vitro*

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Introduction: Liver fibrosis continues to be a significant health concern, as effective therapies are currently lacking [1]. An Adverse Outcome Pathway (AOP) has been developed to summarize the molecular and cellular processes that lead to liver fibrosis [2]. *In vitro* studies have demonstrated the replicability of the liver fibrosis AOP [3,4]. However, quantifying the cellular responses that lead to either pathological fibrosis or adaptive liver responses remains a major challenge.

Methods: This study presents the development of a novel microphysiological system, the AOP-MPS, designed for the quantitative investigation of liver fibrosis. The design allows for on-demand switching between separate mono-cultures that are interconnected by sequential perfusion. The AOP-MPS was compared to a 3D printed PLA chip that allows continuous cell-cell contact by conditioned medium to compare sequential perfusion to continuous medium sharing. Human cell lines representing hepatocytes (HepaRG), Kupffer cells (THP-1), and hepatic stellate cells (hTERT-HSC) were cultured on both systems to recapitulate the key events of the liver fibrosis AOP. Cellular responses were quantified using electrochemical sensors incorporated in the AOP-MPS for in-line monitoring of glucose, lactate, pH, and levels of reactive oxygen species. Supernatant from the PLA chip was manually transferred onto the sensors. The cell cultures were examined by immunostaining to assess cellular markers, including albumin for hepatocellular function and αSMA for fibrotic stress fibers.

Results: The AOP-MPS and the PLA chip were biocompatible. The cellular characteristics of albumin expression for hepatocytes and low expression of stress fibers in stellate cells were maintained over 7 days in both systems. Treatment with pro-fibrotic stimuli for 7 days resulted in a fibrotic phenotype detected by immunostaining. Perfusion with non-fibrotic hepatotoxins resulted in damage to the HepaRG, without activating the HSCs. The individual cellular responses were quantitatively monitored with electrochemical sensors.

Conclusion: The AOP-MPS is a "plug & play" system that facilitates the investigation of cellular interactions that lead to liver fibrosis. It provides quantitative data that can support *in vitro* to *in vivo* extrapolation, making it a valuable tool for advancing liver fibrosis research, including toxicological investigations and drug discovery.

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P05 | Computational toxicology

P05-01
Ensuring *in silico* models for toxicology are FAIR

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The FAIR (findable, accessible, interoperable and reusable) principles are now well-established for the management, stewardship and sharing of data – with a focus on toxicological information, properties and effects being highly relevant to chemical safety assessment. Recent efforts have applied and adapted the FAIR principles to *in silico* models that may also be utilised for predictive toxicology, with a focus on quantitative structure-activity relationships (QSARs) [1]. In total, 18 FAIR principles for *in silico* models were proposed, with the intention of increasing the use and acceptance of QSARs, in particular. The aim of this investigation was to apply these principles to previously reported machine learning QSARs for toxicological endpoints [2], to extend the FAIR principles to other types of *in silico* models and to understand the requirements to ensure such models are FAIR. Each of the six models reported by Belfield *et al.* [2] was evaluated according to the FAIR principles. Currently, the models would not be deemed FAIR, with a number of areas where further improvement is required. Some of the common areas where more work is needed to comply with the FAIR principles include agreement on model identifiers, definition of community standards for reporting and description, as well as storage platforms. Many of these issues can be addressed by including models in a common database, with the QSAR Database (https://qsardb.org/) being an excellent example. This applies a document object identifier (doi) to QSARs as well as making them accessible in a searchable database. The FAIR principles can also be placed in the context of other *in silico* approaches such as pharmacokinetic (PK) models and quantitative Adverse Outcome Pathways (qAOPs) with similar outcomes. Agreement on how to progress must be sought at the user community, or stakeholder, level. Without global agreement of standards for the FAIRification of models, it is very unlikely to be achieved. This project received funding from the European Union's Horizon 2020 Research and Innovation program under Grant Agreement No. 964537 (RISK-HUNT3R), which is part of the ASPIS cluster. The European Commission is not responsible for any use that may be made of the information it contains.

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P05-02
Optimization of the betamethasone and dexamethasone dosing regimen during pregnancy: a combined placenta perfusion and pregnancy physiologically-based pharmacokinetic modeling approach

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Background: Antenatal betamethasone and dexamethasone are prescribed to women who are at high risk of premature birth to prevent neonatal respiratory distress syndrome (RDS). The current treatment regimens, effective to prevent neonatal RDS, are based on limited data. Recently, concerns have been raised regarding possible adverse long-term neurological outcomes due to high fetal drug exposures. Data suggest that maintaining a minimal fetal response threshold above 1 ng/mL for 48 hours is sufficient to retain efficacy.

Objective: We aimed to re-evaluate the current betamethasone and dexamethasone dosing strategies to assess estimated fetal exposure and provide new dosing proposals that meet the efficacy target but avoid excessive peak exposures.

Study design: A pregnancy physiologically-based pharmacokinetic model was used to predict fetal drug exposures. To allow prediction of the extent of betamethasone and dexamethasone exposure in the fetus, placenta perfusion experiments were conducted to determine placental transfer. Placental transfer rates were integrated in the PBPK model to predict fetal exposure and model performance was verified using published maternal and fetal PK data. The verified pregnancy physiologically-based pharmacokinetic models were then used to simulate alternative dosing regimens to establish a model-informed dose.

Results: Our *ex vivo* data showed that both drugs extensively cross the placenta. For betamethasone 15.7±1.7% and for dexamethasone 14.4±1.5% of the initial maternal perfusate concentration reached the fetal circulations at the end of the 3-hour perfusion period. Pregnancy physiologically-based pharmacokietic models that include these *ex vivo*-derived placental transfer rates, accurately predicted maternal and fetal exposures resulting from current dosing regimens. The dose simulations showed that betamethasone intramuscular dose reduction from 2 dosages 11.4 mg 24 hours apart, to 4 dosages 1.425 mg 12 hours apart would avoid excessive peak exposures and still meet the fetal response threshold. For dexamethasone, the dose may be reduced from four times 6 mg every 12 hours to 8 times 1.5 mg every 6 hours.

Conclusion: A combined placenta perfusion and pregnancy physiologically-based pharmacokinetic modeling approach adequately predicted both maternal and fetal drug exposures of two antenatal corticosteroids. Strikingly, our PBPK simulations suggest that drug doses might be reduced drastically to still meet earlier proposed efficacy targets and minimize peak exposures. We propose the provided model-informed dosing regimens are used to support further discussion on an updated antenatal corticosteroid scheme and design of clinical trials to confirm the effectiveness and safety of lower doses.

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P05-03
Read-across of the genotoxicity of active ingredients and residues in pesticides/pesticidal products

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Crop protection products are used for prevention of crop infestation by disease and pests. The active ingredient and its residues must not cause direct or indirect pernicious impact on human health, particularly relating to genotoxicity. Unadopted European Food Safety Authority (EFSA) guidance proposes a workflow to estimate the genotoxic potential of residues of the active ingredient. This guidance suggests predicting genotoxicity of substances without sufficient available data by read-across. A key aspect to identify analogues for read-across is metabolic similarity, although its definition is very difficult.

Datasets have been compiled consisting of active ingredients and their residues for several pesticide chemical classes and their residues. These datasets consist of structural information and corresponding genotoxicity study results, i.e., Ames, *in vitro/in vivo* chromosomal aberration, and *in vitro/in vivo* micronucleus. Importantly, some chemicals in these datasets have an incomplete set of these data – enabling data-gaps to be filled via read-across. These data have been extracted from the EFSA Draft Assessment Report/Renewal Assessment Reports (DARs/RARs). The information in these DAR/RAR documents was used to define *in-silico*profiling schemes.

Read-across case studies were undertaken. These case studies developed initial groupings based on the defined metabolic transformations within the profiling scheme, followed by subsequent *in silico* definition of the domain of the category in terms of DNA reactivity. In addition, these case studies also demonstrated how this information could be used to increase confidence in the read-across predictions. Implementation of the profiling schemes into the OECD QSAR Toolbox will also be discussed.

This work has demonstrated how a combination of metabolic similarity, covalent chemistry profiling and physical-chemistry properties can be used to predict the genotoxicity of pesticide residues via read-across, including for the *in vivo* micronucleus test. The method presented represents a robust and repeatable approach to such read-across predictions, with the potential to reduce unnecessary testing.

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P05-04
Development of an open-source high throughput QSAR-parameterized PBTK prediction model workflow using httk, OPERA and the KNIME Analytics Platform

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Introduction: Humans are exposed to chemicals through consumption of food and water, cosmetics, agrochemicals, and other consumer products and unfortunately many of these substances do not have sufficient toxicity data. Characterization of these chemicals can be achieved by combining *in vitro* bioactivity data from high throughput screening efforts with physiologically based toxicokinetic (PBTK) modelling to form *in vitro* to *in vivo* extrapolation (IVIVE). Computational workflows for these PBTK models, such as TKPlate, can be used by non-coding expert toxicologists and regulators but are often limited to single compounds. To rapidly screen substances in a high throughput manner, we developed a KNIME workflow that gathers the necessary compound specific physicochemical and ADME parameters for a list of substances which can then be run through PBTK models to obtain *in vivo* dose equivalents for *in vitro* assays.

Methods: We selected an EU list of substances as a proof of concept for this workflow. This list was then used to batch search the US EPA CompTox Chemicals Dashboard to obtain QSAR-ready SMILES and OPERA Model prediction of intrinsic clearance (CLint) and fraction unbound (Fub). All workflows were generated using the KNIME Analytics Platform. The next step in the workflow resulted in the generation of an output file containing the non-standardized SMILES structures, the QSAR ready SMILES, the OPERA prediction for human Plasma Fup, the OPERA prediction for the Octanol-water partition coefficients (LogP), the OPERA prediction for the Henry's Law constant, the OPERA prediction for human hepatic intrinsic clearance, and the OPERA prediction for pKa acid and pKa basic for each compound in the list. This output file was used as the input to run the oral, inhalation, and buccal PBTK models in both rat and human using the httk R package.

Results: Running the KNIME workflow on the roughly 2,500 EU substances resulted in the generation of predicted Cmax for each compartment specified in parameters, the plasma AUC, and the half-life of the substances for each model. Comparisons of predicted plasma Cmax for the three different models for both species provides evidence for the animal to human extrapolation, which could be used in risk assessment, such as estimating derived human NOAEL from rat POD data. These results also provide insight into which compounds fall outside of the applicability domain for the different models.

Conclusions: The use of this PBTK workflow allows for the standardization of a method to predict *in vivo* dose information to assess the potential risk of large sets of chemicals with limited data in a reproducible and user-friendly way for non-coding expert toxicologists.

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P05-05
Use of model ensembles to fill labelling gaps for compounds used in textile and leather industry

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Achieving a green and sustainable industry involves enhancing the chemical safety of products and processes, necessitating the identification and classification of hazardous compounds in a cost-effective manner prior to market entry. INDITEX, one of the worldwide leading textile and leather industry (TLI) companies, is aligning with EU mandates to enhance product safety and sustainability. The large number of products used in the TLI industry makes the exhaustive use of experimental methods impractical, leading to the adoption of in silico approaches. However, the heterogeneous nature of TLI compounds presents challenges for traditional in silico methods like QSAR. Previous efforts to predict compound labels such as CMR (Carcinogenicity, Mutagenicity and Reprotoxicity), PBT (Persistent, Bioaccumulative and Toxic), vPvB (very Persistent, very Bioaccumulative) and ED (Endocrine Disruption) were somewhat successful but left many predictions uncertain. To address these limitations, we propose a novel approach

utilizing separate models for individual label components combined with logical rules. This ensemble approach aims to improve the predictive quality of the models and their interpretability. In the present communication, we compare the performance of direct QSAR models with ensemble QSAR models, demonstrating the effectiveness of the ensemble approach in filling knowledge gaps and expanding the applicability domain. The pros and cons of both approaches are presented and discussed in detail using case-study models on real datasets. This research, funded by INDITEX's sustainability department, can contribute to advancing chemical safety in the TLI sector, with potential implications for broader industrial sustainability efforts.

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P05-06

Conazoles' health risk prediction: integrating knowledge graphs and machine learning predictive models

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The impact of conazole fungicides on human health has become a subject of concern due to their widespread use in agriculture, industry, and medicine. The possibility to integrate large and diverse publicly available datasets using knowledge graphs provides an unprecedented chance to develop predictive *in silico* methodologies to support Next-Generation Risk Assessment (NGRA). In this context, we present our strategy to predict the toxicity of a selection of conazoles, classify them based on health risk, and characterize the predictions using enrichment analysis and disease ontologies.

Our strategy is based on toxicity predictions obtained using the Chemical Effect Predictor (CEP). CEP is a machine learning classifier that predicts human diseases and phenotypes associated with the effect of a chemical or drug. CEP is based on a knowledge graph containing drugs, proteins, diseases, and biological functions (56,250 nodes, ~1,5M edges). The machine learning classifier is trained on several features obtained from applying network propagation algorithms. CEP achieved an ROC of 0.70 in a benchmark of drug-adverse effect associations.

Eight conazole compounds (Epoxiconazole, Fenbuconazole, Propiconazole, Prothioconazole, Cyproconazole, Tebuconazole, Itraconazole and Ketoconazole) and the metabolite 1,2,4-Triazole were analyzed using CEP. A Toxicity Score (TS) was defined to classify compounds as low (LT) or high toxicity (HT) based on the number of predictions and their probability values. Once the compounds were classified based on the TS, a hazard characterization was performed using a new approach, the Disease Set Enrichment Analysis (DSEA). The DSEA allowed the identification of disease ontology classes from the ADReCS ontology significantly enriched in the CEP predictions.

CEP predictions allow classifying epoxiconazole and metabolite 1,2,4-triazole as LT and the remaining seven as HT. We found that HT conazoles might affect a wide range of disease classes and systems (17 ADReCS classes), being specially enriched for Neoplams, Endocrine, and Reproductive System Disorders. To a lesser extent, Hepatobiliary, Metabolic, Nervous, and Psychiatric disorders showed significant enrichment. These results are in agreement with reports from animal studies (hepatobiliary, reproductive and endocrine disorders), except for nervous system and psychiatric disorders.

Overall, our approach has proved to be efficient for assessing and classifying the risk to human health of conazoles. The predictions recapitulate results from *in vivo* animal studies and pinpoint new potential hazards for human health that deserve further study. By leveraging these predictions, we can prospectively explore specific diseases in greater depth and utilize the knowledge graph to identify key genes and pathways, unraveling underlying mechanistic insights into the effects of conazoles on human health.

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P05-07

Physicochemical properties and transcriptomic pathways analysis of TiO_2 nanoparticles exposure through computer-aided approaches to provide a new öook at their pulmonary safety

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Inhalation exposure to TiO_2 NPs primarily occurs in occupational settings during the production of TiO_2 NPs and the manufacturing of products containing them. Nanotoxicology research indicates that TiO_2 NPs have an enhanced ability to interact with biological membranes, penetrate deep lung tissue regions, and interact with signaling proteins. The topic of acute pulmonary responses in mice exposed to TiO_2 NPs has been extensively explored. This includes discussions on the influence of nanosized titanium oxide properties on toxicity. However, there is limited evidence regarding the relative importance of these properties, and most studies focus on phenotypic responses following TiO_2 NP exposure. Thus, we aim to investigate whether the toxicity of TiO_2 NPs, measured at the molecular level through transcriptomic-based responses, is influenced by their properties. In this study, we consider the inhalation route as the primary exposure route to TiO_2 NPs. Our research endeavors to integrate computer-aided approaches with transcriptomic responses to elucidate the molecular events initiating lung toxicity in mice, quantitatively relating it to the structural features of nanomaterials that induce it.

To achieve this, we analyzed genome-wide transcriptomic profiles of mouse lung tissue exposed to seven different TiO_2 NPs, alongside benchmark dose (BMD) responses, using a chemometric approach. Principal component analysis and hierarchical cluster analysis were conducted to explore potential relationships between BMD and the physicochemical parameters of the TiO_2 NPs under study.

In results, this study has shed light on the pulmonary toxicity of TiO_2 NPs examined at the transcriptomic level and its relationship with their physicochemical attributes. Our results emphasize that properties such as zeta potential, aspect ratio, BET surface area, ROS production, crystalline structure, and surface modification significantly influence TiO_2 NP toxicity at the transcriptomic level, while nanoparticle size demonstrates limited impact. Moreover, distinct nanoforms of TiO_2 NPs, characterized by varying properties, can elicit diverse responses observed at the molecular level, indicating differing modes of transcriptomic pathway perturbation. Our analysis also suggests the existence of additional properties beyond those investigated here that play crucial roles in shaping the pulmonary pathologies triggered by TiO_2 NP exposure. Consequently, this analysis provides a basis for further research efforts aimed at unraveling the intricate interplay between TiO_2 NP properties and pulmonary toxicity mechanisms. Future investigations should encompass a broader array of nanoparticle characteristics and leverage quantum chemistry to provide a more comprehensive characterization of the materials under scrutiny, thus enhancing our understanding of nanoparticle-induced pulmonary pathologies.

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P05-08
Bridging uncertainty analysis with NGRA workflows through computational tools

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The transition from traditional *in vivo* safety assessments to the Next Generation Risk Assessment (NGRA) requires the application of an extensive spectrum of New Approach Methodologies (NAMs) and the successful integration of their results. To support this paradigm shift, the ASPIS cluster developed the Alternative Safety Profiling Algorithm (ASPA) workflow, which orders the required analytical tasks and expert decisions in a logical sequence that constitutes the NGRA backbone.

To facilitate its practical application, we encoded the ASPA workflow in NAMASTOX; an open-source software that can be installed in most operative systems and environments. The dashboard-like structure of NAMASTOX graphically interconnects key resources of the ASPIS projects, including the NAM- and the compound databases, with the corresponding workflow steps. Additionally, NAMASTOX supports the generation of informative risk assessment reports, which comprise the documented results and expert decisions.

However, as the ASPA matures, so does the awareness of the critical importance of considering uncertainty at different workflow steps. In this context, the RISK-HUNT3R Uncertainty Working Group is currently developing a framework for characterising, quantifying, and combining uncertainty for various types of NAM results. In this communication, we want to present the implementation of a support software for this framework, aiming to provide interactive uncertainty management for NAMASTOX. This new feature set supports users with i) the collection of uncertainty characterisation results at each single workflow step, ii) the review of the collected uncertainty characterisation results from relevant upstream nodes at the decision nodes, and iii) the execution of uncertainty quantification and combination methods, once provided and validated.

More than a closed system, the proposed infrastructure is designed to be a placeholder for more uncertainty analysis methods. At baseline, the system presents all relevant uncertainty characterisation results collected at a certain point in tabular format to facilitate a classical "in the brain" expert assessment. Elicited expert knowledge or judgement is actively collected and stored for the final report, facilitating the documentation of the decision, and thus increasing its reproducibility. The flexibility of the interface allows for seamless integration of novel uncertainty analysis methods and sophisticated probabilistic approaches developed for specific steps of the ASPA workflow.

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P05-09
NAMASTOX: a computational tool supporting the use of NGRA workflows

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The application of NGRA can be complex, involve many steps, and generate many pieces of evidence that are difficult to integrate. Adopting this methodology would be largely facilitated by using a stable, well-designed workflow, guiding the user on the ordered and systematic application of these steps. An example of such workflow is the ASPA workflow currently being developed by the ASPIS cluster.

In this work, we present NAMASTOX, a computational tool designed to support the practical application of NGRA workflows, which was developed by one of the ASPIS cluster projects (RISK-HUNT3R) specifically as an ASPA workflow complement. In the poster, we will present the tool and illustrate how it can be applied, showing some steps of its application in a case study.

NAMASTOX offers a simple graphical interface which will guide the user in the step-by-step application of the workflow, indicating "what to do next", suggesting the most appropriate NAMs (*in vitro*, in silico) and collecting the NAM results in a systematic and ordered way. Some in silico tools can be run directly from the interface, while others can be accessed using links. The tool keeps track of the uncertainties identified in the process and presents this information at decision nodes, facilitating uncertainty-aware decision-making. The whole process can be easily documented by generating reports in standard formats, facilitating the transparency of the process and its adoption by regulatory bodies. NAMASTOX can be installed as a freely accessible tool on the Internet, in a private server, or locally on a desktop computer, allowing its application to sensitive data. Risk assessments conducted with NAMASTOX can be easily exported and shared.

NAMASTOX is an open-source software that can be installed in most operative systems and environments, from an AWS cloud environment to a Windows laptop. A demo instance is accessible at http://namastox.upf.edu

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P05-10
Screening for Developmental and Reproductive Toxicity (DART) using a suite of *in silico* models contextualised on an Adverse Outcome Pathway (AOP) network

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Developmental And Reproductive Toxicity (DART) is a key area of regulatory significance, for which failing to identify toxic liability can have severe consequences. As a result, testing is necessarily intensive and time consuming and usually focussed toward the end of the product development process. However, this approach can be extremely costly if problems are identified in late-stage development. Therefore, early indication of potential DART liability will enable safety assessors to steer development towards successful outcomes.

The drive to develop new approach methods (NAMs) is not only moving testing towards the 3Rs but is also generating vast amounts of mechanistic evidence through the use of *in vitro* and *in silico* models. To take advantage of these approaches fully for DART assessments, knowledge of the pathways and targets associated with DART is required, along with the methods to test them – enabling appropriate assay selection and utilisation of model outputs in order to drive further hypothesis-based testing.

With this in mind, an AOP network of pathways relating to DART was used to select appropriate targets which can be associated with adversity. Existing structural alerts were associated with these targets and data gathered from public sources relating to the targets of concern used to build new statistical (Q)SAR models. Multiple modelling techniques were investigated and the approach optimised. Performant models were associated with the network and gaps due to insufficient data identified. The model-annotated network was then used to screen a test set of known DART toxicants, using combinations of the individual (Q)SAR models and taking advantage of the AOP network to indicate coverage of the models for predicting the adverse outcomes. Where gaps in model coverage were identified alternative approaches to predict these targets were explored.

The approach showed high sensitivity (average combined models showing over 75% sensitivity), implying good coverage of MIEs relating

to known DART toxicants but limited specificity, indicating that activation of an MIE doesn't always lead to DART. A result which might be expected when combining multiple models and where there are numerous biological events which must be perturbed between MIE activation and an AO.

This approach demonstrates that AOP networks facilitate the appropriate application of relevant (Q)SAR models, with the predictions providing hypotheses that can be confirmed through mechanistic testing, including testing of downstream events as well as appropriate combination with evidence from other sources. Using (Q)SAR models in this manner through integration into *in silico* workflows will empower safety assessors to rapidly and confidently assess the DART liability of chemicals.

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P05-11
Correlation analysis of pathological findings of intrathecally administered ASOs

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It is well known that antisense oligonucleotides (ASOs) can trigger immunomodulatory responses leading to the presence of inflammatory infiltrates which have widely been observed in preclinical species following exposure to these compounds. This study purpose was to evaluate potential correlation between inflammatory responses with other histopathological findings as well as exposure in specific brain regions.

For this analysis, we considered data from nine single and repeat dose toxicity studies in non-human primates (NHPs) following exposure to four different ASOs. In these studies, inflammatory infiltrates were widely observed across the brain in some treated NHPs and to a minor extent in untreated NHPs. The area-under-the-curve (AUC) of concentrations in different brain regions were calculated and correlated to the pathological findings noted in these regions. The log-transformed AUC and the grading of severity level were fitted separately for each type of finding. This is because we assume that the severity levels are not comparable across different categories of pathological findings, but could be compared within the range of the same finding across different studies. Correlations between pathological findings across studies were derived for specific brain region sections, i.e. 1) cortex, 2) thalamus/midbrain and 3) cerebellum sections.

We focused on two major findings in the brain, i.e. inflammatory infiltrates and neuronal cytoplasmic vacuolation, as well as the pattern of overlapping. The combination of the nine studies showed that 31.9% (44 out of 138) animals were detected with cellular infiltration and neuronal cytoplasmic vacuolation simultaneously.

There was hardly any cellular infiltration or neuronal vacuolation in the cerebral or cerebellar cortex/grey matter regions. Seventy-one percent (71%) of the neuronal vacuolation was detected in the hippocampus only (66% of studies) and cerebral cortex (33% studies) in the brain, while 57%, 27.6% and 15.4% of the cellular infiltrates were detected in the meninges, neuropil and choroid plexus, respectively. When correlating the severity level of each type of finding with the tissue AUC, we detected a positive significant effect in the hippocampus when considering the neuronal cytoplasmic vacuolation, with the p-value 0.001. No correlation was seen between inflammatory infiltrates and exposure. These findings indicate that inflammatory infiltrates and neuronal vacuolation develop via different mechanisms of action.

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P05-12
New methods for the early characterisation of drug-induced liver toxicity

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Drug-induced liver injury (DILI) is a complex multi-mechanistic event posing a major challenge in current drug discovery and development as it often leads to clinical trial failures and drug withdrawals. Early preclinical risk assessment for DILI is based on a set of *in vitro* experiments such as bile salt export pump inhibition, glutathione trapping and mitochondrial toxicity [1]. In silico models provide an alternative method to address possible DILI liabilities in early drug discovery. Here, we provide an overview of the *in vitro*/in silico framework we deploy for early estimation of DILI in new drug candidates. We introduce a tier 0 model, a classification method whose features are molecular descriptors and fingerprints calculated from chemical structures. The tier 0 model is built on a dataset of 619 late stage/marketed drugs with FDA annotations (Most-, Less-, No-DILI Concern) [2]. In external validation, the tier 0 model surpasses previously published methods with an accuracy of 76% compared to the state-of-the-art 70% [3]. Domain of applicability analysis and uncertainty in prediction are used to inspect the confidence in predicting different chemical spaces. The tier 0 model can be used as a filter for removing potentially DILI positive compounds at an early stage of drug development projects. Furthermore, we have developed a tier 2 model, which improves on a previously published model built using experimental properties measured from *in vitro* DILI assays as features [4]. Our tier 2 model uses a custom set of DILI experiments which are associated with the *in vivo* DILI risk, a combination of internal and public data to increase the model generalisability and a refined version of the algorithm to augment the model predictive power. The tier 2 model can be used at later development stages, when more DILI-relevant data becomes available, to flag the DILI risk of compounds and identify the possible mechanisms that cause DILI. As a result, the tier 2 model can better inform drug designers about properties that can be further optimised to remove the DILI flag and achieve safer compounds. By developing models that use more DILI-relevant features, we hope to reduce DILI attrition rate and hence improve drug safety.

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P05-13
Refining the high potency category rules to enhance the accuracy of exposure-based waiving arguments within a Next Generation Risk Assessment for skin sensitisation

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In recent years, significant steps have been taken towards shifting from conventional chemical risk assessment, reliant on animal testing, to a more progressive approach known as Next Generation Risk Assessment (NGRA), which emphasises exposure-led analysis, hypothesis-driven methodologies, and the replacement of animal testing. [1,2] In a quantitative NGRA for skin sensitisation, one tool which can help is the Dermal Sensitisation Threshold (DST). DSTs represent thresholds of toxicological concern that can facilitate exposure-based waiving arguments if expected consumer exposure levels are sufficiently low. There are three DSTs: non-reactive, reactive and High Potency Category (HPC), and structure-based rules are used to allocate the appropriate DST to a chemical. [3,4] This allocation can be carried out either manually by a human expert or using an *in silico* tool (such as Derek Nexus), leveraging the sensitisation reactivity domains. [5] Quantitative mechanistic models (QMM) have been developed across various reaction domains using a mechanistic understanding of skin sensitisation, by relating sensitisation potency to reactivity parameters and where appropriate lipophilicity. [6] This study sought to update the HPC rules using both patterns and a QMM to reduce false positive (FP) predictions, thus increasing the accuracy and usefulness of the exposure-based waiving approach.

An expanded DST dataset comprising 1150 chemicals was collected and curated from publicly available LLNA data. 166 chemicals in the expanded dataset were classified as HPC and 984 chemicals were classified as non-HPC by a human expert, based on published structure-based rules. Upon analysis within Derek Nexus v.6.3.0, the highest number of false positives (23) were observed for Class 2a Michael acceptors. The scope of the HPC rules for this class was amended by removing quinone (imine) tautomers, as more than 3/4 of chemicals in this category are reported to be weak/moderate sensitisers or non-sensitisers; this significantly reduced the FP predictions from 23 to 3.

An S_NAr QMM has previously been developed, and this was used to update the scope of the S_NAr HPC alert based on the estimated potency.[6] Following the refinement, the 8 experimental HPC S_NAr electrophiles (EC3≤0.29%) were identified correctly and the number of FP predictions (experimental non-HPC) reduced from 15 to 6.

This study highlights the synergy that can be achieved from human expertise and *in silico* approaches such as structural alerts and QMM, emphasising the significance of updating HPC rules, which can lead to increased confidence in the exposure-based waiving arguments. Combining QMM with structure-based rules to identify high potency category chemicals can provide a robust assessment of the risk of skin sensitisation for chemicals, which is especially significant in the context of NGRA framework for evaluating skin sensitisation of consumer products, including cosmetic and personal care ingredients.

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P05-14
Comprehensive *in vitro/in silico* approach for screening PFAS in terms of their potency of interfering with the thyroid system (TTR- TRβ CALUX assay)

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Recent research indicated that per- and polyfluoroalkyl compounds (PFAS) may have endocrine disrupting properties (EDs) by interfering with hormone action. [1,2] In our studies, we developed a comprehensive *in vitro/in silico* approach for screening a large dataset (~12,000) of PFAS[3] in terms of their *in vitro* toxicity potency to disrupt the thyroid hormone transport (endpoint: RPF – Relative potency factor with respect to PFOA). For this purpose, in PHASE I: a set of 47 PFAS has been tested using the TTR-TRβ-CALUX bioassay. Based on this set of data we developed a classification model, distinguishing active and inactive compounds. The model used 2D descriptors – they are easy to calculate and reproduce (i.e., they do not require the optimization of chemical structures). The model fulfills all good practices for QSAR model validation, and it could be used for predicting if PFAS can, or cannot, disrupt the plasma transport of the thyroid hormone (T4), by interfering with the binding of T4 to its plasma transport protein TTR. Next, compounds that were active during the experimental studies were used to develop regression models to estimate the potency of interference with the T4-TTR binding. Here, two separate approaches were investigated (i.e. multiple linear regression MLR, and the approach aimed at identifying multiple valid QSAR models obtained as a function of different data splitting). The developed models are in line with the OECD recommendations for QSAR model validation and predict with precision conforming to current standards.

After the development of the QSAR models, our main goal was to carry out a comprehensive virtual screening of 12,000 PFAS from recently published studies [3] to further analyze their potency to disrupt the thyroid hormone transport and determine the relationship between the structure and activity, based on obtained predictions. Therefore, in the PHASE II, all three developed modeling approaches (i.e., classifier and MLR) have been applied to a large dataset of PFAS. As a result, we have identified more than 7,500 compounds showing activity in the TTR-TRβ-CALUX assay. The relative potency factors (RPFs) for active compounds were also established based on the regression models. It turned out that more than 100 PFAS-like compounds (having RPFs values above 1) may even pose more negative effects on humans than the reference compound PFOA (RPF=1) and particularly need to be further investigated. Our results confirm that the thyroid pathways investigated in this study should be considered when studying the toxicity mechanisms of PFAS.

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P05-15
Large language models in drug development: deciphering discontinuationc causes to inform future strategies

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Annually, numerous drug candidates are terminated during development due to inadequate safety or efficacy profiles. The pharmaceutical industry acknowledges the critical importance of making use of the knowledge kept with historical preclinical and clinical data to refine drug development, accelerating the delivery of cost-effective and efficacious drugs to patients. However, the wealth of data on mechanisms of action, potential toxicities, and expert risk assessments are often hidden within various lengthy and siloed documents. This dispersal of relevant information creates a significant challenge for the reverse translation of knowledge in the drug development pipeline.

We evaluated the utility of Large Language Models (LLMs), and particularly GPT4-Turbo, in identifying specific reasons for the discontinuation of drug candidates by extracting and analyzing data from internal documents, presentations, reports, and meeting minutes. We utilized Roche's internal clinical and pre-clinical databases as the primary data source for the LLM. Our primary objective was to focus on toxicity and more specifically to elucidate the scientific hypotheses leading to discontinuation. Additionally, we aimed to identify the main organs affected by toxicity and to detect evidence of clinical toxicity within preclinical data and to answer questions regarding possibilities for refinement and reduction of experiments.

We conducted a case study on 50 molecules that were discontinued between Phase 0 and Phase 2 of drug development (1997–2023). Initially, the scientific reasons for discontinuation of the drug candidates, on a subgroup of molecules, were collected by interviewing the responsible toxicology project leaders. The collected ground truth was then used to evaluate the performance of the LLM-generated output. Our findings indicate that GPT-4-Turbo can answer the questions with an accuracy ranging from 76 to 89% depending on the retrieval pipeline parameters. The gathered knowledge could be immediately applied to support internal questions regarding frequency of gender-specific toxicity and also in general internal knowledge building .

This work demonstrates the potential of LLMs to significantly contribute to the reverse translation process, enabling the reuse of historical data to inform and improve future drug development strategies and rapid data driven decision making.

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P05-16
Prioritising plant protection products for toxicity testing by *in silico* screening

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With millions of synthetic chemicals and natural toxicants surrounding us, addressing mixture toxicity is inevitable. To overcome the sheer complexity of the task there is an urgent need for good prioritisation strategies. Qualitative or quantitative structure-activity relationship (QSAR) tools provide the advantage of minimal costs and high-throughput. They enable the rapid grouping of substances potentially sharing similar modes of action or target organs as well as the identification of possible metabolic or active transporter interactions. As intentional mixtures, plant protection products (PPPs) provide an excellent case study to test *in silico*-based prioritisation strategies. Using QSARs, we aim to screen co-formulants and active substances in PPPs to prioritise them for further testing focusing on potentially unknown toxicants and kinetic interactions.

We investigated the endpoints genotoxicity, hepatotoxicity, and nephrotoxicity using Leadscope Model Applier, Derek Nexus, and Sarah Nexus. Scores were attributed based on the concordance of statistical and expert model predictions. Additionally, we predicted active transporter (P-glycoprotein) and metabolic enzyme (CYPs) interaction with the ADMET Predictor and ADMETlab. Where available, SMILES codes for all co-formulants in approximately 750 PPPs authorised in Germany were retrieved and included. Moreover, pesticides grouped into the cumulative assessment groups (CAGs) for liver and kidneys were added.

Of 1048 co-formulants, 488 could be attributed to distinct SMILES codes. Others were, for example, extracts, polymers, or minerals. Of the 488 co-formulants, 69 were prioritised based on genotoxicity, 124 based on nephrotoxicity, and 143 based on hepatotoxicity. Altogether 325 PPPs contained prioritised co-formulants above 0.1% or 10%, depending on the substances' priority scores. In addition, 244 PPPs were flagged based on potential metabolic enzyme and active transporter interactions between co-formulants and active substances. Limitations of this process include the limited and sometimes unclear applicability domain of QSAR models, the exclusion of non-distinctive substances, and the individual models' predictive capacities.

In conclusion, few PPPs were prioritised based on genotoxicity and nephrotoxicity. This can be attributed to adequate regulation of known toxicants but also to the limited power of extrapolation beyond the training data set of available QSARs. In a next step, prioritised PPPs will be investigated *in vitro* and *in vivo* threshold values for PPPs will be extrapolated using physiology-based kinetic (PBK) modelling.

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P05-17

Predicting nitrosation of individual Amines in drug molecules using statistical (Q)SAR Models

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Since NDMA was discovered in pharmaceuticals such as Valsartan in 2018, there has been a need for tools that can predict which APIs are likely to produce nitrosamines and to determine what risk these individual nitrosamines (NAs) pose to human health. In 2023, the CPCA was established to satisfy the latter need. The CPCA groups NAs into one of five categories, each with its own assigned acceptable intake, using a straightforward workflow based on the molecule's structure. While this tool accomplishes risk assessment for known NAs, there still remains a gap in predicting the formation of nitrosamines, which can occur during the synthesis, storage, or even digestion of pharmaceuticals. The most straightforward way to detect these impurities is in the lab, either by testing for NAs as part of manufacturing or following the WHO's Nitrosation Assay Procedure (NAP) test. The NAP test specifies conditions such as pH range, temperature, drug and nitrite concentrations, and reaction duration, all of which are chosen to favor nitrosation. However, benchtop testing cannot match the efficiency of high-throughput methods like an *in-silico* workflow. Ideally, (Q)SAR tools would be able to predict if a particular amine is likely to be nitrosated, but since statistical models rely almost entirely on available experimental data to build their decision algorithms, a lack of consistent data makes building a reliable model difficult. The limited published results can vary widely in experimental conditions and NA detection methods, making it challenging to assign quantitative NA yields in the model training set. One way in which we minimized these discrepancies was by limiting our initial training set to literature that adhered to the NAP test. Where there were still multiple differing yields for a single amine, the higher yield was chosen. To train classifier models, the experiments' NA yields were simplified to binary results of whether NA had been formed at yields of $>0\%$, $\geq 1\%$, or $\geq 10\%$. In the cases where multiple amine groups were present in one molecule, individual yields were assigned to each amine. This included cases where there were multiple products formed or when a single product had been nitrosated at more than one site. We trained multiple machine learning classifiers using structural descriptors favoring nitrosation, such as methylamines and nucleophilic hydrazines. As deprotonation is a critical step in the nitrosation of secondary amines, pK_a was calculated using MolGpKa when possible. Since pK_a notably improved the accuracy of predictions, pK_b was also computed as a stand-in descriptor for tertiary amines. It was found that the Nearest Neighbors classifier was the best model when $pK_{a/b}$ was not available and that the tree-based classifiers (Decision Tree and Random Forest) were the most reliant on $pK_{a/b}$. Overall, the tree-based and SVM classifiers were the most consistent, with cross-validated accuracies over 0.7 when $pK_{a/b}$ was included.

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P05-18

Chemical classification and predictive models for the assessment of extractables and leachables

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The toxicological evaluation of extractables and leachables (E&L) includes an assessment of mutagenicity and sensitization. Understanding the distribution of potent sensitizers and mutagenic compounds is a foundational assessment to setting qualification limits for E&L compounds. In this regard, computational approaches used to assess mutagenicity and skin sensitization play an important role. In this study, ~783 unique E&L structures from a combined Extractables and Leachable Safety Information Exchange (ELSIE) and Product Quality Research Institute (PQRI) dataset were used to determine the prevalence of mutagenic and potent skin sensitizers among E&L chemicals. 9% (69 out of 783) of chemicals were flagged as potential mutagens with prevalent alerts including polycyclic aromatic hydrocarbons, unhindered epoxide, and primary aromatic amine. Moreover, results from this study corroborated previous findings that strong/extreme sensitizers are not routinely observed in leachables, as the prevalence of strong/extreme sensitizers in the E&L dataset is low (~3%). However, the identification of potent sensitizers remains an important aspect of assessing the risk posed by E&L compounds. Recent knowledge derived from proprietary databases mapping skin sensitization data to chemical structures have been used to improve the performance of computational models predicting skin sensitization potency. Such *in silico* model enhancements reduce the number of out of domain predictions and increase the prediction of potent sensitizers; for example, in an analysis of 925 structures not in the model's training set, an updated model predicted 83% of sensitizers based on an EC3% (concentration inducing a 3-fold increase in lymph node cell proliferation) threshold of 1%.

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P05-19

Interpretable graph neural networks trained on OECD test guideline data for screening inhalation toxicity of environmental chemicals

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Machine learning has become increasingly prevalent in various domains, including drug development and toxicity assessment. Additionally, it holds promise as an alternative approach for the regulatory screening chemical substances. In this study, we focus on inhalation toxicity and collected datasets potentially relevant to inhalation exposure from regulatory lists. There is a shortage of inhalation toxicity data adhering to standardized methodologies, specifically OECD Test Guidelines (TG) 403 (Acute inhalation), 412 (Sub-acute inhalation), and 413 (Sub-chronic inhalation). To bridge these data gaps, we trained a classification model using interpretable Graph Neural Networks (GNNs) and identified core subgraphs of chemicals that may implicated in inhalation toxicity. The OECD TG 403, 412, and 413 data were initially collected, preprocessed, and used for model training. Employing the model with the highest F1 score, we screened 1185 chemicals lacking inhalation toxicity information and explored their presence in consumer products. Our study highlights the potential of employing machine learning models to address data gaps and prioritize chemicals to further evaluate their potential risks.

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P05-20

A new large-scale benchmark knowledge graph leveraging the comparative toxicogenomics database

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With increasing societal concerns about environmental and health issues, research and understanding of the impact of chemicals on the human body are becoming increasingly important. Knowledge graphs are gaining attention as an important tool, particularly in modeling biological systems, as they provide a way to model complex interactions among entities. Numerous studies have been conducted on toxicity prediction through machine learning, but comprehensive research is still lacking. To facilitate extensive research, large-scale knowledge graphs are necessary. While several biological knowledge graphs have been proposed, existing ones have primarily been developed for the purpose of screening new drugs or chemical compounds for treating diseases. These knowledge graphs are not suitable for screening chemicals that induce diseases, and there is a lack of knowledge graphs for toxicity prediction. In this study, we propose a new knowledge graph CTDKG, utilizing the CTD database to screen chemicals related to disease occurrence. CTDKG incorporates diverse relational information between chemicals, genes, diseases, and pathways. Furthermore, CTDKG contains a large-scale dataset, which will be beneficial for comprehensively understanding interactions between chemicals and diseases.

We compiled the knowledge graph by extracting relational information from the CTD database. To ensure consistency in analysis, we preprocess the relational information and provide negative samples for consistent evaluation. We conducted various benchmark experiments on the proposed knowledge graph. These will serve as baselines for developing models for future toxicity prediction. CTDKG is expected to contribute to a better understanding of the impact of chemical compounds on human health and to the development of models for future toxicity prediction.

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P05-21
Machine learning for rodent liver toxicity prediction: leveraging drug substructure properties

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Purpose: Assessing liver toxicity in rodents (rats, mice) is crucial in various drug development processes and is important for safety assessment, regulatory compliance, cost and time savings for pharmaceutical companies, necessitating robust predictive models. This study showcases the efficacy of machine learning in predicting rodent liver toxicity, offering a potential solution for compound-level toxicity assessments and informed drug development strategies.

Method: The study utilized toxicity data from Tg-Gates and Livertox categories A&B drugs, while non-toxic data comprised Livertox category E drugs. Training, validation, and testing datasets were created, and data preprocessing was performed. Features were extracted using structural characteristics, chemical properties, and molecular patterns of drugs. Additionally, advanced computational techniques were employed to derive more than 2000 diverse sets of features. Following this, feature reduction was carried out using statistical techniques. Logistic regression, alongside other machine learning models like XGBoost and ANN were employed and evaluated using 5-fold cross-validation for different sets of hyperparameters. ROC-AUC and confusion matrix was used as the evaluation metrics. Threshold selection for confusion matrix was based on maximum F1-score on validation dataset.

Results: Logistic regression emerged as the most stable and interpretable model among various algorithms tested. The final logistic regression model comprised of nine informative features, using which an ROC-AUC of 72% on internal test data (130 compounds) and 71% on external data (27 compounds) was achieved. The model attained an F1 score of 67% on the testing data and 77% on an external dataset obtained from a global pharmaceutical company.

Conclusion: This work showcases the efficacy of machine learning in accurately predicting drug-induced liver toxicity at an early stage. The model is embodied in AIRATox, a unified platform for fast and efficient compound evaluation. The adaptability of the methodology suggests its potential application to predict a wider range of toxicities and adverse effects at the compound level.

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P05-22
Link prediction of the knowledge graph in the CTD database

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The CTD is a database aimed at enhancing understanding of how environmental exposure impacts human health.

This database provides curated information obtained by researchers manually reviewing literature on the interrelationships among chemicals, genes, diseases, and more [1]. Leveraging associations between selected chemical-disease (C-D), chemical-gene (C-G), and gene-disease (G-D) pairs, it offers new insights into potential causal relationships through interaction information [2]. Our research proposes a methodology for predicting hidden relationships within a knowledge graph composed of CTD data [3]. By applying state-of-the-art methodologies utilized in artificial intelligence, we embed heterogeneous components of the knowledge graph, and train a model learning the relationships among their phenotypes. Our research findings confirm the utility of screening latent relationships within the knowledge graph and discovering them as valuable tools.

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P05-23
Learning by doing: modelling and simulation for risk assessment in the open-source Open Systems Pharmacology Suite (PK-Sim® and MoBi®)

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Training the next generation of modelers in physiologically based kinetic (PBK) and quantitative systems toxicology (QST) remains a press-

ing need in addressing the high demand for skilled professionals. Herein, the training platform developed at ESQlabs is introduced as a self-paced online education program. It aims to equip learners with hands-on competencies to navigate the complexities of PBK and QST using the open-source Open Systems Pharmacology (OSP) Suite.

A high-quality curated 20+ real-world applications of PBK and QST were developed into online courses by instructors with proven credentials and extensive industry experience. The curriculum was designed with examples translated into PK-Sim® and MoBi®. The training materials were aimed at various levels, beginners and advanced learners, to help them gain practical skills and develop critical thinking. The platform is accessible on desktop laptops and mobile phones.

Over 400 learners have chosen to study PBK and QST modeling and simulation in OSP Suite using the training platform developed at esqLABS. In addition, 112 active learners have completed at least one course and are better skilled, and over 800 certificates were issued. Courses include developing a simple PBK model, model qualifications and reports, PBK models for special populations, dermal absorption modeling, and scaling models across species rat, dog, and monkey, to name a few.

As the applications of mechanistic models expand across industries, fostering expertise ensures aspiring modelers stay at the forefront of research and innovation. The ESQlabs' training platform serves as a companion for toxicologists who wish to build and advance their careers in the field of PBK and QST and assist them in effectively developing such models for chemical safety and risk assessment. The learners gain confidence, are able to build a portfolio and earn certificates to share with prospective employers.

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P05-24
Development of a generic pregnancy PBPK model for mouse: application to neurotoxic molecules

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An increasing number of studies are raising concerns about the potential effects of environmental pollutants on neurodevelopment through maternal exposure. Mice are commonly used in neurodevelopmental studies, but the observed effects are rarely linked to internal concentrations of substances in the fetal brain. It is therefore relevant to account for pre-gestational and maternal chemical exposures to better characterize the fetal exposure, particularly during the critical window of fetal brain development. This can be done using Physiologically Based Pharmacokinetic (PBPK) models which are useful tools to predict the pharmacokinetic behavior of xenobiotics in an organism. A few PBPK models exist for the pregnant mouse, but these often assume constant maternal weight throughout pregnancy and do not include pre-pregnancy exposures. We have developed a generic pregnancy PBPK model for mouse to determine the concentration in the fetal brain resulting from maternal oral exposure to various chemicals. This model considers seven compartments for the pregnant mouse and five for the fetus including a brain compartment. The model describes the growth of maternal weight gain since birth, organ volumes and changes in blood flow. A sensitivity analysis was carried out using the Sobol method which shows that physico-chemical parameters are the most sensitive. The generic aspect of the model, i.e., its applicability to a wide range of chemical families, was demonstrated on five molecules with different physico-chemical properties: PCB 153, PCDD, dieldrin, DDE and arsenic. These molecules have been identified in a recent study as effect drivers of neurodevelopmental and thyroid risk. The model predicts the internal chemical kinetics of these five chemicals in the fetal brain throughout the neurodevelopmental window. In combination with the data obtained from *in vitro* studies on human neuronal cells, the *in vitro* to *in vivo* extrapolation with this p-PBPK model could be used to investigate developmental neurotoxicology.

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P05-25
Evaluation of comprehensive read-across assessment of compounds with limited toxicological data using *in silico* tools

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Efficient strategies are needed to assess the general biological and toxicological profiles of novel compounds with no or limited experimental data. In silico approaches have emerged as indispensable for addressing this challenge. This study presents an in silico methodology that uses predictive pharmacology, toxicology profiling, target-based cluster analysis, chemical fingerprinting, and metabolic similarity assessment to support read across with weight of the evidence by selecting more suitable target analogues with available toxicological information.The first component of the approach uses Chemotargets Clarity to predict the potential biological targets for compounds of interest. Chemotargets Clarity is a commercially available statistical software tool to predict biological targets using six independent methods. A target-based cluster analysis of group compounds based on their predicted biological targets was employed. This analysis facilitates the identification of structurally diverse compounds that could potentially share common mechanisms of action, aiding in the evaluation of read across. Additionally, the target-based prediction grouping was combined with chemical fingerprinting techniques using RDKit to assess structural similarity among compounds. Next, metabolic similarity analysis was performed using tools like QSAR OECD Toolbox to evaluate potential metabolic pathways shared among compounds. This step enhances the understanding of the compounds' potential metabolic fate and interactions within biological systems. Finally, we combined the similarity predictions from these approaches to assess overall compound similarity. This approach assists in the identification of a potential suitable read-across substance for compounds with limited toxicological information. We present a case-study using this approach via carvone and structurally related substances used as food ingredients.

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P05-26
A comprehensive set of structural keys for N-Nitrosamine fingerprinting and determining surrogate relevance in Carcinogenic Potency Assessments

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Purpose: This study aims to identify a set of structural features for efficiently searching and identifying nitrosamine surrogates that mimic the reactivity and structure of complex nitrosamine drug substance related impurities (NDSRIs). Emphasizing the significance of CYP-450 mediated α-hydroxylation, these features also reflect the importance

of other metabolic pathways and carbocation stability in evaluating the carcinogenic potential of NDSRIs.

Methods: We assembled structural features relevant to N-nitrosamines' carcinogenic potency. These features formed the basis for building fingerprints used in computing a quantitative relevance index and surrogate selection. Three distinct feature sets were created: basic atom types surrounding the >N-N=O group, Carcinogenic Potency Categorization Approach (CPCA) [1] derived features, and metabolic α-CH2 hydroxylation modulators from our recent research work [2]. The rationale for surrogate selection was their structural and reactivity similarity to facilitate read-across analyses for data-deficient NDSRIs.

Results: Expert visual assessment and comparison with regulatory agency surrogate choices confirmed the effectiveness of the fingerprints in identifying appropriate surrogates for NDSRI intake limit-setting. The study demonstrates that fingerprints based on identified specific structural features notably outperform traditional structural similarity methods in identifying suitable surrogate nitrosamines for carcinogenicity assessment.

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P05-27
Using expert knowledge to re-evaluate the sensitisation potential of quaternary ammonium salts

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Quaternary ammonium salts (quats) are cationic chemicals with several applications, including as surfactants, detergents, and antistatic agents. They are commonly added to cosmetics and personal care products for these useful properties and as such must be assessed for the risk of any potential toxicity after dermal exposure, including skin sensitisation and skin irritation. While experimental testing is likely to be used to inform any such a risk assessment, computational models can also form part of the overall weight of evidence. This study sought to investigate and improve the *in silico* prediction of the skin sensitisation potential of quats within the knowledge-based model Derek Nexus, based on an analysis of the existing *in vivo* data, expert knowledge about their mechanism of action, and calculations of their reactivity and lipophilicity.

A dataset of 55 quats with associated skin sensitisation data was assembled from the public domain, and the toxicity data were analysed in detail to conclude on their sensitisation and irritation potential. Expert knowledge and a thorough literature review were used to examine the skin sensitisation potential of this chemical class. Theoretical calculations were also employed, by assuming that quats sensitise via an S_N2 mechanism and calculating the reactivity and lipophilicity of a worst-case example chemical to estimate its potency. [1] Proprietary sensitisation data for 14 mixtures containing quats were also analysed to help validate the findings.

While 29 of the quats had historical positive *in vivo* data suggesting sensitisation potential, upon review these data were better explained by considering these chemicals' ability to cause skin irritancy. The proposed mechanism of action for this class involves non-covalent ion pair formation with, and subsequent disruption of, lipid membranes, which would be a very unusual mechanism to cause sensitisation but is consistent with skin irritation. These findings were corroborated by the theoretical calculation of the reactivity and lipophilicity for a worst-case C18 chain quat, which was predicted to be a non-sensitiser with a calculated EC3 value greater than 100%. This was supported by an existing QSAR for predicting the aquatic toxicity of quats which uses lipophilicity alone without the need to include a reactivity parameter, [2] again suggesting that these chemicals are not reactive toxicants. The analysis of the 14 proprietary mixtures containing quats further supported the above conclusions, as 13 of them were negative. The remaining mixture was positive; however, a database and literature search suggested that the sensitisation potential is caused by compounds other than quats in the mixture. Overall, it is likely that this class of chemicals can be skin irritants depending on dose, but they are not true skin sensitisers. The knowledge within the structural alerts in Derek Nexus has been updated to reflect this.

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P05-28
Developing an open-source harmonized Physiologically Based Kinetic (PBK) model for 4 PFAS (PFOS, PFOA, PFNA, and PFHxS) to assess the toxicological profile of forever chemicals

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Concerns regarding PFAS-induced health effects, including hepatotoxicity and immunotoxicity across various age groups, have grown steadily over time. Consequently, regulatory bodies like the European Food Safety Authority (EFSA) have continuously reduced the tolerable weekly intake (TWI) for 4 key PFAS compounds (PFOS, PFOA, PFNA, PFHxS) to the current level of 4.4 ng/kg BW/week based on immune effects in infants. Establishing these limits, often necessitates the use of physiologically based kinetic (PBK) models which leverage mechanistic information to convert blood PFAS levels into reconstructed daily exposure. Through systematic search, we found that existing PBK model for PFAS predominantly rely on the Loccisano model which was developed decades ago and lacks comprehensive mechanistic and biological perspective. Considering the current human biomonitoring data and in-vitro results, an advanced harmonized human model adhering to PBK OECD criterion for 4 priority PFAS compounds was developed. This model incorporates physiological and biochemical parameters along with mechanistic information. The liver was considered as the primary target tissue with compounds like PFOS binding to L-FABP protein with values extracted from in-vitro cell lines, defined using the Michaelis-Menten equation. Hypothetical filtrate compartment as assumed in previous models have been replaced by proximal tubule lumen and cells mediated by organic anion-transporting polypeptide (OATP) transporters, differentiated based on sex. Enterohepatic recirculation was introduced, wherein compounds are transported to intestinal tract via gastric emptying from stomach followed by transport to liver and subsequent biliary secretion back into the small intestine. Excretion

occurs through feces and urine from gut and kidney respectively, with unabsorbed dose appearing in feces. Model evaluation and validation were conducted both in rats and humans at multiple doses to enhance prediction confidence. We found that compounds like PFOS and PFOA have higher renal resorption compared to other PFAS contributing to their extended half-life. Interestingly, PFHxS and PFNA exhibited sex-dependent toxicokinetics with faster elimination in females compared to male rats. This may be attributed to increased excretion through menstrual blood or reduced renal resorption. The model demonstrated good agreement with human biomonitoring data with predicted organ concentration within 2 times of observed data. The final model has been transformed into web-based user-friendly visualization platform, facilitating the application of PBK model for exposure reconstruction and estimating tissue concentration. This open-source harmonized PBK model (deepika060193/PFAS-PBPK-Model (github.com)) can serve as a backbone for regulators and other authorities to evaluate human health risk and establish regulatory guidelines.

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P05-29

Refining toxicokinetic models for enterohepatic recirculation of toxins undergoing hepatic glucuronidation

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Enterohepatic recirculation (EHR) plays a crucial role in toxicokinetics (TK), substantially influencing the internal exposure of chemicals and their glucuronidated metabolites. Apical clearance of glucuronides from hepatocytes to bile canaliculi is a critical component of this process, relying on active transport by efflux transporters. However, a notable challenge in TK modeling of EHR persists due to the lack of computational methods for predicting glucuronide clearance via the multidrug resistance-associated protein 2 (MRP2) receptor. To address this gap, we compiled a dataset containing known kinetic values concerning clearance via MRP2 of 104 different glucuronides. These measures were derived from a range of experimental settings, such as *in vivo* studies employing mutant rats exhibiting defective MRP2 expression, *in vitro* cell permeability assays, and adenosine triphosphate-dependent uptake by membrane vesicles expressing human MRP2. We standardized these values by adjusting them to account for discrepancies resulting from diverse experimental conditions. Next, we developed a Quantitative Structure-Activity Relationship (QSAR) model to predict MRP2 clearance values for glucuronides. The model was constructed based on relevant molecular descriptors and physicochemical properties from the training portion of the dataset, and subsequently validated against the test set to evaluate its robustness and predictive accuracy. Finally, we integrated the predicted MRP2 clearance values into the parameterization of human TK models. Subsequently, we compared the newly generated TK model predictions of *in vivo* systemic concentrations with non-EHR refined models and validation data to assess our method's capacity to improve the accuracy of prediction. These results offer valuable insights for understanding how metabolism impacts internal exposures and provides a useful strategy for refined simulation of enterohepatic recirculation dynamics, advancing our capacity for performing risk assessments using New Approach Methodologies.

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P05-30

The impact of notational inconsistencies in SMILES for QSAR prediction with chemical language models

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In recent years, chemical language models utilizing string representations of chemical structures, such as SMILES notation, have gained prominence in computational toxicology, leveraging natural language processing techniques. Specifically, QSAR based on chemical language models offers comparability with existing models by utilizing SMILES as input, enabling seamless integration within the field. However, despite Canonical SMILES notation being the predominant input format, the lack of standardized processing methods across generation systems introduces certain notational inconsistencies. Does this 'dialect of SMILES notation' influence the application of chemical language models in toxicology? To address this question, the present study examines the following inquiries.

Initially, we investigated the root causes of variability and discovered that many datasets contain a mix of SMILES notations with and without consideration for stereoisomerism. Among 42 datasets examined, 52.5% of compounds lacked enantiomer information that should have been assigned, whereas only 1.09% lacked cis-trans isomer information. The disparities in stereoisomer data across datasets are likely to impact the effectiveness of chemical language models. To address the impact of SMILES notation dialects, we developed two novel models tailored to handle SMILES with notational inconsistencies, and compared the following 3 models: (1) standard preprocessing without changing original SMILES notation, (2) explicit assignment of stereoisomerism information through 3D structure calculation, and (3) deliberate exclusion of stereoisomerism data. These models were then evaluated on the Ames mutagenicity test dataset, composed of 6,512 chemicals, as a case study for the QSAR based toxicity prediction. Evaluation metrics included translation performance (ability to encode/decode compound structures, [0, 1]) and prediction performance (Area Under the Receiver Operating Characteristic, AUROC of predicted values, [0, 1]). Results revealed a substantial improvement in translation accuracy with models (2) and (3) compared with model (1): (1) 0.306, (2) 0.843, and (3) 0.827. On the other hand, prediction performance exhibited slight enhancement: (1) 0.839, (2) 0.846, and (3) 0.863.From the above, we found that (1) compound databases have many notational inconsistencies, and (2) taking stereoisomerism into account would contribute to improving the performance when applying chemical language models. We are currently working on analyzing the substructures that contribute to the prediction by the attention mechanism, which learns the weights of each token in token-based data representation such as texts. It is expected that the operation of an appropriate chemical language model will contribute to the improvement of QSAR tasks in toxicology such as Ames mutagenicity prediction.

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P05-31

PBK modelling concepts for a user friendly generic bovine kinetic modelling platform to predict transfer of compounds from feed, veterinary medicines and supplements to food

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Understanding the transfer of various compounds from feed, veterinary medicines and supplements into cattle tissue or cattle-derived products such as meat and milk is essential to assess possible health risks of

contaminants in feed and for the efficacy and safety testing of veterinary drugs and supplements. Physiologically-based kinetic (PBK) models can predict tissue residues by simulating the kinetics of a compound in an organism and is increasingly used for cattle. Although most PBK models are tailored for expert use and specific to a certain compound, there is also an increasing demand for bovine-specific models that are easily accessible and can be applied by a wide group of users for a variety of (new) compounds. Moreover society, industry and the government promote the development and adoption of animal-free methods. However, the current lack of bovine-specific *in vitro* and in silico methods for predicting absorption, distribution, metabolism, and excretion (ADME) properties poses a challenge for non-animal based PBK model parameterization. A comprehensive tool, integrating all the abovementioned requirements, is currently lacking. Therefore, the Artificial Cow Model project aims to develop a modeling platform that seamlessly integrates PBK modeling with new bovine-specific *in vitro* and in silico methods. This platform is envisioned to be applicable for a broad group of compounds and accessible world-wide by different users. A central element of the platform is the generic PBK model. A first step was to develop a conceptual PBK model, which incorporates model features tailored to needs that were expressed by risk assessors, risk managers, and representatives from the feed and livestock industry. Furthermore, a tiered modelling concept is included with the ability to provide increasingly higher levels of compound-specific ADME information, applying a worst-case approach in case little compound-specific data is available. Furthermore, the conceptual model is developed to accommodate ADME parameters derived from both simple *in vitro* systems and in this project newly developed advanced bovine organoid-based *in vitro* systems, of which the outcomes will be translated to *in vivo* relevant values. The generic bovine kinetic modelling platform will be supplemented with support resources, including model documentation, an extensive PBK model help tool and standardized protocols for conducting bovine *in vitro* assays. Additionally, the model interface will be developed in consultation with the stakeholders, ensuring accessibility to both novice users and modelling experts.

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P05-32

S²CIE: semantic, syntactic, and context-based information extraction for AOP development

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Adverse Outcome Pathways (AOPs) are a conceptual framework for understanding and encoding the cascade of mechanistic events occurring at different levels of biological organization, between an exposure and an adverse outcome in an organism. AOP development begins with hypothesis generation – formulating a skeleton of a proposed AOP based on existing evidence and knowledge. Hypothesis generation therefore requires intensive literature curation, which may be aided by text information extraction methods. Currently, available tools facilitate the curation of literature based on keyword searches. These approaches, however, are limited in both sensitivity and specificity, and therefore usefulness: they do not return relevant literature not containing pre-assigned keywords, and do return irrelevant literature containing the pre-assigned keywords. Implementing modern artificial intelligence approaches over academic literature (35 million articles) to more accurately extract relevant literature proves challenging. Firstly, the flexibility constraint arises when the model is fine-tuned for a specific domain, making it less interoperable for other tasks. Secondly, the computational cost requires searching within a larger contextual space and ranking. To address this challenge, we propose a hybrid (rule-based and machine learning based) real-time information extraction platform supporting semantics, syntactic, and context-based curation of literature irrespective of the domain for hypothesis generation in AOP development. S²CIE is an information extraction component of AOP-BOT (an AI-assisted AOP development pipeline developed by the Partnership for Assessment of Risks from Chemicals), that provides an interface for researchers to interactively define rules over surface token and syntactic graphs to facilitate accurate information extraction from literature at sentence level, followed by a filtering and ranking process to provide additional useful context. A pre-defined grammar templating system for capturing natural language expression of AOP key events was developed to mitigate the necessity for technical grammatical expertise. Moreover, an intuitive visualization will be provided for analyzing the distribution of biological concepts. This includes generating a heatmap and network visualization showcasing concepts occurring at an abstract level. Furthermore, literature space visualization will be enhanced with topic modeling. With horizontal scaling design, this tool enables developers to extract information from large literature sources in real time. It can cover various sources like policy documents and in-house documents without compromising on speed and recall. SC²IE will be accessible both as an integrated AOP-BOT and as a standalone tool.

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P05-33

Physicochemical QSAR model of P-glycoprotein efflux ratio and its application to predicting brain penetration

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Human P-glycoprotein (P-gp) is a major carrier protein expressed in a variety of tissues including the blood-brain barrier. P-gp is responsible for removal of xenobiotics from the cells and protecting the tissues from the potentially toxic action of chemicals. At the same time P-gp efflux may preclude delivery of pharmaceuticals to their site of action and contribute to loss of efficacy. Numerically, P-gp effect is commonly described by the compound's efflux ratio (ER), *i.e.*, the ratio of BA to AB permeation rates in polarized transport assays.

Due to the lack of accurate quantitative measurements, computational studies traditionally treat P-gp efflux as a binary endpoint and only attempt to classify molecules as P-gp substrates or non-substrates. However, recently we have proposed a QSAR model that can produce quantitative predictions of ER values [1]. The model was parameterized using a minimal set of key physicochemical descriptors (log*D*, pK_a, molecular size, etc.) and a censored regression-based machine learning methodology that can use experimental data characterized as either exact data points or ER intervals (censored values). In the current study we extend this approach by applying an estimate of passive permeability in Caco-2 cells [2] to split measured ER values into the contributions of passive and active transport routes, and subsequently fitting the model to represent pure P-gp efflux effect. The new approach enabled us achieving similar predictivity on the qualitative classification task (>75% overall accuracy at a threshold of ER>2 for substrates), while having better interpretability compared to the previous model. Practical utility of quantitative predictions is demonstrated by incorporating predicted ER values into the model characterizing drugs' accessibility to CNS [3]. The respective model supplemented with P-gp efflux estimates was able to predict one of the key brain penetration characteristics, the unbound brain/blood distribution ratio ($K_{p,uu}$) with $R^2>0.5$.

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P05-34
Classification & Labelling Assessment for Skin Sensitisation (CLASS) update: a mechanistic *in silico* model to predict skin sensitisation potential

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The innovative, mechanistic model iSafeRat® CLASS (Classification & Labelling Assessment for Skin Sensitisation) is designed to predict skin sensitisation (SS) expected in the mouse local lymph node assay (LLNA) through four sub-models predicting skin absorption, hapten, pro-hapten or pre-hapten (*i.e.* protein adduct formation) and positivity in the LLNA test only. The latter is determined by comparison with results from *in vivo* tests (*e.g.*, GPMT or Buehler) and could be considered akin to a false positive. The first version which predicted only the potential for positive or negative sensitisation results was developed in 2023. Since then, major improvements increasing confidence in predictions and widening the applicability domain have been integrated. These modifications improved the prediction of skin sensitisation potential and satisfy the QSAR Assessment Framework (QAF) requirements.

A larger dataset of LLNA was gathered to extend the applicability domain. Data were extracted from the extended Cosmetics Europe (CESSD), ECHA REACH and LLNA NiceATM databases, together with proprietary data. All studies were validated according to OECD Guideline No. 429, No. 442a and No. 442b requirements, to select only high-quality data. Then, a splitting method was developed to separate the dataset into training and external test sets. This splitting was based on the mechanism of action structural alert scheme (iSafeRat® MechoA Premium), structural similarity, and the LLNA results. Next, structural alerts, defined in the sub-models, were refined and some exclusion rules were added to accurately define the applicability domain of the model and the substances in the test set fragmented to improve the structural domain evaluation.The new dataset contains 590 validated sensitising or non-sensitising substances which were split into a training set of 467 substances and a test set of 123 substances. By comparison, the former training set contained 398 substances. Accuracy, sensitivity and specificity were increased respectively from 74% to 79%, 82% to 84% and 58% to 73%, based on the training set. Statistics based on the new test set were 78% accuracy, 85,7% sensitivity, and 59% specificity. iSafeRat® CLASS performances as sensitiser or not sensitiser are sufficient for the prediction to replace experimentation for skin sensitization. This new version of CLASS provides analogue search and a better insight into the applicability domain since the test item fragments provide relevant information on the reliability of the prediction. This was developed in order to be fully compliant with the QAF and ECHA guidance. Furthermore, the sensitisation potency through EC3 prediction will be implemented in the upcoming version of the model. Finally, iSafeRat® CLASS is following the "me too" process for its inclusion in the OECD guidance of Defined Approach for Skin Sensitisation (OECD 497).

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P05-35
Molecular modelling study on CYP2C19 mediated biotransformation of organophosphorothioate pesticides: insights on a possible mutational landscape

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Organophosphorothioates (OPTs) are low molecular weight compounds, typically weighing between 250 g/mol to 450 g/mol. Widely used as insecticides globally, they are essential in pest control and improving crop yields. They share the presence of phosphoric acid ester derivatives referred to as phosphorothioates, wherein at least one oxygen atom is substituted by a sulfur atom. OPTs are weak acetylcholineesterase (AChE) inhibitors; however, they are bioactivated by a desulfuration reaction to their phosphate triesters or oxons, powerful inhibitors of brain and serum AChE. Despite showing selective toxicity towards insects over mammals, OPTs are potentially toxic to humans and the environment, posing risks to non-target organisms and ecosystems [1]. The metabolism of these pesticides, largely dependent on cytochrome P450 (CYP) enzymes, plays a crucial role in their toxicity. Certain CYPs promote the bioactivation of OPTs to their oxon counterparts, while others catalyze their detoxification into alkylphosphate and corresponding alcohols [2]. Despite their importance, the mechanisms at the basis of these diverse outcomes remain substantially unknown. In this context, our focus was on understanding the metabolism of phosmet (PHO), an OPT pesticide. We aimed to shed light on its bioactivation pathway, mediated mainly by CYP2C19, distinguishing it from other OPTs such as chlorpyrifos, which is detoxified by CYP2C19 instead. Starting from a validated computational pipeline [3], we unveiled the molecular basis for the differential metabolism of PHO compared to some others OPTs serving as controls. Our analysis emphasized the distinct binding pocket occupancy as a key determinant of their varying transformation capabilities. Furthermore, we explored the mutational landscape of CYP2C19, providing insights into how genetic variations may influence the metabolism and toxicological outcomes of PHO and other OPTs at an inter-individual level. Taking advantage of 3D molecular modelling techniques, including molecular docking and dynamics simulations, we aimed to dissect the structural rationale behind CYP-mediated metabolism. By considering the known genetic variability and polymorphisms of CYPs within the human population, our analysis encompassed the variability of CYP2C19 sequences and associated activities over PHO and others OPTs describing single point mutations likely able to alter the pesticides bioactivation. Understanding how these genetic variations impact the OPTs metabolic fate is crucial as CYP polymorphisms can significantly alter the internal dose of the toxic species [4]. This study offers valuable insights into the molecular mechanism underpinning OPTs metabolism, paving the way for future studies exploring the interplay between CYP polymorphisms and OPT toxicity.

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P05-36
May depsipeptide mycotoxins interfere with heme? An *in silico* case study on the possible impact of enniatin B and beauvericin on Atlantic salmon

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Enniatin B (ENNB) and beauvericin (BEA) are two emerging depsipeptide mycotoxins produced by *Fusarium* species which frequently contaminate cereal-based food and feed. ENNB and BEA can be found in the liver and fillets of fish species intended for human consumption, including Atlantic salmon (*Salmo salar*) [1], as well as in feed for aquaculture [2]. ENNB and BEA are still not regulated, and they may have potential chronic effects on salmon health with an impact on the production yield of this species when farmed with contaminated feed. From a mechanistic point of view, ENNB and BEA display ionophoric properties though they are no longer considered pivotal for their toxicity. In fact, they may disrupt the regulated transport of certain ions [3] resulting in the alteration of normal cell and organelle functions [1], though additional and more specific mechanisms are likely to occur. In this respect, clues have led to suspect specific mechanisms targeting heme-containing proteins [4], which are in line with a certain degree of structural similarity between depsipeptide mycotoxins and heme. This may be at the basis of the huge impact these mycotoxins may have on salmon "smoltification", i.e. the physiological changes young salmonid species undergo while adapting from fresh water to seawater. The present work frames into the MYTOXA project (Norwegian Research Council, grant number 34401), a project integrating *in vivo*, *in vitro* and *in silico* approaches to investigate how and to what extent feed-borne exposure to emerging mycotoxins during the freshwater phase affect salmon growth and the smoltification process. Specifically, this communication reports the development of a computational pipeline including molecular modelling, docking and dynamics simulations to: i) investigate whether ENNB and/or BEA can act as inhibitors of the enzymes involved in the heme biosynthesis; and/or ii) investigate whether ENNB and/or BEA might compete with heme within heme-binding proteins.

Concerning the effect on heme biosynthesis, ENNB and BEA were found likely to bind and possibly inhibit three enzymes involved in the late phase of heme biosynthesis. These results prioritized these enzymes for further dedicated investigation and suggest that ENNB and BEA may act as inhibitors of heme biosynthesis at different levels.

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P05-37
Graph attention networks using knowledge graphs, for predicting novel points of departure for brominated flame retardants

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Brominated Flame Retardants (BFRs) are present in everyday products and materials, to improve fire safety [1]. Various studies have identified BFRs that are neurotoxic, teratogenic and reprotoxic in animals, yet other BFRs continue to lack relevant *in-vivo* Points of Departure (PODs) [1]. *In-vivo* studies pose ethical, scalability and validity concerns, which *in-silico* methods such as Quantitative Structure-Activity Relationship (QSAR) modelling may address [2]. This study hence aims to develop a novel artificial intelligence driven QSAR model, using Graph Attention Networks (GATs) acting on Knowledge Graphs (KGs) of molecules, to predict new BFR PODs relevant to neurotoxicity, teratogenicity and reprotoxicity.

Datasets of PODs for each endpoint (over a consistent species, exposure route and POD type) were obtained via curation of the Toxicity Value Database (ToxValDB) [3], each containing 532-2022 molecules. PODs included Median Effective Concentrations (EC50s) for water flea neurotoxicity, No Observed Adverse Effect Levels (NOAELs) for rat teratogenicity, while both No Observed Effect Concentrations (NOECs) and Lowest Observed Effect Concentrations (LOECs) for water flea reprotoxicity. Conflicting POD values for certain molecules, often varying by several orders of magnitude, were averaged over interquartile ranges. All PODs followed log-normal distributions and so were logarithmised to enforce normal distributions. Furthermore, 432 BFR descriptors were aggregated from relevant literature [4–7], with a majority absent from the endpoint-specific datasets.

KGs were created for each endpoint, encoding dataset chemicals and BFRs as nodes, with shared substructures as edges; substructures of molecular graphs were computed via the Girvan-Newman algorithm [8] and then used in graph isomorphism searches. Node feature vectors were included, containing physicochemical metrics relevant to Lipinski's rule of 5 [9]. The QSAR Applicability Domain (AD) was uniquely defined as any molecule connectable to the KG, via the substructure search method. GATs were trained to perform node regression of log-POD values over each KG, implemented in PyTorch Geometric (Python 3) [10], with a Mean Absolute Error (MAE) loss function. MAEs converged to minima on the testing data for all KGs, corresponding to uncertainties of 22%–51% on mean log-POD values for each endpoint. Exponentiation into PODs propagated these into error factors ranging from 3–16; nonetheless significantly lower than the variation of several orders of magnitude in the original PODs data.

Overall, the novel QSAR methodology explored was found to be an effective approach for predicting ranges for *in-vivo* PODs to occur within, over the relevant endpoints. Predicted ranges were sufficiently specific to aid in prioritisation of BFRs of greater concern, for future research. An open-source dataset of novel BFR POD predictions is planned, following enhancements to the model such as inclusion of edge features.

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P05-38
DoseRider: a multi-omics approach to study dose-response relationships at the pathway level using mixed models

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Traditional toxicogenomics approaches focus on the mechanisms of action (MoA) and benchmark dose (BMD) at the gene level, using dose-response models for individual genes. This method, however, misses the complex gene interactions within biological pathways. To address this gap, we aimed to develop DoseRider, a more comprehensive method that employs mixed models with cubic splines for studying nonlinear dose-response relationships at the pathway level. This method overcomes the limitations of classical dose-response modeling and is adaptable to multi-omics experimental designs. DoseRider is available as an R-package and a web application. All molecules within a pathway are studied simultaneously and DoesRider not only provides a trend for the pathway but also identifies the trend change dose (TCD), the concentration at which significant changes in pathway activity occur. In conclusion, DoseRider marks a significant advancement in toxicogenomics by enabling a comprehensive analysis of dose-response relationships at the pathway level and facilitating the identification of the biological effects of a compound at specific concentrations.

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P05-40
Extracting hepatotoxicity-related insights through analysis on animal toxicity database – towards improving *in silico* prediction accuracy

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The liver is a major target organ for a variety of xenobiotic substances. Past studies have suggested that hepatotoxicity is affected by distribution, presence of reactive functional groups, and metabolic properties. Various prediction tools have been developed to predict hepatotoxicity, but their accuracy remains to be improved. In this study, we aim to offer improved knowledges for hepatotoxicity prediction. We constructed a hepatotoxicity database of rat 90-day subchronic studies, mainly conducted with pesticides. About 200 substances were grouped into three by the hepatotoxic level: High toxicity ("high") group, which showed low lowest-observed-effect level (LOEL) values and severe hepatotoxicity-related findings, low toxicity ("low") group, which showed high LOEL values and minor findings, and the intermediate group. In the following, we analyze the characteristics of the "high" and "low" groups. First, we subjected them to two known Structure-Activity Relationship (SAR) tools, and found more than half showed false-negative results, suggesting the difficulty of structural alert-based hepatotoxicity prediction. To improve prediction accuracy, further knowledge and considerations are needed in various aspects, including localization, metabolic properties, reactive functional groups, and biochemical specific interactions. Therefore, we collected various information, such as, the structural features, physicochemical properties, absorption, distribution, metabolism, and excretion (ADME) and pesticide mode of action (MoA). We, then, explored possible hepatotoxicity-related factors useful for prediction in terms of the above four aspects. It turned out that some structural features associated with hepatic accumulation, metabolic persistence, and metabolic burden are more common in the "high" group. Two groups were also differed in their physical properties (molecular weight (MW) or log P) and ADME parameter values (T1/2, Tmax, etc.). Moreover, we found that combining criteria of MW and log P could be a possible screening indicator for hepatotoxicity. On the other hand, we found that the "high" group contains more pesticides that target mitochondria or cytochrome P450, both are abundant in the liver. This implies MoA-based categorization could be a valid option for hepatotoxicity prediction. Furthermore, considering these factors enabled to detect hepatotoxicity of a part of false-negative substances in the SAR models. Our findings suggest that these types of consideration, along with the existing SAR models, could work as a useful step of hepatotoxic risk assessment workflow in near future. Also, we will discuss the performance of our findings in external validation.

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P05-41
Simulations of antibiotics in dairy cattle in PKSim: a case study of sulfadiazine and trimethoprim

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In combination with trimethoprim, sulphonamides are commonly used for broad-spectrum antimicrobial therapy in veterinary medicine. The main indications in cattle are alimentary and urinary tract infections, mastitis and metritis. Pharmacokinetics for both chemicals are described for various life stages of cattle, including dairy cattle. Antimicrobial therapy for cattle can be complicated by several factors, and the rationale for optimising antibiotic combinations is influenced by sustained therapeutic concentrations for both agents. On the other hand, residues in edible tissues and milk are of concern due to possible adverse effects on human health. Physiologically based kinetic (PBK) models can be applied to simulate and predict pharmacokinetics and concentration-dependent activities to identify strategies to minimise antimicrobial resistance risks. We have developed a PBK model for dairy cattle in PKSim based on a mechanistic description of all the key ADME processes, including the drug penetration into tissues such as muscle, liver, kidney, and udder, and a permeability-limited approach to estimate milk concentration. The simulations were conducted for

trimethoprim and sulfadiazine for the intravenous, intramuscular, and subcutaneous administration routes and validated with plasma and milk concentrations from the literature. Overall, model simulations were within a factor of two for the included administration routes of sulfadiazine and the iv administration of trimethoprim. However, it was not possible to reproduce trimethoprim plasma and milk concentration from literature after intramuscular and subcutaneous administration. A possible explanation may be the infection after administration leading to a prolonged release of both pharmaceuticals into the plasma. Nevertheless, the model performed well when administering sulfadiazine and trimethoprim (intravenous administration) and could be extended to other chemicals.

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P05-42
Comparison of Acceptable Daily Intake (ADI) and *in vivo* doses extrapolated from *in vitro* point of departure obtained with cell painting on U2OS

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The Acceptable Daily Intake (ADI) is the dose of a chemical compound that can be ingested daily by a human without eliciting any adverse effect. The ADI is derived from *in vivo* experiments. The highest dose without any adverse effect from the most sensitive species, for any type of study, is used with typically a safety factor of 100 (10 for intra specie variability and 10 for inter specie variability).

In the context of Next Generation Risk Assessment (NGRA), there is a pressing need to employ new alternative methods (NAMs), such as *in vitro* assays, to reduce or even replace animal studies. One such assay, is Cell Painting, an *in vitro* assay developed by the Broad institute, which generates morphological profiles of cells perturbated by chemicals. It uses 6 dyes to reveal 8 cell compartments, to form after image analysis a robust and unbiased morphological profile describing the morphology of cells.

Inspired by results from the US EPA, where Cell Painting was used with reverse dosimetry to extrapolate an *in vivo* dose and to prioritize risky compound testing, we ran a Cell Painting campaign on chemical compounds with known ADIs to compare ADIs with doses extrapolated from *in vitro* point of departure (PODs) obtained with the Cell Painting assay.

We performed Cell Painting on U2OS (human osteoblast cell line) on 71 compounds at 8 concentrations (from 0.03 µM to 100 µM). We determined their *in vitro* Point of Departure (POD), the concentration for which the cell morphology started to defer from the negative controls. Out of the 71 compounds, 49 had a POD.

For the reverse dosimetry, we used the US EPA R httk package. The parametrization was done using ADME *in vitro* measures: Human Clint, fraction unbound and human blood to plasma ratio, along with unspecific binding ratio on plastic and media for the 384-w plate, to estimate the free concentration and refine the POD.

We computed the Administered Equivalent Dose (AED) from the *in vitro* POD using httk.

We obtained AED ranges: the 5th, 50th and 95th quantiles. The results of this comparative analysis will be presented and discussed in the context of the development of NGRA.

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P05-43
What does it take to make a PBK for a pesticide in a NGRA framework? Evaluation of the kinetic space of pesticides and how to model it using open-source tools

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Physiologically-based kinetic models (PBK) represent a mechanistic modeling approach to predict the systemic availability and organ concentrations over time for chemicals through external exposure. These model can be used to link *in vitro* hazard characterization data and external dose estimations hence, constituting a cornerstone in Next Generation Risk Assessment (NGRA). To be amenable for NGRA framework, PBK models have to be more bottom-up and mechanistic. While there is a common generic structure and set of input parameters for PBK models, special characteristics of the test chemical might require the inclusion of specific processes in the PBK models. In the absence of new *in vivo* data to understand whether such specific processes are needed, we rely on read-across of PBK models of similar chemicals.

PBK (Physiologically Based Kinetic) models have found widespread utility in the safety evaluation of therapeutic agents. Conversely, the application of PBK models to pesticides poses unique challenges due to the broader spectrum of physicochemical properties associated with pesticides, coupled with the scarcity of comprehensive ADME (Absorption, Distribution, Metabolism, and Excretion) and *in vivo* kinetic data. Thus, modelling pesticides kinetics requires more tailored approaches. The primary objective of this study was to assess the feasibility of leveraging existing knowledge and conducting systematic PBK model read-across within the domain of pesticide chemistry.

We reviewed literature on PBK models for organic pesticides, focusing on phenoxy herbicides, organochlorine insecticides, and pyrethroids. Then we identified key aspects of the compounds kinetics and how they are modelled in the PBK models, including parameterization and evaluation approaches. Finally, we assessed the suitability of open PBK software (e.g. PK-sim and TKplate) to integrate some of these kinetic specificities but also the capacity of algorithms of chemical grouping (e.g. KWAAS) to pair target chemical with relevant analogues Certain pesticides exhibit high lipophilicity, challenging standard PBK assumptions. To address this, some models incorporate a separate blood compartment for lipoproteins, and may include deep liver compartments or lymphatic routes of oral absorption. The low solubility of some pesticides leads to uncertainty in *in vitro*-derived ADME parameters, which could be mitigated by integrating quantitative structure-property relationships to correct for the fraction unbound. Some pesticides also exhibit bile excretion, formation of bioactive metabolites, and toxicodynamic effects, which should be considered in modeling.

This study contributes to the broader goal of analyzing how kinetic information is reported in academic literature and regulatory documents and explores its integration into systematic, automated PBK read-across methodologies, setting the stage to integrate AI tools in the near future.

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P05-44
SAFEPATH: Using AI to understand the molecular mechanisms causing safety failures, enabling drug optimisation and turnaround

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Each year, the pharmaceutical industry is forced to abandon hundreds of drug projects due to safety concerns unearthed in pre-clinical or Phase I trials. These trials, while crucial, often act as a 'black box', indicating the presence of safety issues without delving into the mechanistic causes of toxicity or suggesting how the drug could be altered to mitigate these issues.

Enter SAFEPATH, our advanced artificial intelligence (AI) platform, designed specifically to address this gap in drug development. Its core use case is to decipher the underlying mechanisms of safety problems, assess the feasibility of salvaging a drug, and chart a course for its optimisation. SAFEPATH leverages the combined power of cheminformatics, bioinformatics, deep learning, and our proprietary datasets to unravel the complex biological interactions that lead to drug safety issues, moving beyond mere prediction to provide actionable solutions.

The foundation of SAFEPATH's approach is the use of cutting-edge deep learning models to elucidate the interactions between drugs and biological targets, including unintended receptors or *in vitro* safety markers. It incorporates equivariant graph neural networks (EGNNs) within its analytical framework to pinpoint the specific atoms responsible for adverse interactions, thereby guiding the structural modification of the drug to eliminate these toxic effects. Next, these interactions are analysed through a novel Causal Knowledge Graph methodology, which integrates diverse data types including multi-modal 'omics data, heterogeneous relationship types, *in vitro* toxicity endpoints, and ADME/PK properties, to generate robust, testable hypotheses. This method effectively connects predicted off-target effects with observed toxicity, through clear mechanistic pathways.

SAFEPATH has demonstrated its utility in not just predicting but understanding drug-induced liver injury (DILI), as seen in a case study with the tyrosine kinase inhibitors Erlotinib and Gefitinib, which are known to cause hepatotoxicity. The platform recalled a known UGT1A1-mediated hepatotoxicity in Erlotinib [1] and proposed a novel mechanistic link to Gefitinib-induced hepatotoxicity via sphingolipid metabolism [2] and the PRKD1/PRKD3 pathway.

The real value of SAFEPATH lies in its ability to dissect the intricate web of biological cause and effect, offering not just a predictive model but a comprehensive solution-oriented approach to drug safety. This makes it an indispensable tool for turning around drug projects with safety concerns, thereby reducing the rate of abandonment in drug development.

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P05-45
Ontology maps: data integration tools for applications in toxicology

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Developed within the H2020 ONTOX project, ontology maps are computational tools that allow integration, organization and visualization of relevant biological, toxicological, chemical and kinetic data coming from various sources. Organ-specific ontology maps are designed for 6 adverse outcomes: cholestasis and steatosis (liver), tubular necrosis and crystallopathy (kidney), neural tube closure and cognitive function defects (developing brain).

The foundation of each ontology map is a biological layer of information, i.e. a physiological map (PM) which graphically represents biological mechanisms. For all case studies, we designed organ-specific PMs, standardized with the Systems Biology Graphical Notation (SBGN) using the CellDesigner software and visualized and annotated using the MINERVA platform.

These computational tools have the capacity to integrate and annotate data from a variety of sources. They allow for an easy-to-interpret visual representation as well as for machine readability and compatibility with other platforms. On top of the biological layers, ontology maps will integrate standardized AOP networks as well as tables with chemical information (structural and physico-chemical properties) and kinetic data (e.g. absorption, distribution).

Beyond structuring diverse concepts and data and showing their properties and relations between them, ontology maps aim to serve as a basis for setting up *in vitro* test batteries and *in silico* models (e.g. Boolean modeling) to evaluate specific types of toxicity. We also aim to make them interoperable with other tools and platforms (e.g. Biobricks, WikiPathways) to facilitate the (re)use of data.

Ontology maps are dynamic tools that should be updated when new data become available and are the result of a collaborative effort by domain experts and biocurators, using standardized guidelines for comprehensive annotation and documentation. Their development is expected to support and accelerate the generation of New Approach Methodologies for next-generation risk assessment, only possible through collaboration between the toxicology and systems biology communities.

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P05-46
Transcriptomic and metabolomic integration of tributyltin-exposed adipocytes reveals associations with neurological disorders

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Neurological disorders (ND) are a prevalent cause of death and disability worldwide and have been associated with various environmental pollutants and endocrine disrupting compounds (EDCs). NDs are characterised by complex pathophysiological processes and can manifest in a wide range of disruptive symptoms. Most research of associations between ND and EDCs focuses on neurological tissues while ignoring the complex interplay of various physiological tissues in maintaining homeostasis. Adipose tissue is a highly dispersed tissue that plays a considerable role in the regulation of metabolism and energy homeostasis and is recently being intrinsically considered an organ. Further, EDCs may impact the normal development and function of adipose tissue, such as adipocytes, which make up the vast percentage of adipose tissue per volume. To explore further, Simpson-Golabi-Behmel syndrome (SGBS) pre-adipocytes were grown to near confluence and incubated in differentiation medium for four days, followed by cultivation in maintenance medium for six days. The differentiation medium of TBT-exposed cells was additionally supplemented with 25nM tributyl-tin (TBT) during the initial four days. TBT is a well-known obesogen with other endocrine disrupting capabilities. Cells were harvested at day 10 of differentiation and TBT-exposed and differentiated controls were compared. Transcriptomic analysis was performed using Agilent microarrays to determine differentially expressed genes (DEGs) between treatment groups. Samples for untargeted metabolomics were analyzed using Reversed Phase (RP) and Hydrophilic Interaction (HILIC) Liquid Chromatography in positive and negative ionization modes. Data preprocessing, cleaning, and statistical analyses were conducted in R using limma for transcriptomics, and xcms, IPO, PMCMRplus, and xMSannotator packages for metabolomics. Differentially expressed genes (DEGs) and metabolites (DEMs) were collectively mapped onto signaling and metabolic pathways using MetaboAnalystR. This approach elucidates nuanced disturbances in adipocyte responses and the role in neurological mechanisms. It also highlights both individual molecular changes and their integrated effects on cellular pathways. Overall, perturbations in pathways linked to neurological dysfunction, including Parkinson's and Alzheimer's, were identified. The identification of key features, including amino acid disruption, and perturbed pathways, such as AMPK, Adipocytokine, and AGE-RAGE signalling pathways, provides a foundation for further mechanistic investigations and potentially informs strategies for mitigating the adverse effects of EDC exposures. Future studies will delve deeper into the adipocyte 'ome', seeking indications of susceptibility or early warning signs specific to health outcomes in the realm of neurological syndromes.

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P05-47
An ontology of Physiologically Based Pharmacokinetic Model (PBPK) for harmonization and automation of modeling framework (PBPKO)

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Physiologically Based Pharmacokinetic Models (PBPK) are extensively employed in pharmaceutical and environmental fields to evaluate xenobiotic kinetics. Despite numerous publications, a major challenge lies in the inconsistent vocabulary used, hindering automation potential. Harmonization techniques like ontology which offer a formal, explicit specification of a shared conceptualization, can help to facilitate models FAIRification solution. Presently, while several biological domain ontologies exist on the Bioportal platform, a dedicated one for PBPK is lacking. This work aims to fill this gap by developing PBPKO, a dedicated PBPK ontology, to benefit the wider kinetic community. PBPKO encompasses potential mechanisms, kinetic terms from various databases, and expert terminology. Its development adheres to open-source principles, with the initial version (V1.0) published following FAIR guidelines at OBO Foundry via ODK (https://github.com/Crispae/pbpko). Future iterations will include multiple species and additional kinetic terms through expert collaboration. PBPKO annotates both physiological and biochemical parameters pertinent to PBPK modeling. Currently, a few example PBPK models converted into SBML, are being linked to PBPKO for automating computational modeling and facilitating text mining of PBPK parameters and model output. To facilitate the automation of code conversion of PBPK model, we'll leverage sbmlutils, a Python library for ontology-based SBML model annotation, offering comprehensive functionality. A generic PBPK use case study using PBPKO will demonstrate the utility of ontology terms and aid in harmonizing existing PBPK models for regulatory acceptance.

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P05-48
Computational New Approach Methodologies: a strategic chess-inspired approach for the Next Generation Risk Assessment

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Next-generation risk assessment for chemicals involves leveraging advancements in technology, data science, and toxicology to enhance the accuracy, efficiency, and comprehensiveness of chemical risk assessment processes. Such data-driven procedures are called New Approach Methodologies (NAMs) and the most widely accepted ones include read across methods, *in vivo in vitro* extrapolations (IVIVE), High-Throughput Screening (HTS), omics technologies, Integrated Testing Strategies (ITS), exposure science, modelling, Adverse Outcome Pathways (AOPs) development, Data Integration and Sharing, Non-Animal Testing Methods, Systems Biology Approaches, and Artificial Intelligence (AI). In this paper, we introduce an integrated bottom-up strategy for managing chemicals, taking into account the dose-to-outcome continuum. Our approach involves the application of pharmacokinetic models (PBPK/PBTK), artificial intelligence, Natural Language Processing (NLP), and quantitative Adverse Outcome Pathways (qAOPs). PBTK

models are utilized to predict exposure and intake and assess the absorption, distribution, metabolism, and excretion (ADME) processes of chemicals within the human body. NLP is employed to collect comprehensive data from the literature and facilitate the development of AOPs, as well as *in vivo/in vitro* studies necessary for qAOPs development. Recognizing the critical demand for robust NLP models, we have introduced four self-trained and finely tuned transformer models to effectively gather data. These models are built based on the RoBERTa approach, ensuring a strong foundation for their architecture. Our models have the capability to retrieve information concerning chemicals/stressors, genes, proteins, DNA, RNA, diseases, and endogenous metabolites. Dose-response modelling and response-response modelling were conducted with the employment of advanced self-trained machine learning models. These models were trained in an unsupervised manner with the adoption of parallel computing methodologies as well. In situations with limited data availability, generative AI methodologies are employed to enhance existing datasets. In this paper, we present the New Approach Methodology developed through a case study focusing on Liver Fibrosis. Our work is completely aligned with the 3Rs principles.

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P05-49

External validation of alert profilers for genotoxicity hazard within the OECD Toolbox

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Objective: Genotoxic hazard identification is a key aspect of regulatory decision-making in many countries. Computational toxicity prediction is a useful first-step approach to hazard assessment of pesticide impurities and metabolites. In addition to validated Quantitative Structure-Activity Relationship (QSAR) models, general mechanistic and endpoint specific profiler information from the OECD QSAR Toolbox can be used for chemical grouping. They contain expert knowledge about structure-activity relationships, however, their general predictive performance is only available for the internal dataset. To better understand alert performance, genotoxicity-relevant profilers in OECD QSAR Toolbox were compared to experimental results from the Case Ultra database.

Methods: As external training data served the almost 30000 compounds from the Case Ultra AMES mutagenicity database and the commercial *in vivo* micronucleus test (MNT) database trained with further data from EFSA evaluations on pesticides and their metabolites (1059 compounds, 330 actives/729 inactive). The compounds were profiled through the OECD QSAR Toolbox by the relevant profilers with and without consideration of metabolic processing.

Results: Positive predictive performance varied from 40–78% for AMES and from 29–55% for MNT, indicating substantial differences in the relative performance of profilers for predicting genotoxicity. Consideration of metabolic simulation for chemicals without an alert improved positive predictive performance slightly by 2–6% points for AMES and by 3–20% points for MNT, resulting in a positive predictive performance of 46–82% for AMES and 37–67% for MNT profilers. Considering expert-derived deactivating rules implemented with CASE Ultra GT_EXPERT model provided an additional improvement of 3–7% points for bacterial mutagenicity profiling.

Conclusion: Understanding the predictive performance of OECD Toolbox profilers is important to calibrate the scientific confidence one should apply in a weight of evidence assessment for genotoxicity. The external validation clearly demonstrates that the OECD toolbox profilers should not be used for genotoxicity prediction or assessment purposes but are considered for building chemical categories for subsequent grouping. An in depth analysis and rework of the profilers using a larger compound space is advisable.

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P05-50

Using ontology map and physiological map to improve predictive models for kidney toxicity

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The kidney filters blood and maintains chemical balance in the whole body. It performs its activity through the nephron, the vital functional unit of the urinary system. The understanding of toxicities affecting the nephron is crucial, particularly in chemical and drug safety assessments. Recent advances in machine learning, specifically QSAR and read-across approaches, have led to predictive computational models for various toxicological endpoints, comprising nephrotoxicity. However, these models often lack explanatory power regarding their predictions, notably concerning biological mechanisms of action. To address this gap, we developed a systems biology approach within the H2020 ONTOX project *(Vinken, M. et al. 2021)*, building a nephron Physiological Map (PM) and two associated disease ontology maps. They serve to collect relevant toxicological data and display them in a user-friendly graphical interface.

The PM is a comprehensive representation of biological processes and interactions constructed in CellDesigner software using the standardized Systems Biology Graphical Notation. We designed a workflow facilitating PM creation and enhancement, inspired by the Disease Maps community *(Mazein, A. et al. 2018)*. This method involves data extraction from literature and databases, followed by review of experts and curators. As a result, the PM of the human nephron includes genes, proteins, and metabolites within pathways for urine production and vitamin D metabolism, among others. Notably, transporters for drugs, as well as other transporters, play a significant role.

The ontology maps are developed as a multilayer extension of the PM, in order to study two conditions that affect the normal physiology of the nephron: kidney crystallopathy and tubular necrosis. By systematically organizing knowledge, ontologies aid in identifying gene-disease associations, drug targets, and pathways, driving toxicological discoveries. In this way, they are useful for suggesting new *in vitro* tests and for developing new adverse outcome pathways and Boolean models. Moreover, our ontology maps are a New Approach Methodology (NAM), with the aim to avoid animal testing. We visualize the PM and ontology maps using the MINERVA platform, an interface that allows easy navigation and facilitates the identification and understanding of possible toxicities.

Finally, as the PM and ontology maps provide a detailed, interactive view of cellular and molecular processes associated with specific functions, they can improve the understanding of toxicological mechanisms. This innovative perspective can enhance toxicological predictions qualitatively and quantitatively, offering new insights into human toxicities and improving Next Generation Risk Assessment .

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P06 | Omics in toxicology

P06-01
Cadmium telluride quantum dots induce changes in mouse neural behavior: analysis of inflammatory response from a single-cell transcriptomic perspective

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Cadmium telluride (CdTe) quantum dots (QDs) are prized for their simple synthesis, high quantum yield, monochromaticity, and quantum-limited domain effect, making them promising for drug delivery and medical imaging. However, understanding their neurotoxic effects is crucial for safe applications. This study investigated CdTe QDs' impact on C57BL/6J mice after a single-tail vein injection. Behavioral experiments revealed CdTe QDs induced behavioral changes related to learning and memory in mice. Transcriptomic analysis of 49,829 hippocampal single cells identified 32 subclasses, representing major cell types. Disorganization, particularly in microglia and endothelial cells, was observed, involving inflammatory responses and cytoskeleton reorganization. Exposure to CdTe QDs triggered inflammation, oxidative stress, and endoplasmic reticulum stress in these cells, associated with cytokines. Oligodendrocytes, choroid plexus cells, and smooth muscle cells exhibited mitochondrial dysfunction and endoplasmic reticulum stress. These findings suggest complex effects on the mouse nervous system, emphasizing the need for comprehensive studies to understand CdTe QDs' neurotoxicity mechanisms fully. This study lays the foundation for exploring safe biological applications through insights into CdTe QDs' gene responses in various hippocampal cells.

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P06-02
Distinct transcriptomic differences between chicken and turkey in molecular toxicity of salinomycin

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Introduction: Salinomycin (Sal) is commonly used as an anticoccidial drug in poultry. The biotransformation of Sal takes place in the liver. Even though the mode of action of Sal is identical irrespective of the treated animal, the clinical data indicate that there is a difference in Sal's toxicity between chicken and turkey. Chickens are resistant to Sal's toxicity, whereas turkeys are susceptible. Yet the molecular differences between chicken and turkey in response to Sal intoxication are unclear.

Objectives: We aimed to determine the transcriptomic responses to Sal in chicken and turkey, which may help understanding the molecular toxicity of Sal in both species.

Methods: Animals were classified into Sal-exposed (n=6; 0.9 mg/kg b.w/day) and control group (n=6). Chickens and turkeys received Sal with feed for the last 2 weeks of rearing, and liver tissue was collected at week 5 (chicken) and 15 (turkey). Total RNA was extracted with Maxwell RSC (Promega). NGS data was created with the Illumina NovaSeq 6000 system. FastQC, Fastp, and rnaseqc were utilized for quality control (QC) and trimming. Alignment and quantification of gene expression levels were conducted using the RSEM supported by the STAR aligner. A comprehensive set of statistics was gathered using MultiQC. The DESeq2 package ($log2FC \geq 1$; $p\text{-}adj < 0.05$) was used to identify the differentially expressed genes (DEG) within species. Gene Ontology (GO) and Reactome databases were used to evaluate altered molecular signaling pathways.

Results: A total of 3049 DEG (1526 up-regulated; 1523 down-regulated) were found in the chicken and 2337 DEG (1169 up-regulated; 1168 down-regulated) in the turkey. In chicken, DEG were involved in positive regulation of transcription, DNA repair and functioning in protein kinase activity. In turkey DEG played a role in signal transduction and mitochondrial translation. Reactome pathway analysis showed enhanced cell division-related pathways and diminished ECM organization and protein translation in the chicken liver. The increased ECM organization and signal transduction pathways were determined in turkey. All down-regulated pathways that we found in turkey were related to mitochondrial translation. Additionally, we determined that 795 DEG common for both chicken and turkey were more distinctly altered in turkey than in chicken. These DEG were involved in the immune system, protein metabolism and modification pathways.

Conclusion: Sal intoxication possibly impairs energy metabolism and triggers mitochondrial dysfunction in the turkey liver. On the other hand, Sal toxicity may trigger cell division via up-regulation of cell cycle-related pathways in the chicken liver. These results suggest that Sal toxicity in the liver differs at the cellular level between chicken and turkey.

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P06-04
Advancing bladder cancer detection: unveiling a novel urinary biomarker panel through metabolomics

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Introduction: Bladder cancer (BC) remains a significant global health challenge and its incidence continues to rise. Invasive and late diagnosis often leads to advanced disease stages and poorer prognosis. Therefore, there is an urgent need to explore new diagnostic approaches that allow for a timely intervention. By examining the metabolic profiles of urine samples, metabolomics enables the identification of unique volatile organic compound (VOC) signatures associated with BC. The dynamic nature of VOCs, influenced by cellular metabolism and the tumor microenvironment, holds great potential for capturing subtle changes indicative of early-stage BC. Our study aimed to use a metabolomics approach to identify volatile biomarkers that can accurately differentiate non-muscle invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) from cancer-free individuals.

Methods: Urine samples were collected from 196 participants, including 98 BC patients (67 NMIBC and 31 MIBC) and 98 cancer-free controls. Ethical approval was obtained, and the informed consent was signed by all participants. Volatile profiling in urine samples was performed using headspace solid-phase microextraction coupled to gas chromatography-mass spectrometry (HS-SPME-GC-MS). Data analysis included principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), receiver operating characteristic (ROC) analysis, and univariate analysis to identify potential biomarkers for BC detection.

Results: Data analysis revealed 19 metabolites that significantly discriminated BC from controls. Among these, a panel of 10 potential biomarkers was identified for overall BC detection. These biomarkers included two aromatic compounds, three ketones, three alcohols, one pyran-like compound, and one fatty acid. This biomarker panel had a sensitivity of 81%, a specificity of 90% and an accuracy of 85%. For NMIBC cases compared to controls, the panel showed sensitivity, specificity, and accuracy rates of 83%, 87% and 84%, respectively. In contrast, for MIBC cases compared to controls, the panel revealed sensitivity, specificity, and accuracy rates of 90%, 77% and 87%, respectively. No discrimination was observed between the urinary volatile profiles of NMIBC and MIBC patients.

Conclusions: Our study highlights the potential of urinary volatile biomarkers for the detection of early and advanced stages of BC. These results highlight the value of metabolomics in uncovering novel diagnostic approaches for BC and underscore the need for further research to improve our understanding of the heterogeneity of BC and to refine diagnostic strategies.

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P06-05
Exposomics analysis of agricultural workers at risk for Mesoamerican nephropathy

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Chronic kidney disease of unknown etiology (CKDu), also known as Mesoamerican nephropathy (MeN) in Central America, is a global epidemic of kidney disease that is primarily impacting young otherwise healthy agricultural workers. Those most impacted in Central America are sugarcane workers of which many are dying from this disease and for which the cause remains unknown. It has been hypothesized that exposures ranging from metals to pesticides along with heat stress and dehydration may be major contributors to development of Mesoamerican nephropathy. In the current study, we utilized untargeted metabolomics and elemental analysis to determine potential toxicological exposures and metabolic changes in sugarcane workers from Guatemala who are at risk for developing Mesoamerican nephropathy. Urine samples were collected from these workers at the beginning of a harvest season (November) and at the end of the harvest season (April) (n=20). We found an increased concentration of silicon and silica nanoparticles across the harvest season along with several pesticides (diquat, paraquat, carbofuran and metalochlor) while heavy metal exposures remained low. In addition, we observed changes in phosphorous levels indicative of chronic kidney disease. Metabolically, these workers developed mitochondrial stress related changes including impaired fatty acid oxidation leading to accumulation of fatty acids as well as altered amino acid metabolism. Overall, these results confirmed that multiple exposures are occurring in sugarcane workers and may provide insight into early warning signs of kidney injury and may help explain the increased incidence of CKDu among agricultural workers.

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P06-06
Omics approach for pathways involved in cisplatin-induced nephrotoxicity

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Purpose: This study addresses the critical need for early renal injury detection to mitigate renal disease progression. Leveraging an omics approach, this research endeavors to advance toxicological understanding of markers and pathways involved in cisplatin-induced nephrotoxicity. This approach aims to detect early biomarkers of nephrotoxicity, typically identified in later stages.

Methods: Cytotoxicity was evaluated utilizing the MTT assay subsequent to 24-hour exposure to cisplatin in HK-2 and Hek-293 cell lines. The chosen concentration was applied for subsequent analyses. Caspase 3/7 activity was quantified employing Promega's Caspase 3/7 Glo kit, with luminescence measurements conducted utilizing a GloMax Discover multimodal plate reader. RT-qPCR was executed utilizing the Illustra Ready-to-GoTM Beads kit, with RNA (100 ng) and oligo dT, conducted on the Applied Biosystems StepOne-Real Time PCR System. Flow cytometric analysis was performed on the Cytek Aurora platform, with data acquisition facilitated by SpectraFlo software and analysis undertaken using FlowJo software. Statistical analyses were carried out using GraphPad Prism 8.0.

Results: Cytotoxicity assay selected the concentration of 6.6 µM of Cisplatin. Caspase 3/7 Glo assay showed an increase in Cisplatin treated HK-2 cells. Gene expression in HK-2 treated cells showed upregulation for AXL (2.10**), BCL2L1 (3.04**), CDH1 (1.87), HSP27 (3.03**), HSP70 (3.28**), TLR2 (2.76**), and VIM (4.80*). In Hek-293 treated cells was observed an upregulation of AXL (1.14), CDH1 (1.50**), HSP70 (1.18), TLR2 (1.07), and VIM (9.02**), while ABCB5 (0.00**) was downregulated. Additionally, a subtle, non-significant decrease was observed in HSP27 (0.84) and BCL2L1 (0.93). All values represent fold changes compared to untreated cells. Protein expression through FACS analysis demonstrates significant upregulation in KIM1 for HK-2

treated cells ($p<0.01$). Also an increase of BCL2L1 (1.04), HSP27 (1.02), and VIM (1.02). AXL was downregulated (0.45). In Hek-293 treated cells, there was upregulation of AXL (1.66), BCL2L1 (1.01), HSP27 (1.21), and KIM1 (1.03). There was no change for VIM (1.00). The study unveils significant caspase 3/7 activation in HK-2 cells post-cisplatin exposure, indicating apoptosis involvement via caspase. Additionally, inflammatory pathways (AXL and TLR2), kidney injury, and fibrosis (AXL, KIM1, and VIM) were elucidated. Notably, adaptive responses were triggered at the tested concentration, evidenced by upregulation of BCL2L1 (cell survival), CDH1 (EMT, fibrosis), HSP27, and HSP70 (cell stress, protein misfolding, and damage). These findings highlight multiple mechanisms involved in the nephrotoxic response to cisplatin. In summary, this study elucidates molecular pathways involved in cisplatin-induced nephrotoxicity, offering insights crucial for early detection and targeted interventions in renal injuries, underscoring the importance of omics in managing renal diseases.

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P06-07
The role of major biochemical parameters and key gene polymorphisms in the one-carbon pathway in Parkinson's disease

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Parkinson's disease (PD) is the second most common neurodegenerative disease. The cause of PD is shown to be the decrease or degradation of dopaminergic activity in the brainstem region, which is thought to be triggered by environmental factors, aging, infectious agents, and other pathological conditions. Abnormalities in the folate-mediated one-carbon pathway may play a role in the pathophysiology of PD as it increases total homocysteine levels. We aimed to show possible associations between folate, homocysteine, cysteine, and vitamin B12 levels, which function in the one-carbon pathway, and PD risk. Furthermore, the effect ofgenetic polymorphism of *MTHFR* and *MTR,* which are involved in the one-carbon pathway, on PD risk was investigated. In our study, 108 patients diagnosed with Parkinson's disease and 97 healthy volunteers participated. The biochemical parameters were measured by ELISA, and genetic polymorphisms were analyzed by PCR-RFLP. After adjustment for confounding factors such as age, smoking habit, and gender, PD risk increased statistically significantly with increased folic acid levels and decreased vitamin B12 and homocysteine levels. These findings suggest that extra folic acid intake in the patients' diets may have been the cause of these findings. Higher homocysteine levels were observed in PD patients with the *MTHFR C677TTT* genotype and the *MTR A2756G GG* genotype. However, this difference was not significant. No effect of cysteine levels on PD risk was observed. The *MTHFR C677T* and *MTR A2756G* gene polymorphisms were not found to be risk factors for PD.

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P06-08
The short-term toxicity and metabolome of Dicyclopentadiene

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Background and Purpose: The EU REACH legislation stipulates the information requirements of what is required for assessment of industrial chemicals. It was anticipated, as exemplified in the preamble to REACH, that alternative methods would be developed to minimise animal usage. Currently, only a few New Approach Methods (NAMs) have achieved regulatory acceptance. This study was intended to develop and demonstrate an approach that combined animal testing with state-of-the-art metabolomics to provide valid information with a reduction in animal use.

Methods: Wistar rats received Dicyclopentadiene (DCPD) daily for 14 days via gavage at dose levels of 0 (corn oil vehicle), 50 and 150 mg/kg bw and standard endpoints were addressed. Blood samples were taken from animals to be used in metabolomic analyses. Metabolites were identified in blood plasma using GC-MS and LC-MS/MS methods. The software MetaMap®Tox was used to develop metabolomic profiles for DCPD based upon the data collected above.

Results: The highest dose induced transient clinical signs of toxicity and in males only reduced body weight gain. High-dose liver changes were characterized by altered clinical chemistry parameters in both sexes and pathological changes in females. In high-dose males, an accumulation of alpha-2u-globulin in the kidney was noted. Comparing the DCPD metabolome with previously established specific metabolite patterns in the MetaMap®Tox data base suggested that the high dose would result in liver enzyme induction leading to increased breakdown of thyroid hormones for males and females which confirms similar findings in the published 90-day studies. An indication for liver toxicity in males was also noted. Metabolomics also suggested an effect on the functionality of the adrenal glands in high dose males, which together with published data, is suggestive of a stress related effect in this organ. A match with the alpha-2u-globulin metabolome pattern could not be established as the effect at the high-dose level was too weak.

Conclusions: The results of the present 14-day combined toxicity and metabolome investigations were qualitatively in line with existing literature data. Importantly no other types of organ toxicity, or hormone

dysregulation beyond the ones associated with liver enzyme induction and stress were indicated, again in line with results of the 90-day studies. It is therefore suggested that similar short-term "smart" studies, combining classical toxicity endpoints with "'omics" technologies, could be a 2R (refine and reduce) new approach method allowing for the reduction of the *in vivo* higher-tier toxicity testing that is required as a part of REACH and other regulatory legislations globally. Ultimately, these smart NAMs would lead the way from traditional studies, with high animal burden associated to more efficient techniques that do not compromise on the ability to characterise mammalian toxicity and thus safety.

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P06-09
Toxicogenomics to analyze systemic toxicity of salicylic acid as part of a NGRA ab initio case study

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Toxicogenomics is often used in combination with other methods such as pharmacological profiling or cell stress assays for assessing systemic toxicity in ab initio Next Generation Risk Assessment (NGRA) of unknown compounds. In the present study, toxicogenomics was applied to analyze the benzyl salicylate metabolite salicylic acid in an ab initio case study of benzyl salicylate [1]. The aim of the study was to evaluate the systemic effects of salicylic acid at the transcriptome level according to two analysis methods and to derive a point of departure (POD) based on the benchmark dose (BMD) analysis.

HepG2 and MCF7 cells were incubated for 6 and 24 hours, and HepaRG cells for 24 hours, with 5 concentrations of salicylic acid and trichostatin A as positive control. The samples were analyzed using Illumina RNA-sequencing and data analysis was performed by two different companies according to their statistical best practice approaches. The data were analyzed both qualitatively with a differentially expressed gene analysis (DEG) and quantitatively with a BMD analysis. BMD median values of the lowest pathway BMD median were used for POD derivation, as recommended by the National Toxicology Program omics guideline [2].

Trichostatin A caused a dose-response effect in all analyzed cell lines and concentrations in the DEG analysis, whereas salicylic acid only resulted in a dose-response effect at the two highest concentrations in HepG2 cells at the 24 h timepoint. This was confirmed by a high overlap between the up- and down-regulated genes at the two highest concentrations. There was a lack of a dose-response effect by salicylic acid in MCF7 and HepG2 cells at the 6 h timepoint in the DEG analysis and in HepaRG at the 24 h timepoint. Therefore, the BMD analysis of the HepG2 cells at 24 h was used to derive a POD. The BMD median values for the lowest pathway BMD median were 213 µM (method 1) and 10.6 µM (method 2). The more conservative value of 10.6 µM was used for the final safety assessment. The different BMD values are probably a result of different analysis steps during the BMD analysis. This study highlights the importance of using standardized analysis methods in order to use toxicogenomics as a reliable tool to assess systemic toxicity of compounds in a NGRA.

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P06-10
NGS-based discovery of circulating microRNA biomarkers of drug-induced pancreatic injury (DIPI)

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Available biomarkers of DIPI including serum amylase and lipase lack sensitivity, pancreas specificity, and proportionality to injury, and are therefore inadequate to support safe development of potential therapeutics that carry a risk of DIPI. MicroRNAs (miRNAs) are short non-coding RNAs with important regulatory functions. High cross-spe-

cies conservation, tissue-specific transcription, and stable presence in liquid biopsies have sparked high interest in the application of miRNAs as drug safety biomarkers.

A fit-for-purpose validated small RNA-sequencing workflow was applied for analysis of plasma from normal healthy volunteers (NHVs, n=155) and a prospective acute pancreatitis (AP) patient research biobank (University of Liverpool) consisting of 145 mild, 93 moderate, and 45 severe AP patients according to the Revised Atlanta Classification (RAC) of AP severity.

Prior to statistical analysis, miRNAs were filtered from ~2500, which were detected at least once, to those with ≥4 read counts in ≥31 samples, resulting in a set of 1108 remaining miRNAs. To identify miRNAs that correlated with AP severity, the data were split into a training (70%) and test set (30%). Differential abundance analysis was performed with DESeq2 on the training set, adjusting for age and sex, and mean abundance differences with FDR-adjusted $p<0.05$ were considered significant. miRNAs that exhibited a consistent change in abundance (based on the sign of $\log_2$FC) between NHV vs. mild AP and mild AP vs. severe, were selected, resulting in a set of 10 miRNAs. These miRNAs were further used to develop multivariable predictive models. A model consisting of three miRNAs differentiated AP from NHVs with a area under the receiver operating characteristic curve (AUROC) in the training set of 0.936 (95% CI 0.91–0.963) and test set 0.961 (0.934–0.988). A model with two of these three miRs differentiated uncomplicated (RAC mild) from complicated AP (RAC moderate or severe) with AUROC in the training set of 0.751 (0.683-0.819) and test set of 0.879 (0.806–0.953). A multiplex RT-qPCR assay was developed for targeted analysis of selected miRNAs to confirm NGS results and perform prospective validation of the clinical utility for diagnosis of DIPI.

We conclude that by minimising the number of miRs to reduce overfitting we have identified a panel of three miRs that simultaneously detect and predict the severity of AP with sufficient accuracy to justify further clinical assessment.

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P06-11

A vision for the use of molecular points of departures (PODs) for chemical testing

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The scientific community has long anticipated the use of molecular data in human and ecological health risk assessments of industrial chemicals and agrochemicals. Despite this, such data are rarely utilized in risk assessments. In this abstract, we propose a logical framework to explore the feasibility and potential development of transcriptomic methods as an alternative to the current apical endpoint-based regulatory toxicity testing paradigm. We outline four foundational principles that must be accepted by stakeholders for this transformative vision to be realized. First, transcriptomics is a reliable tool for detecting alterations in gene expression caused by internal or external factors in the test organism. Second, these alterations in gene expression are indicative of adverse or adaptive biological responses to stressors. Third, transcriptomics can be employed to establish a benchmark dose-based point of departure (POD) from short-term, *in vivo* studies at a dose level below which a concerted molecular change (CMC) is not expected. Fourth, the use of a transcriptomic POD, set at the CMC dose level, will support a human health-protective risk assessment. If these four principles are substantiated, this vision is expected to transform aspects of the industrial chemical and agrochemical risk assessment process that are focused on establishing safe exposure levels for mammals across numerous toxicological contexts. Additional considerations need to be standardized with respect to bioinformatics pipelines and study design. However, this approach would result in a significant reduction in animal use while providing equal or greater protection of human health. Importantly, these principles and approaches are also generally applicable for ecological safety assessments.

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P06-12

Deconvolution of multiple effect signatures in plasma metabolomics data using BMD modelling, concordance and complementarity of molecular points of departure derived from *in vivo* transcriptomic and metabolomic endpoints

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Background and Purpose: Molecular Points of Departure (PODs) from short term *in vivo* studies for risk assessment purposes have great potential to reduce the need for traditional animal-based chronic toxicity studies. While benchmark dose modeling (BMD) of transcriptomic (Tx) data and transcriptional POD (tPODs) are becoming commonplace, the use of metabolomics (Mx) for POD (mPOD) assessment is still a relatively new approach. A major challenge in plasma Mx data interpretation arises when a test substance induces multiple effects in multiple target organs, which can all be reflected in the plasma Mx profile. Here, we use BMD modelling to deconvolute multiple effect signatures in plasma Mx and to compare Tx and Mx based PODs from a short-term *in vivo* study with an HPPD inhibitor herbicide in rat.

Methods: Male and female rats were exposed to 5 concentrations of the compound in diet for 14 days. Mx data (polar and lipid) were generated from terminal plasma and liver samples by LC-HRMS. Tx data were generated using whole genome TempO-Seq on liver, kidney, thyroid, adrenal gland, testis and ovary. Benchmark dose modeling was performed in BMDExpress. PODs were derived as the most sensitive GO biological pathway (tPODs only), or as individual feature accumulation plot maximum curvature points (tPODs and mPODs).

Results: There were kidney histopathology findings at the top dose, as well as altered hematology and clinical chemistry results. Bioanalysis confirmed tyrosinemia at all doses. For Tx, only the liver and kidney had sufficient data to generate tPODs.

The plasma Mx BMD predicted 3 candidate mPODs. The first plasma mPOD was 1.57 mg/kg/day, driven by tyrosine-related metabolites. Interestingly, there was no measurable Tx response to HPPD inhibition or tyrosinemia. The second plasma mPOD was 8.65 mg/kg/day, driven by liver-associated metabolites. The third plasma mPOD was 352.4 mg/kg/day, driven by kidney toxicity biomarkers. By comparison, the liver tPOD by pathway method was 10.97 mg/kg/day, and the kidney tPOD was 317.7 mg/kg/day.

Conclusions: The collection of predicted mPODs reflected the findings of the study: HPPD inhibition induced tyrosinemia, liver and kidney effects, at doses that aligned with the bioanalysis and Tx results. Mx and Tx produced similar molecular POD for the liver and kidney findings.The results of this study indicate that BMD can aid the interpretation of plasma Mx data by grouping metabolites based on target organ effects and thus increase the accuracy of target organ identification when multiple effect signatures are present in a plasma Mx profile. More work is needed to assess if plasma mPODs could potentially serve as a triaging tool in toxicology studies to identify tissues for additional Tx and/or Mx analysis.

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P06-13
Mitoxantrone disrupts brain glutathione defence and the metabolic pathways in CD-1 mice

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Long-term cognitive dysfunction caused by chemotherapeutic agents is commonly referred to as 'chemobrain'. Mitoxantrone (MTX) a topoisomerase II inhibitor used in the treatment of multiple sclerosis and several cancer types by binding and intercalating with DNA [1]. Despite its widespread clinical use, MTX can potentially cause chemobrain. However, its neurotoxic mechanisms are still poorly understood. This study aimed to elucidate the adverse outcome pathways (AOP) activated in the brain following exposure to a clinically relevant cumulative dose of MTX. Three-month-old male CD-1 mice received bi-weekly intraperitoneal administrations of MTX for three weeks until reaching a total cumulative dose of 6 mg/Kg. Euthanasia was performed two weeks after the final administration, with the left-brain hemisphere subjected to targeted profiling of the glutathione metabolism and the right hemisphere to an untargeted metabolomics approach. Our results demonstrated a significant impact of MTX treatment in the thiolomic profile by decreasing the free and total levels of cysteine (Cys), cysteinylglycine (CysGly), and reduced glutathione (GSH). Metabolic analysis revealed alterations in pathways related to phosphatidylethanolamine and unsaturated fatty acids biosynthesis, as well as glycerolipid metabolism. These findings provide insights into MTX-induced neurotoxicity through omics analyses, uncovering previously undescribed pathways.

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P06-14
Single-nucleus RNA-sequencing of the testis in dibutyl phthalate-exposed mice

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Phthalates are widely used as a plasticizer in plastic products and known endocrine disruptors. Studies show that phthalate exposure may affect male reproductive system, but additional experimental evidence *in vivo* for the exposure-driven male infertility is needed. To mechanistically evaluate male reproductive toxicology of phthalates, we conducted single-nucleus RNA-sequencing (snRNA-seq) of testes from dibutyl phthalate exposure (DBP) mice. In a pilot study, single nuclei were isolated from frozen testes of 4 adult mice (2 control mice, 1 low- and 1 high-dose exposure mouse). The whole procedure was carried out at 4°C within 1 hour and isolated nuclei were sequenced using the 10x Chromium single-cell 3' reagent kit. A total of 17889 nuclei passed QC with an average of 1242 genes and 1996 unique molecular identifiers (UMIs) per nucleus from 4 mice. Unsupervised clustering identified all major testis cell types including 5 germ cell types in different differentiation stages and 5 somatic cell types including Leydig cells, Sertoli cells, macrophages in all 4 samples. Furthermore, myoid and fibroblast cells showed similar in expressing Pdgfrb, but a distinct pattern in contractile gene expression. All known testicular cell-type specific markers in annotated clusters were detected, supporting the method's reliability and robustness. Preliminary differential expression analysis between controls and exposures showed that 31 abundantly expressed genes in macrophages were downregulated in the two exposure groups, compared to only 1 in both Leydig and Sertoli cells. Replication with more samples is needed to confirm these DBP-induced effects. In conclusion, snRNA-seq provides a powerful tool in toxicological assessment *in vivo* with single-cell resolution and mechanistic insights and is especially suited for biobanked complex tissues such as the testis.

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P06-15
Temporal transcriptomic analysis reveals Parkinson's disease-related perturbations upon increasing polychlorinated biphenyl levels in human iPSC-derived dopaminergic neurons

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Parkinson's disease (PD) is a second most common neurodegenerative disease which is characterized by progressive, unintended movements, such as shaking and stiffness. Prevalence of PD is increasing with age, and currently more than 1% of the population above 60 years are affected. Sporadic PD represent about 90% of the PD cases and risk factors comprise next to heredity, age, and gender also exposure to pesticides and herbicides. Moreover, epidemiological and experimental studies have identified exposure to polychlorinated biphenyls (PCBs) as a potential risk factor for Parkinson's disease. PCBs are synthetic chemicals primarily used as coolants and insulators in electrical equipment. Although banned for several decades, PCBs persist in the environment for long periods and therefore humans are still exposed mainly as a consequence of dietary intake.

The molecular mechanism of the potential involvement of PCB exposure in the etiology of PD are unknown. The objective of this work was to determine the PD-related transcriptomic perturbations upon PCB exposure in human induced pluripotent stem cell (iPSC)-derived dopaminergic neurons.

Human iPSC-derived dopaminergic neurons were exposed to multiple physiological relevant concentrations of PCBs (10nM, 500nM and 10.000nM). For each concentration biological triplicates were generated. Resulting cells were examined using temporal transcriptomic analysis by RNA sequencing at 24h and 72h after exposure. A Wald test was used for the statistical analysis of differential expressed genes (DEGs) in the PCB group against the untreated control group. For a

gene to be considered differentially expressed, the threshold of false discovery rate was set to <0.05.

The analysis revealed no significant DEGs at 10nM, 99 significant DEGs at 500nM and 182 at 10.000nM PCB exposure. Gene overrepresentation analysis for the observed DEGs featured PD relevant pathways such as 'Potassium Channels', 'Signaling by Receptor Tyrosine Kinases' and 'Neurotrophin Signaling'. Moreover, the analysis revealed PCB-induced transcriptional changes in 48 genes which were directly related to the development of Parkinson's disease. These transcripts included dose-dependent downregulation of well-known PD-genes such as vitamin D receptor (*VDR*), insulin like growth factor 1 (*IGF1*) and heme oxygenase 1 (*HMOX1*) at 24h after PCB exposure.To our knowledge this data shows, for the first time, the potential involvement of PCB-induced transcriptional changes in the etiology of sporadic PD in human iPSC-derived dopaminergic neurons. Moreover, the study highlights iPSCs as a promising tool to improve our understanding on gene expression dynamics in the development of neurodegenerative diseases.

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P06-16
Application of transcriptomic analysis to an MPP+-induced *in vitro* 2D model system for studying Parkinson's disease in search of potential drug modulators

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Parkinson's Disease (PD) is a neurodegenerative disorder, characterized by a complex interplay of genetic and environmental factors, including exposure to toxicants. However, little is known about protective risk factors for PD development. SH-SY5Y 2D dopaminergic cells, treated with the neurotoxicant MPP+ provide an accessible model, capable of reproducing important PD-specific pathological changes.

The aim of this work is to apply a bioinformatic approach to identify relationships between pathological processes, modelled in MPP+-treated SH-SY5Y cells and the transcriptomic fingerprints of drugs.

The RNA-Seq data analyzed were obtained from the series GSE203522, accessible from the Gene Expression Omnibus and selected due to the high-quality of the reads. It has been previously used to highlight the difference between 2D and 3D cultures on the development of dopaminergic neuronal markers while reducing proliferation markers' expression. The current study builds upon this notion, by further exploring the transcriptomic profile of the more commonly used 2D model. RNA-Seq reads were pseudo-aligned by Kallisto and the result was analysed for differential gene expression (DGE via DESeq2 R Package). Then, a gene set variation analysis (GSVA R Package) was done with the reference gene set LINCS L1000 "Library of Integrated Network-based Signatures", validated on a broad range of cell lines and drug perturbagens.

Our results show that there are similarities between the transcription profiles of the model system and two substances, estradiol and methylprednisolone. This outcome could be related to the notions of an inflammatory component to the disease and of lower risk of PD in women. It confirms that MPP+-treated SH-SY5Y cells in 2D culture are an appropriate model for researching the effects of disease modulators, affecting glucocorticoid- and estrogen-related transcriptional regulation.

In conclusion, our bioinformatic approach highlights the applicability of the 2D model systems in representing aspects of mitochondrial toxicant-induced PD. It hints at potential modulators, influencing its development, and provides direction for further confirmatory and exploratory research.

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P06-17
Assessment of genome-wide methylation changes caused by TiO_2 nanoparticles on human intestinal caco-2 cells

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Titanium dioxide nanoparticles (TiO_2NP) are widely used in industry and biomedicine (e.g., engineering, cosmetics, pharmaceutics, and food industry) due to their highly interesting properties, such as brightness, refractivity and UV radiation absorption capacity. In the food industry, food-grade TiO_2 (E171) is commonly used to whiten and make food products better-looking, while enhancing their flavours. Although E171 has been banned from all food products inside the EU due to its possible genotoxicity, ingestion of TiO_2NP may still occur in other countries, or even within EU through contaminated food or water, consumer products (e.g., toothpaste, lipstick) or pharmaceuticals. Although there are many studies on the toxicological effects of TiO_2NP, very few focused on its effect in DNA methylation, an epigenetic mechanism that regulates gene expression by the addition of methyl groups to the cytosine of CpG dinucleotides, which show increased concentration in gene promoters. Moreover, none of these studies used genome-wide approaches that allow uncovering which genes have their methylation pattern altered by TiO_2NP, and consequently exploring the consequences of TiO_2NP exposure on molecular pathways. This study aimed to investigate potential effects of three different types of TiO_2NP (anatase, rutile or brookite phase) on the DNA methylation pattern of intestinal cells.

Caco-2 epithelial intestinal cells were exposed to each TiO_2NP or exposure medium (control) for 24h. After genomic DNA extraction, DNA libraries were generated using the Premium Reduced Representative Bisulfite Sequencing kit (Diagenode) and sequenced on the NextSeq 550 system (Illumina). The Galaxy platform was used for read treatment and mapping, methylation calling and assessment of differentially methylated regions between exposed and non-exposed cells. Pathway analysis was performed using Reactome, and gene ontology analysis was conducted with the ClueGO plugin in Cytoscape.Our results demonstrated significant differential methylation of 92 genes (21 hyper- and 71 hypo-methylated), 70 genes (12 hyper- and 58 hypo-methylated) and 88 genes (21 hyper- and 67 hypo-methylated) for the anatase, rutile and brookite phase TiO_2NP, respectively. Functional pathway analysis of the genes with differences in DNA methylation identified enrichment of several relevant cellular pathways that may affect cell proliferation, differentiation and survival, with some of those being associated with colon cancer. Moreover, although some dysfunctional pathways are common to the three types of TiO_2NP, many are type-specific, suggesting different molecular mechanisms of action for each TiO_2NP. This study highlights the relevance of assessing genome-wide methylation patterns to uncover new genes and pathways impacted by nanoparticle exposure in human cells.

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P06-18
Enhancing prediction and priorisation of adverse effects: TOXsIgN as a comprehensive resource for toxicogenomics

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In a context where there is a need to systematically determine the toxic risk of chemicals that are ubiquitous in our environment and to which humans are constantly exposed [1] while limiting the use of animal testing (3Rs principle), the need for alternative strategies based on toxicogenomics and in silico approaches to complement traditional toxicology in the identification of potentially harmful contaminants has never been more urgent. Here we present TOXsIgN [2], a web-based toxicogenomics resource specifically designed for the storage, dissemination, and comparison of toxicogenomic signatures (up/down regulated gene lists) across species and technologies.

Since the first publication, the system architecture has been redesigned by using modern web technologies to provide a more stable and user-friendly experience. This major update greatly facilitates the submission process and introduces ML-based predictive toxicology tools, such as the Chemical Prioritisation System (ChemPSy). The expanded database now includes a large collection of signatures from public repositories and emerging technologies, including TempO-seq and BRB-seq [3], underlining the platform's commitment to supporting the evolving needs of toxicology research. TOXsIgN allows users to compare their own toxicogenomics signatures with the 637,000 signatures already hosted in the system. Users can also predict the toxicological effects of a given compound based on their toxicogenomics signature, making TOXsIgN a valuable tool for predicting chemical toxicity and reducing reliance on animal testing, in line with the 3Rs. TOXsIgN's comprehensive update not only advances toxicogenomics research, but also provides a central resource for regulatory agencies, providing a data-driven foundation for safer chemical regulation and public health protection.

TOXsIgN's represents a significant advance in the field of toxicogenomics, providing researchers with a more powerful tool for comparing and predicting chemical toxicity, with a user-friendly interface and expanded database content, reinforcing its position as a cornerstone of modern toxicological research.

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P06-19
Murine liver mRNA expression analysis of repeated exposure to chemicals by Percellome Project protocols

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The Percellome Project aims at reinforcing and replacing the safety factor in toxicology by comprehensively identifying the transcriptomic networks induced by xenobiotics in murine organs. "Percellome" method was developed [1] to generate absolute copy numbers of mRNAs in a "per one cell" basis. Data from the Affymetrix MOE430 2.0 GeneChip are absolutized and visualized in 3-D graphs (time x dose x copy number). Datasets of mouse liver (4 time points x 4 dose levels, triplicate, 48 GeneChip data per chemical/organ) on 160 chemicals are compiled [2,3].

In addition, newly designed repeated dose study which is insipred by a single dose study on gene knockout mice, was developed. This new protocol uses wild type mice. To all 48 mice, an equal dose of a chemical is repeatedly exposed for 14 days to create a "chemically-induced transgenic state". Then, on the 15th day, a single dose of a chemical was given as decribed above and the liver was sampled at 2, 4, 8 and 24 hours thereafter (designated as [14+1] protocol). Now we have data on thalidomide and methylcellulose in addition to CCl_4, clofibrate, valproic acid, and corn oil in terms of Affymetrix GeneChips for the 48 mice and ChIP-seq data of H3K4me3, H3K27me3, H3K27Ac, and H3K9me3 for control and 14 day exposed mice. A shorter version, i.e. 4-day repeat plus one single dosage or [4+1] is developed and performed for more than 10 chemicals including estragole, DEHP and PFOA.

The effect of repeated dosing on mRNA expression can be interpreted as a combination of two elements, i.e. baseline response (BR: gradual shift of the basal mRNA expression level by repeated dosing) and transient response (TR: alteration of the magnitude and/or pattern of the quick response in 2 to 24 hours). The BR and TR were generally linked in a way that lowering of the BR is linked to the suppression of the TR, and vice versa. And ChIP-seq analysis reveals that BR and TR of mRNA of some characteristic genes were explained by the histone modification induced by the repeated dosing; enhanced mRNA response with increase in H3K27Ac and/or H3K4me3 (activation marks), and suppressed mRNA response with increase in H3K9me3 and/or H3K27me3 (repression marks). Analysis on [14+1] and [4+1] data including thalidomide and PFOA will be briefly presented, along with the development of supportive analytical tool using AI technologies [4]

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P06-20

OMICs approaches linking effects of endocrine disrupters on developing rat hippocampus with impaired memory function in adult offspring and human stem cell-derived *in vitro* models

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To develop novel approaches of developmental neurotoxicity (DNT) testing, effects of known or suspected endocrine disrupters on OMICs in developing rat hippocampus were investigated as a link between *in vivo* and *in vitro* data. The chemicals chosen had been found to be associated with impaired children's behavior in the SELMA study: Bisphenol F (BPF, 3.6 or 0.036 mg/kg), butylbenzylphthalate (BBzP, 200 or 20 mg/kg), cyclohexane dicarboxylic acid diisononyl ester (DINCH, 300 or 30 mg/kg), perfluorooctanesulfonic acid (PFOS, 0.75 or 0.3 mg/kg), permethrin (PMT, 3.6 or 0.36 mg/kg), or triphenylphosphate (TPHP, 20 or 2 mg/kg) was administered in chow to F0 rat dams from pre-mating until lactation. The higher dose was chosen from reprotox data. Hippocampus was taken on postnatal day 6 (PND 6) for transcriptomics, epigenomics and metabolomics performed in the same tissue sample. One pup/sex/litter of the higher dose group was raised to adulthood for testing of activity, memory function, emotional and social behaviors.

Memory function (Morris water maze) was impaired by BPF and BBzP in adult male offspring and by DINCH and TPHP in female offspring, while PFOS was ineffective (the PMT group could not be tested for behavior). Transcriptomics of developing hippocampus at PND 6 was correlated with behavioral outcome in adult offspring of the same litters. Weighted gene co-expression network analysis (WGCNA) identified gene modules in PND 6 hippocampus that were significantly linked with chemical treatment and behavioral outcome in a sexually dimorphic manner. Impaired memory function was linked with developmentally relevant signalling pathways, including proneural factors (Ascl1, Neurog2) as well as factors inhibiting neuronal differentiation, Wnt and Notch signalling, and genes involved in cell cycle regulation, differentiation and synaptogenesis, and epigenetic mechanisms. Specification of interneuron subtype was also affected, in medial ganglionic eminence-derived interneurons by BPF and in caudal ganglionic eminence-derived interneurons by BBzP in males. Comparative analyses with metabolomics data of PND 6 hippocampus are ongoing.

Preliminary data of comparative transcriptomic analysis of human stem cell-derived brain organoids and PND 6 hippocampus suggest that it may become possible to characterize treatment effects on corresponding functional modules, with differential responses according to sexual phenotype and genotype.

Our study revealed gene expression patterns induced by different environmental chemicals in developing hippocampus that are linked with adverse behavioral outcome. The combination of transcriptomics, epigenetics and metabolomics is expected to identify molecular targets that could be tested for their predictive value.

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P06-21

Multiparametric *in vitro* assays combined with high-throughput RNA sequencing for cardiotoxicity risk assessment and mechanistic insight

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Cardiotoxicity remains one of the most common adverse drug reactions reported leading to drug attrition in pre-clinical and clinical drug development[1]. Development of non-clinical models with better predictive value are required to significantly improve cardiac safety. A previous pilot study using 42 reference compounds provided proof of concept of the synergism of our multiparametric *in vitro* assay and high-throughput RNA-sequencing. Here, an expansion of the compound set was used to further validate our cardiotoxicity assessment approach.

A cardiac safety database of 163 reference compounds, including the CiPA compound panel[2], was built by selecting chemicals across several therapeutic indications. The database covers a broad range of mechanisms of action including ion channel blockers (Na^+, K^+, Ca^{2+}), receptor modulators (adrenergic, dopamine, serotonin, histamine, acetylcholine, glucocorticoid, sulfonylurea), enzyme activities (COX, phosphodiesterase) and DNA metabolism. The effects of all compounds were investigated in human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) with a combined risk assessment strategy which integrates high-content imaging (HCI), kinetic monitoring of calcium transients (CaT), release of lactate dehydrogenase (LDH) and whole genome high-throughput RNA-sequencing (ScreenSeq™). The safety database contains data from all 4 high-throughput assays for a single treatment timepoint (24 h) of an 8-step dose-response range.

Differential gene expression and pathway enrichment analysis showed that cardiotoxicants induce responses at lower concentrations in relation to the maximum plasma concentration (C_{max}) than non-cardiotoxicants. Dose-response analysis with BMD Express 2 confirmed that cardiotoxicants had generally lower gene and pathway points of departure. Cardiotoxicity prediction methods based on minimal effective concentrations of all assay responses and C_{max} revealed that a combined HCI, CaT and RNA-sequencing approach provided the strongest cardiotoxicity prediction metrics, confirming a 42-compound pilot study[3]. Clustering analysis grouped compounds by mechanisms of action and toxicity mechanisms. Transcriptional signatures of compound mechanism of action and cardiotoxicity were established, which allowed quantification of distinct target-related and toxicity responses.

In summary, the developed combined multi-endpoint approach could detect and predict a diverse range of cardiotoxicity mechanisms and allowed the early de-risking of New Chemical Entities (NCEs) in drug discovery.

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P06-22
Prevalidation of Epi²SensA, an assay using gene expression with the EpiDerm RHE model to predict skin sensitization *in vitro*

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The Epidermal Sensitization Assay (EpiSensA) developed by Kao Corporation was validated by JacVAM for integration into the OECD TG 442D. It addresses the key event 2 of the AOP (1) and uses the LabCyte reconstructed human epidermis (RhE) as experimental system. Thanks to the air liquid interface of the RhE and to their metabollic activity (2), the method showed high performances for pre/pro-haptens as well as for lipophilic substances.

The Epi²SensA is a similar method using the EpiDerm™ RhE model from MatTek as experimental system. EpiDerm™ is already validated in the OECD TG431, TG439, TG498 and is available in a large number of OECD member countries. As part of the first stage of the OECD "me-too" validation, we conducted a pre-validation of Epi²SensA in two laboratories.

Epi²SensA is based on gene expression quantification of four biomarkers related to the induction of skin sensitization: activating transcription factor 3 (ATF3) and interleukin 8 (IL-8) which reflect the inflammatory response of keratinocytes; glutamate-cysteine ligase, modifier subunit (GCLM) and DnaJ (Hsp40) homolog, subfamily B, member 4 (DNAJB4) which reflect the induction of cytoprotective gene pathways. The prediction model of the assays is based on the modulation of the expression of the four target genes quantified by quantitative real-time PCR analysis after topical exposure of test chemicals. The chemical is classified as skin sensitizer if the fold induction of the expression of at least one of the genes exceeded the respective cut-off value: 15-fold for ATF3, 2-fold for GCLM or DNAJB4, and 4-fold for IL-8.

For the prevalidation study, 10 chemicals covering the whole range from weak to extreme sensitizing compounds were tested in two laboratories, Mattek Corporation in the US and VitroScreen in Italy. Compared to the validated reference method (VRM), the protocol was optimized. To take account of the surface area of the EpiDerm™ model the volume of test chemical applied was increased from 5uL to 10uL. We evaluated the 6h exposure length as proposed in the VRM and a shorter time: 1h exposure stopped by rinsing step and followed by a 5h post-incubation. Reducing the duration of exposure to the chemicals tested minimizes the risk of cytotoxicity induced by certain compounds, and the post-incubation period allows the gene induction response to develop. This proved to be important for some cytotoxic compounds, particularly weak sensitizers which require a higher concentration to induce a positive response in the EpiDerm™ model. It is then possible to test higher concentrations while respecting the acceptance criteria of 80% cell viability.

Based on the set of chemicals tested, the accuracy of the method and its reproducibility with the EpiDerm™ RhE model meet the requirements of the VRM performance standards. The next step is now to transfer the method to two additional laboratories to carry out the multicenter validation study this year.

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P06-23
Integration of metabolomics and transcriptomics for improved mechanism-based hazard characterization of drug-induced liver injury

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RISK-HUNT3R, an EU-funded project, addresses multidisciplinary aspects of toxicological studies with the aim to integrate human centric next generation testing strategies promoting the 3Rs for risk assessment. In one case study of the project, we investigate the mechanisms of DILI by conducting an integrative metabolomic (µMIV system, ~300 metabolites)-transcriptomics (TEMPO-Seq, >14000 genes) study in an *in vitro* liver test system (HepG2) exposed to a set of 10 DILI compounds with various severities in 7 concentrations for 6 and 24 hours. Further, CALUX (activity of certain receptors and transcription factors) and mitostress (mitochondrial respiratory rates) assays are being used to complement the data.

To select test concentrations from IC0.1 to IC70, we performed ATP-based cytotoxicity CellTiterGlow assays. The following OMICS study itself exhibited a dose-response and time-response patterns. We noticed concordance between metabolomics and transcriptomic perturbations in several compounds indicating similarity in the mechanisms of these compounds. Our results also indicated that the mitochondrial respiration was perturbed at lower concentrations of the compounds. In addition, the CALUX assay enabled us to decipher the activity of tested compounds towards receptors and transcription factors linked to the mechanisms of liver injury. Further analyses based on correlation of metabolites and transcripts resulted in an integrated network of metabolites and transcripts and revealed subnetworks of gene-module/metabolites indicative of cellular responses linked to the mechanisms of liver injury. Additionally, benchmark concentrations were derived from metabolites and transcripts by two different approaches and resulted in similar concentrations. Altogether, our OMICS findings apparently characterize the hazard that leads to liver injury, which may result in increased predictivity of DILI.

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P06-24

Chemical and metabolomic profiling of zebrafish (*danio rerio*) embryos exposed to DDE, DEHP and PFOA

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Zebrafish (Danio rerio) is a commonly used vertebrate model in the study of environmental toxins and their effects on human health. One such toxin, p,p'-Dichlorodiphenyldichloroethylene (DDE), is a byproduct of the prohibited pesticide DDT. DDE is widely present in the food chain and can accumulate in the tissues of living organisms, leading to various metabolic disorders. Di-2-ethylhexyl phthalate (DEHP), a member of the phthalates family, is a hydrophobic plasticizer which can be harmful to human reproduction and development. Perfluorooctanoic acid (PFOA), a perfluorinated compound with a long chain structure, is known for its carcinogenic properties and its toxic effects on reproduction and the immune system. PFOA can also interfere with thyroid and lipid metabolism. By combining chemical and untargeted metabolomics analyses, the objective of this study was to assess the metabolic responses of 5 days post fertilization (5dpf) zebrafish embryos exposed to two different concentrations of DDE, DEHP, and PFOA and connect them with metabolic disorders. Global untargeted metabolomics analysis was performed using an Agilent 6540 Ultra High Definition Accurate-Mass QTOF instrument in positive and negative ionization modes. Moreover, the samples were analyzed using Reversed Phase (RP) and HILIC analytical columns to increase the coverage of the detected metabolites. The Agilent MassHunter Software v.B.06.01 was used to collect the data, followed by the data pre-processing and processing steps (data cleaning, log transformation, normalization, and batch effects correction), through the Bioconductor R-based package XCMS, and IPO. The xMSannotator R package was used for Network-Based annotation, retrieving information from HMDB, KEGG, and Lipid Maps. Statistical analysis was performed using a Mann-Whitney U test with FDR post hoc in order to determine the statistically significant differential metabolites between treatment groups. A p-value cutoff of <0.05 and a FC>2 were applied. The majority of the annotated metabolites belonged to the family of lipids and more precisely to Glycerophospholipids, Glycerolipids, Sphingolipids, and Sterol lipids. Fatty acyls and carboxylic acids were also significant categories with a high number of annotated metabolites. Pathway analysis revealed perturbations in lipid-related pathways (Sphingolipid and Glycerophospholipid metabolism), and other essential biological and metabolic processes, including metabolism and synthesis of amino acids, such as tryptophan metabolism. Overall, the metabolomics analysis in the 5dpf zebrafish samples exposed to DDE, DEHP, and PFOA allowed for the detection and identification of various differentially metabolized compounds, with DDE exhibiting the highest impact on the metabolism of the zebrafish, which can also be confirmed by chemical analyses.

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P06-25

The plasma metabolome of Genistein and Daidzein in rats

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Although there is limited evidence on the relevance of potential adverse effects of the endocrine effects of soy isoflavones (mainly Genistein and Daidzein), guidance values have been proposed based on epidemiological data, intake surveys as precautionary measures.

We investigated plasma metabolomes using GC-MS and LC-MS/MS techniques at days 7, 14 and 28 of the isoflavones Genistein (1000 and 300 mg/kg bw gavage and 1000 and 200 ppm dietary) and Daidzein (1000 and 300 mg/kg bw gavage) in young adult male and female Wistar rats. The heteroscedastic t-test ("Welch test") was used from statistical analysis at $p<0.05$. Similarity analysis of the compounds metabolic profiles with predefined patterns in MetaMap®Tox (>110 patterns, currently covering 42 modes of action) was determined with an algorithm using a median r value metric.

For males and females, gavage dosed with high dose (HD) Genistein, matches with previously defined patterns for sex hormones (estrogens) and for ovarian changes (days 7 and 14, less pronounced day 28) were noted. At the low gavage dose (LD) this was only noted for females. In addition, there were some indications of an effect on liver metabolism. For the feeding study there were no such matches at any of the doses. HD Daidzein matched with estrogenic effects and changes in liver metabolism at day 7. At LD an estrogenic effect was suggested for females only at day 28. Comparing the entire metabolome profile of Genistein with >1000 other profiles in the MetaMap®Tox data base showed good correlations with other estrogenic compounds for both gavage treatments at the HD. For the feeding studies such matches were not apparent. Overall, the data suggest the absence of an estrogenic response for Genistein in the feeding studies at 1000 ppm (approx. 90 and 100 mg/kg bw for males and females respectively) and lower. In 1000 ppm males, there were some weaker correlations with compounds influencing liver and liver metabolism. For the gavage studies, HD Daidzein females showed good correlations with many estrogenic compounds, for males with only one. For LD males there were more such matches, however, as Daidzein and those estrogenic compounds for which an association was noted, were all administered in corn oil as a vehicle, a bias based on the vehicle cannot be excluded. Other compounds associated with the Daidzein profile, particularly in males suggest an activated PPAR alpha like potential.The metabolome profiles of the two compounds showed commonalities but also significant differences. This study showed that metabolomics is suitable for mode of action identification and for determination of point of departure.

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P06-26

Application of a validated Q-FISH method for telomere length estimation to assess lifestyle exposure effects in young adults

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Purpose: Age-related disorders (ARDs) involve mechanisms of accumulated molecular changes over time, often due to environmental exposures. Approaches to exposome research require a holistic assess-

ment of lifestyle effects on overall health and wellness. Especially for ARDs, early assessment of biomarkers quantifying lifestyle effects on physiology is essential. Telomeres (TL) are nucleoprotein structures at chromosome ends regulating cell growth. TL shortens with each cell division, serving as a hallmark of cellular senescence and aging, influenced by lifestyle exposures, oxidative stress, and inflammation. Analyzing TL characteristics for the estimation of biological age (BioA) and deviations from chronological age (ChronA) is established in exposome research, particularly when combined with lifestyle data. This work presents preliminary results from evaluating telomere dynamics via Quantitative Fluorescent In-Situ Hybridization (Q-FISH) in young adults. Q-FISH determines essential TL parameters (e.g., mean or median length, proportions of critically long or short TLs, etc.) and allows the estimation of BioA, which enables the association of biomarkers such as TLs with lifestyle features.

Methods: A cohort of young adults (n=21), aged 20–40, participated in this study. Participants underwent comprehensive assessments of lifestyle and environmental exposures, including self-reported physical activity, body mass index (BMI), dietary supplement usage, sleep patterns, smoking, alcohol consumption, anxiety/stress, and working hours. Blood samples were collected for TL length analysis through Q-FISH. The results were analyzed considering the estimation of BioA in relation to the lifestyle report.

Results: The mean (± S.D) TL length (base pairs, bp) in the cohort was calculated to be 11351 bp (±1,343), with a cutoff (20th percentile of short TLs) at 5956 bp (±900). The mean BioA was estimated to be 29.7 years (±6.5), similar to the cohort's mean ChronA of 30.6 years (± 6.0). The cohort can be subdivided into two subgroups: 11 individuals with a shorter BioA (27.0±6.6 years) than ChronA (31.5±6.2 years), and 10 with a higher BioA (32.6±5 years) compared to ChronA (29.5±5.2 years). Individuals with a shorter BioA preserved longer TLs (12061±701 bp) and critical short TLs (6665±497 bp) compared to those with a higher BioA (TLs 10076±1003 bp and cutoff 5175±426 bp). They also exhibited better BMI, stress management, exercise habits, avoided smoking or alcohol, healthier diets, and supplement usage. Those with a higher BioA had higher anxiety/stress, often smoked or consumed alcohol, were less active, and used fewer supplements. Conditions like chronic inflammation-associated diseases, sleep quality, and workload were similar in both subgroups.

Conclusions: This work demonstrates the applicability of TL dynamics in exposome studies as a biomonitoring method for real-life exposures to predict exposome impacts on health and wellness.

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P06-27
Transcriptomics data to inform risk assessments by extensive gene set enrichment approaches linking to Adverse Outcome Pathways

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The integration of high-throughput omics data, particularly transcriptomics, holds promise for advancing risk assessment methodologies in toxicology. Despite its potential in elucidating toxicological mechanisms, interpreting transcriptomics data within risk assessment frameworks remains challenging. [1] This study explored multiple approaches to implement transcriptomics data by using gene set enrichment analyses closely tied to a complete Adverse Outcome Pathway (AOP) network for liver toxicity based on the AOP-Wiki. By leveraging gene set enrichment methods based on biomarker genes, ontologies, and pathways [2], we aimed to elucidate the complex molecular mechanisms underlying AOPs, enhancing our understanding of toxicological pathways and quantifying Key Events. The integration of enriched gene sets with the AOP network was expected to improve the interpretability and relevance of transcriptomics data in risk assessment.

Using transcriptomic datasets of known toxicants, we aimed to define the applicability domain of each methodology tested. While these methods enabled the identification of activated Key Events by chemical exposure, our findings also highlight inconsistencies in KE activation throughout AOPs. Moreover, our study demonstrated that the different scopes of the annotated gene sets provide widely varying results, indicating the necessity for careful selection and validation of gene sets. Within the liver AOP network, comprising 31 AOPs and over 150 Key Events, transcriptomic data were utilized to quantify Key Events based on enrichment scores using different types of gene sets. Collectively, the results emphasized the importance of refining gene set enrichment analyses to improve the accuracy of risk assessments.

Our study underscored the potential of transcriptomics data in informing more accurate and predictive risk assessments, contributing to enhanced chemical safety evaluations and regulatory decision-making processes.

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P06-28
On the way to Safety-by-design: using artificial intelligence for de novo design of small molecules

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The discovery and development of novel molecules with biological activity can be a costly and long process. A significant part of this R&D process is designing a molecule with a desired safety profile. In the ideal scenario every molecule that is selected for undergoing the development pipeline is safe by design. Generative AI is a tool that could be used for generation of molecules with a decreased probability of undesired effects. As the design of molecules is a target-oriented approach, the first focus when constructing a novel algorithm is on the generation of de novo compounds inducing the desired biological effect. Thus, it makes sense to use the biological data as the input on which the model learns to produce a biologically active compound. To date, a few models were designed to sample chemical space primed with a target biological response. One of data modalities used to train these models were imaging data collected in cell painting assay. This method allows assessing changes in cellular morphology after applying a perturbation (treatment with a perturbing agent) thanks to fluorescent staining of cell compartments.

As a proof of concept, a conditional generative adversarial network has been trained on a set of 30,000 small molecules linked to their cell painting morphological profiles [1]. With the model learning the link between the chemical structure and biological response, we wanted to test how well the model performs in a realistic drug development scenario.

In this poster we present the results of generating molecules using morphological profiles of 10 overexpressed genes. Generated compounds were filtered to retain only valid, drug-like, and synthesizable compounds. Of the remaining compounds 76 were synthesized and

submitted to testing. Additionally, 39 controls were selected to compare to molecules already available on the market. First, cell painting data was acquired, and the morphological profiles of the generated compounds were compared to the overexpression profiles used to condition them. On average *de novo* compounds induce a cellular response closer to the parent profile than expected at random (p value 0.0065 with the highest tested concentration). Next, biochemical tests were performed to show that the response is elicited through the pathway regulated by the overexpressed gene. These tests revealed that 18% of generated molecules affect the target overexpressed in the parent profile.

Thus, we showed that conditional generative adversarial network can be trained to produce compounds that elicit the biological response of interest, and the generated compounds generally exert their effect through the cellular pathways that are modified in the parent condition. In conclusion, conditional generative adversarial networks represent a promising tool for *de novo* molecule design, paving the long-term way for safety by design development of biologically active molecules.

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P07 | Biomarkers of effect/exposure

P07-01
Monitoring of mercury release in dental amalgam wearers by measuring salivary and urinary mercury

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Background: Mercury amalgam remains the most effective and least expensive dental filling material. Mercury is considered by the World Health Organization as one of the ten chemicals or groups of chemicals that are extremely concerning for public health. The aim of our study is to assess exposure to mercury from dental amalgams through the measurement of salivary and urinary mercury.

Population and methods… This is a prospective, analytical study carried out at the National Toxicology Center of Algiers during the year 2004. The study included 48 subjects with dental amalgams and 12 subjects without amalgams. Mercury levels in saliva and urine were measured by cold vapor atomic absorption spectrometry using the "Flow Inject Mercury System".

Results: Compared to other studies, the urinary mercury values in our study are relatively high. This observation is also true for our controls. For salivary values, our subjects have levels (after chewing) that remain within the same range. The comparison of urinary and salivary means shows a significant difference between subjects with three or more amalgams and controls. There is also a significant correlation between the number of fillings and salivary concentrations. Among all amalgam bearers, there is no significant correlation between urinary and salivary concentrations. Elevated levels were noted in our controls compared to foreign studies, indicating probable general exposure (food, atmosphere, etc.).

Conclusion: There is a real exposure to mercury from dental amalgams, but is this exposure harmful to the health of the bearers? This risk remains "acceptable" as long as we stay below the accepted limits. It is necessary to protect at-risk groups (pregnant and breastfeeding women, children, and adolescents) from this exposure.

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P07-02
Effects of *Ericaria selaginoides* extracts on antioxidant status in Caco-2 cells

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Purpose: In the last few years, biomedical, pharmaceutical and nutraceutical industries have shown growing interest in novel compounds from marine organisms, including macroalgae. In this research, we provide a comprehensive overview of the cytotoxicity and antioxidant potential of the *Ericaria selaginoides* extracts using human colon adenocarcinoma cells (Caco-2), as a model system. Reactive oxygen species (ROS) are continuously produced in aerobic organisms as a natural by-product of oxygen metabolism and act as subcellular messengers in complex cellular processes, such as mitogen signal transduction, gene expression, and regulation of cell proliferation. Excess ROS is involved in various pathological conditions, including aging, cancer, and inflammation. Therefore, antioxidants to reduce ROS have been proposed to the prevention of diseases associated with oxidative damage. In this study, the antioxidant ability of brown seaweed extracts, *Ericaria selaginoides* was investigated.

Methods: The seaweed *Ericaria selaginoides* was collected from Comillas (Cantabria region, Spain) in the Atlantic coast of northern Spain. Algae samples were mixed with a mixture of hexane-isopropanol-water (10:80:10). Extracts were evaporated to dryness under vacuum conditions at room temperature. The residue was stores at –20ºC until the analysis. The phenolic content of algal extracts was determined according to the method of Wang *et al.* (2009) with minor modifications. The phenolic content of extracts called 115 and 117 were 23.73 µg phenolics/mg dw (dry weight) extract and 48.69 µg phenolics/mg dw extract, respectively (expressed as equivalents of phloroglucinol). Cytotoxicity induced by *Ericaria selaginoides* extracts was assessed determining cell viability by (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) MTT assays, and lactate dehydrogenase (LDH) leakage. Antioxidant activities of *Ericaria selaginoides* extracts were assessed by determination of malondialdehyde (MDA) levels as a marker lipid peroxidation; generation of ROS and nitric oxide (NO) production were also evaluated in human colon carcinoma cell line, Caco-2 cells.

Results: The phenolic content/mg extract was correlated to the antioxidant effect. The results of the biomarkers analyzed show that treatment of Caco-2 cells with *Ericaria selaginoides* extracts enhance antioxidant defences, which imply an improved cell response to an oxidative challenge.

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P07-03
Preliminary exposure data on neonicotinoid pesticides and their metabolites from the 2018–2019 Canadian Health Measures Survey

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Background: Neonicotinoids are a class of broad-spectrum insecticides used primarily in agriculture, horticulture and forestry. They are also used in household pest control to kill insects and control fleas on pets. The widespread use of neonicotinoids has led to their presence in food and water. As a result, diet is a potential route of human exposure to neonicotinoids.

Objective: To quantify biomarkers of neonicotinoid exposure in human urine and provide some initial measures of exposure in people living in Canada.

Method: A novel and sensitive UPLC-APCI-MSMS method was developed by the *Centre de Toxicologie du Québec* (CTQ), in partnership with Health Canada, to measure 13 biomarkers of neonicotinoid exposure in urine. Biomarkers include clothianidin (CLO) and its metabolite desmethyl-CLO (DCLO), imidacloprid (IMI) and its metabolites 4-hydroxy-IMI (4-OHIM) and 5-hydroxy-IMI (5-OHIM), thiamethoxam (THIAM) and its metabolite desmethyl-THIAM, acetamiprid (ACE) and its metabolite desmethyl-ACE (DACE), as well as nitenpyram, thiacloprid, dinotefuran, and sulfoxaflor. This method was employed on 114 stored urine samples from the 2018–2019 Canadian Health Measures Survey (CHMS). Detection rates were calculated for each biomarker. For those detected in at least 60% of samples, geometric means were calculated for the total population, as well as by sex, age group, season of collection, region of Canada and time of day of collection. Owing to the small sample size, survey weights were not considered for the analyses.

Results: Seven biomarkers (CLO, DCLO, IMI, 4-OHIM, 5-OHIM, THIAM, DACE) were detected in at least 60% of samples, while the remaining six were detected in fewer than 40% of samples. Concentrations for the seven biomarkers ranged from a geometric mean of 0.18 µg/L for CLO to a geometric mean of 0.028 µg/L for IMI. Concentrations of DACE were significantly higher in children and adolescents compared to adults ($p=0.004$). People living in the West region of Canada had significantly lower levels of CLO ($p=0.002$) and its metabolite DCLO ($p=0.002$) compared to people living in Ontario and the East region of Canada. There were no significant differences between sexes, or among season or time of day of collection.

Conclusions: The sensitive UPLC-APCI-MSMS method presents new opportunities for quantifying levels of neonicotinoids in urine samples from the general population. The preliminary results from the 2018–2019 CHMS provide early insight into neonicotinoid exposures in people living in Canada. This method is currently being used to measure neonicotinoids in a more robust, nationally-representative sample from the 2022–2024 CHMS.

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P07-04
Biomarkers of arsenical vesicant lewisite ocular exposure in an *in vivo* rabbit model and effective countermeasures by dexamethasone

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Lewisite (LEW), first synthesized for use in warfare in 1918 during WWI, continues to be a threat during terrorist activities, combat, and accidental exposure from stockpiles/improperly discarded munitions. It is a powerful/fast-acting arsenical vesicant that causes injuries primarily through ocular, dermal, and inhalation routes. Eyes, particularly the outermost transparent corneas, are most prone to LEW toxicity due to their highly aqueous environment/high turnover rate. Injuries can manifest as pain/discomfort, inflammation, neovascularization (NV), clouding, blistering, and vision impairment depending on exposure dose/duration/physiology of exposed individuals. There are no approved/targeted treatments for LEW-induced ocular injuries, due to lack of biomarkers following its exposure and associated mechanisms, which was the focus of our first line of studies. LEW ocular exposure in an *in vivo* rabbit model induced corneal ulceration, opacity, and thickness, as well as NV. Histopathology revealed increased corneal epithelial degradation/thinning and epithelial-stromal separation, increased keratocyte cell death, immune cell infiltration, and blood vessel counts in the stromal region of the cornea. Molecularly, COX-2 (inflammatory biomarker), VEGF (angiogenesis biomarker), and MMP-9 (proteolytic mediator biomarker) increased in cornea following LEW exposures. Establishing these critical biomarkers and associated molecular regulators set us for testing effective countermeasures. Previously, we found that dexamethasone (DEX; FDA approved anti-inflammatory steroid) was effective in treating mustard-vesicant induced corneal injuries in both *in vivo* and *ex vivo* models, thus, here we assessed the efficacy of DEX in treating LEW-induced corneal injuries using above established biomarkers. Using IACUC approved protocols, male New Zealand white rabbits (n=4–6/group/timepoint) were divided into four groups: control (no exposure/treatment), LEW ocular exposure (~0.2 mg/L; 300 ml/min flow rate for ~8 min), DEX 14-day and DEX 28-day treatment following LEW ocular exposure (LEW+-DEX). DEX was delivered 2 h post- exposure, 3x/day, for 14 or 28 days post LEW-exposure. Clinical, histopathological, and molecular biomarkers were assessed extensively in the collected cornea samples at the end of the study period, which showed that DEX effectively reversed LEW-induced corneal injuries at all levels, and that DEX 28-day treatment was more effective than the 14-day treatment, with almost complete reversals in most LEW-induced ocular injuries. Taken together, these outcomes provide novel and valuable *in vivo* model of ocular LEW exposure with established biomarkers of both acute and chronic corneal injury, as well as to evaluate and establish effective countermeasures against LEW-induced corneal injuries that could be valuable in mass causality/combat situations *(supported by U01 EY030405)*.

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P07-05
Miniaturization of haematology sampling in rodents on toxicology studies

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In line with the 3Rs initiative to Reduce, Refine and Replace the use of animals in research, both the number of animals on a study and the number of in-life blood draws is kept to a minimum. Microsampling techniques utilised for toxicokinetic (TK) assessment on rodent toxicology studies have already led to multiple welfare improvements including: reduced restraint times; minimal blood loss; reduced time spent within a warming chamber; and overall reduction of the number of animals included on a toxicology study [1]. Additionally, repeated microsampling has been shown to lessen the impact of blood volume draws on red cell parameters such as red blood cell count, haematocrit and haemoglobin [2]. While TK assessment by microsampling has been widely adopted, clinical pathology blood sampling in rodents is limited by analytical volume constraints. Often the terminal blood sample must be split across multiple endpoints (eg. clinical chemistry, haematology and cytokine assessment), so when temporal profiling of multiple critical parameters is necessary, animal numbers must be increased with the inclusion of satellite groups. In keeping with the 3Rs initiative, we aimed to establish a microsampling workflow for haematology analysis that would avoid the need for additional animals.

Here we compare a dilution protocol for haematological assessment to neat whole blood sampling using the Sysmex XN-V (Sysmex Corporation, Kobe, Japan) haematology analyser. Minimal differences in haematology parameters were observed in 30 µl blood samples from naïve mice and rats when diluted 1 in 7 with CellPack™ (Sysmex) compared to neat samples. Additionally, detectable dose-dependent changes in haematology parameters were observed in rats following a single oral dose of Carboplatin. These were comparable to measurements assessed in parallel on the Advia 2120 instrument (Siemens Healthineers, Erlangen, Germany). By minimising the volume required for haematological assessment, the impact of sampling on the animal is reduced, reduces blood draw time and facilitates concurrent assessment of multiple endpoints from a single sample.

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P07-06
Exposure assessment of children to mycotoxins: A preliminary biomarker study conducted in Ribeirão Preto, São Paulo, Brazil

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Mycotoxins are the toxic secondary products of certain fungi species that contaminate food products, particularly grains and dairy products. Infants and young children are the most vulnerable population groups subjected to mycotoxin exposure, due to their immature metabolism and elimination, and higher growth and development rates, among other factors [1]. Exposure assessments based on specific urinary biomarkers have important advantages, since biomarker excretion correlates well with the intake of some mycotoxins, such as aflatoxin (AF), zearalenone (ZEN), fumonisin (F) B_1 and ochratoxin A (OTA) [2]. In this study, a preliminary investigation on the exposure of infants and children from Ribeirão Preto, São Paulo, Brazil, to multiple mycotoxins in the diet was conducted using biomarkers in urine. Identification and quantification of mycotoxin biomarkers were performed using a previously validated method based on isotopic dilution and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [2]. Sampling procedures were conducted between August and December 2022, aiming at collecting samples of the first morning urine from 34 children, including preschoolers (aged 3–6 years, n=10), schoolers (aged 7–10 years, n=10), and adolescents (aged 11–17 years, n=14) cared in the Vila Lobato Community Social Medical Center of Ribeirão Preto. The study received ethical approval from the Research Ethics Committee (REC) of the School of Animal Sciences and Food Engineering, University of São Paulo. The mycotoxin biomarkers quantified in urine included aflatoxin M_1 (AFM_1) and OTA in 3 samples (8.8%), as well as FB_1 and ZEN in 2 samples (5.9%). The levels of AFM_1 and OTA in urine samples ranged from 0.127 to 1.579 ng/mL and from 0.140 to 1.385 ng/mL, respectively. FB_1 levels found in the two positive samples were 0.178 and 0.369 ng/mL, while ZEN concentrations in those samples were 0.024 and 0.026 ng/mL. Results of this preliminary trial indicate a clear exposure of children from the studied area to dietary AFB_1, OTA, FB_1 and ZEN. In particular, the levels of AFM_1 found in 3 urine samples warrant concern about the occurrence levels of AFB_1 in infant diets, since this genotoxic mycotoxin is classified in Group 1 (carcinogen) by the International Agency for Research on Cancer [3]. This an ongoing investigation that will increase the number of volunteers to provide a more robust data for estimation of probable daily intakes of mycotoxins in the diet of Brazilian children, based on their biomarker levels in urine. As a first study describing the exposure of children to multiple mycotoxins through urinary biomarkers in Brazil, the resulting data may help regulation agencies to better assess the mycotoxin exposure pattern in infant populations.

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P07-07
EFSA dietary exposure assessment related to the presence of Polychlorinated Naphthalenes (PCNs) in food

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Purpose: The European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain assessed the chronic human dietary exposure to Polychlorinated Naphthalenes (PCNs) for different EU population groups in the context of a European Commission request on the risks for animal and human health related to the presence of PCNs in feed and food.

Methods: The assessment was based on analytical data in food reported by European national authorities and research institutions. These were combined, at individual level, with national food consumption data from the EFSA Comprehensive European Food Consumption Database including data from 49 dietary surveys from 22 EU countries considering seven population groups (infants, toddlers, other children, adolescents, adults, the elderly and very elderly). Due to limited data available on other PCN congeners than hexaCNs, the assessment focused on i) individual hexaCNs (i.e., PCN-63, PCN-64/68, PCN-65, PCN-66/67, PCN-69, PCN-70 and PCN-71/72) and ii) a selected group of PCN congeners, i.e., 'mixture scenario', based on a sum of all hexaCNs and PCN-73. Due to presence of one very high PCN-69 concentration reported for an egg sample, scenario A and B – with and without this sample – were calculated. Two additional scenarios, including exposure via fish meat and via human milk consumption, were also assessed and an uncertainty analysis was performed.

Results: The highest mean exposure across the European dietary surveys was estimated for PCN-69 (scenario A) followed by PCN-66/67. The mean and P95 exposures for the PCN-69 scenario A were up to 5.61 and 19.4 pg/kg bw per day (toddlers), respectively. The food categories contributing mostly to the overall exposure to PCN-66/67 were fish and seafood and meat and meat products in the adult population, and milk and dairy products in infants. For other individual hexaCNs, eggs and egg products together with fish and seafood made the most important contribution. Regarding the 'mixture scenario', the highest mean and P95 exposure levels were estimated for the 'mixture scenario A' with up to 11.5 and 29.8 pg/kg bw per day (toddlers), respectively. An exposure scenario for high consumers of fish resulted in 95th percentile dietary exposure to PCN-66/67 being approximately two-fold higher in comparison to total population with the maximum exposure level estimated for toddlers (11.1 pg/kg bw per day). An exposure scenario for breastfed infants resulted in median exposure of 340 pg/kg bw per day for PCN-66/67 and 405 pg/kg bw per day for the 'mixture scenario' when considering high consumption of human milk. Taking into account the uncertainties involved, the assessors were all at least 95% certain that the highest 95th percentile exposure was $\leq$900 pg/kg bw per day.

Conclusions: The highest mean exposure to the individual hexaCNs and the mixture of hexaCNs was estimated for the young age groups, with the tendency to decrease moving to the older age groups.

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P07-08
BIOBRAND II: Interventions to reduce dermal and internal PAH exposure among firefighters and conscripts during education as firefighters

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In a previous study on recruits during firefighting training we found strong correlations between dermal polyaromatic hydrocarbon (PAH) exposure, urinary excretion of the PAH metabolite 1-OH-pyrene and DNA damage in blood cells [1]. Furthermore, the study showed that respiratory personal protection protected the firefighters from pulmonary exposure to particles [2]. This indicates that dermal exposure to PAHs is an important exposure route and that dermal exposure is important for the genotoxic effects.

In an ongoing study – BIOBRAND II – we are now investigating how dermal PAH exposure and PAH uptake may be reduced by different interventions. This is assessed in two study groups: 1) conscripts under education as firefighters and 2) real firefighters.

Conscripts: We recruited 79 conscripts from a rescue specialist educational course of whom 49 participated in all samplings of the study. The intervention tested was the use of inner gloves underneath the firefighting gloves. In addition, the recruits received information on adverse health effects of PAHs. For 38 conscripts, we assessed exposure to PAHs following three consecutive exposure scenarios: 1) Firefighting exercise without intervention, 2) control situation with no PAH exposure, and 3) firefighting exercise with intervention. To evaluate the effect of greater experience, an additional 13 conscripts participated in three scenarios of which the first two were identical to the first group of recruits but without intervention in the third exposure scenario.

Firefighters: We recruited 26 firefighters from three different fire stations of whom 20 completed the study. The firefighters collected skin wipes and urine samples for up to two months without intervention and up to two months with intervention. The interventions tested were 1) the use of fire suits and hoods made of textiles impermeable to particles compared to traditional fire suits, 2) sauna after fire calls compared to no sauna, and 3) extra focus on showering after all fire calls compared to the usual practice. Before the intervention, the participants received information on their individual dermal PAH exposure levels before/after fire calls as well as before/after showering following fire calls.

For both the conscripts and firefighters, dermal exposure was measured as PAH concentrations in skin wipes and total PAH exposure was measured as urinary excretion of PAH metabolites, whereas silicone wristbands were used as personal passive samplers for airborne PAHs. In two of the measurement campaigns for the conscripts, we also did observations on how protective equipment was used.

The design of the study and preliminary results will be presented.

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P07-09

Spatio-temporal multi-omics analysis of lung tissue and matched biofluids identifies circulatory safety biomarkers for monitoring inhaled drug induced lung injury

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Lung inflammatory lesions identified in animal toxicology studies are currently difficult to monitor in the clinic. We designed *in vivo* rat proof-of-concept studies integrating multi-omics to explore candidate circulatory safety biomarkers associated with lung toxicity observed with two tool compounds (AZ1 and AZ2 in two concentrations). Proteomic and metabolomic profile of bronchoalveolar lavage fluid (BALF), plasma and matched lung tissue transcriptomics, mass spectrometry imaging (MSI), and spatial transcriptomics data were analysed. Omics data were correlated with histopathological features at four different time points. Repeated dosing of AZ1 and AZ2 increased neutrophil count and abundance of lung surfactant component phosphatidylcholine in BALF. MSI revealed a temporal pattern of accumulation of AZ1 in lung that spatially correlated with crystalline material on haematoxylin and eosin staining. Applying spatial transcriptomics, we identified a gene expression cluster that exhibited localized overexpression at the site of AZ1 accumulation. A 15 gene lung RNA expression signature associated with inflammation and tissue injury was identified that exhibited dose- and time-dependent changes with both compounds. Interestingly, protein enrichment of several of these markers was detected in BALF and subsequently detected in plasma, suggesting their potential as circulatory biomarkers. In addition, four of these candidate proteins were found to be enriched in BALF from mice treated with an additional six known lung irritants with diverse toxicity mechanisms. Together multi-omics profiling of tissue and paired analysis of biofluids identified 20 candidate biomarkers associated with lung toxicity/inflammation and may potentially be applied in the future to monitor inhalation toxicity that is currently considered non-monitorable

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P07-10

Differential impact of renewable diesel exhaust particles on protein profiles in bronchoalveolar lavage fluid and plasma after instillation exposure in mice

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Exposure to diesel exhaust increases risk of cancer and cardiovascular disease and a proposed common mechanism is inflammation. Blending and substituting petroleum diesel with renewable diesel (*e.g.* hydrogen-treated vegetable oil, HVO; rapeseed methyl ester, RME) can alter both fuel and emission properties but the potential health effects remain less understood. This study aimed to explore and clarify underlying toxicity mechanisms of diesel exhaust from renewable fuels. Using proximity extension assays (Olink), 92 proteins linked to inflammation, cardiovascular function and cancer were analyzed in bronchoalveolar lavage fluid (BALF) and plasma of mice 24 h post instillation to exhaust particles at the doses of 6, 18 and 54 µg corresponding to 0.3, 0.9 and 2.7 mg/kg bw, respectively. Tested particles were derived from combustion of three fuel types at 13% O_2 engine intake: HVO13, RME13 and petroleum Swedish MK1 ultra-low sulfur diesel, DEP13. We also included particles generated from MK1 diesel at 17% O_2 engine intake (DEP17) for comparison. We identified positive dose-response relationships between the exposures and proteins in BALF using linear models: 33 proteins for HVO13, 22 for DEP13, 24 for DEP17, and 12 for RME (p-value <0.05). A set of eleven differentially abundant proteins in BALF (*i.e.* CCL2, CXCL2, CCL3L3, CSF2, IL1A, CCL20, TPP1, GDNF, LGMN, ITGB6, PDGFB) were common for all exposures and formed a distinctive protein fingerprint; however, the effect size of the changes was larger in response to HVO13 and DEP13 compared with RME13. Downstream bioinformatic analysis in BALF revealed activated cytokine signaling, inflammation and cell movement by all tested particles. Several proteins in BALF (*e.g.* CCL2, CXCL2, CCL3L3, CSF2, IL1A) were found to be correlated to previously assessed neutrophil cell count and DNA damage in BALF. Notably, RME13 and to a lesser extent DEP13 also altered plasma protein profiles, suggesting an acute systemic effect, with similar enriched pathways for both plasma and BALF. However, proteins in BALF and plasma had overall a poor correlation with each other. In conclusion, our data shows a large overlap in toxicity between the fuels, but some differences were observed: combustion particles from HVO13 were more potent in inducing local inflammatory effects, whereas RME13 were more potent in inducing systemic effects, compared to DEP13 or DEP17. However, in the context of risk assessment it is important to consider that RME and HVO reduced particle mass emissions (per energy or fuel volume unit) by about a factor of 3 compared to DEP13. Overall, our quantitative protein-based approach proved to be a sensitive method for identifying distinct protein changes in different matrices and to gain mechanistic understanding on the toxicity of exhaust particles. Applying our approach to other combustion emissions and non-exhaust traffic emission may assist in describing toxicity pathways of our most common air pollutants.

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P07-11

Ciprofloxacin alters synaptic and immune proteins in rat brain areas linked to emotional, reward, and cognitive functions

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Ciprofloxacin, a fluoroquinolone used to treat infections, has been associated with a high number of mood and cognitive adverse events (MCAEs), through as yet unspecified mechanisms. Here, we aimed at ascertaining the involvement of specific molecular events on the ciprofloxacin-elicited onset of MCAEs.

To this end, adult male Sprague-Dawley rats were daily administered 20 or 100 mg/kg ciprofloxacin, or vehicle (methylcellulose) by oral gavage, for 4 weeks. The expression of the following targets was analyzed by immunofluorescence in the isolated brain tissue: Synaptosomal-associated protein 25kDa (SNAP-25), interleukin-17 receptor (IL-17R), glial fibrillary acidic protein (GFAP), gamma-aminobutyric acid type A receptor subunit-5 (GABRA5), integrin alpha M (CD11b), nicotinic acetylcholine receptor subunit alpha-7 (nAChRα7), synapsin I/II/III (Syn), cannabinoid receptor 1 (CBR1), 5-methylcytosine (5-mC), and histone acetylation (H3K9ac).

The number of target-positive cells per area (Ca), and the fluorescence intensity per total area were calculated using an in-house ImageJ-based semi-automatic quantification method in the nucleus accumbens (NAc), prefrontal cortex (PFC), and hippocampal formation [hilus, dentate gyrus (DG), CA1 and CA3].

GABRA5 expression was reduced by both ciprofloxacin doses in DG. The lower dose (20 mg/kg) also decreased GABRA5 in hilus, and increased it in PFC, while 100 mg/kg reduced GABRA5 levels in NAc. Both doses increased SNAP-25 expression (100 mg/kg in the DG and 20 mg/kg in the NAc). Syn levels were only altered by 100 mg/kg (decreased in NAc, and increased in hilus). Ciprofloxacin affected immune markers, as both doses increased IL-17R expression in CA3. Yet, only 20 mg/kg increased IL-17R levels in DG and CA1, and decreased them in NAc and PFC. Similarly, 20 mg/kg ciprofloxacin increased CD11b (microglia marker) in hilus, CA1 and CA3, and decreased it in PFC and NAc. Both doses reduced epigenetic markers (5-mC, H3K9ac) in NAc. Only 100 mg/kg reduced 5-mC levels in PFC.

Our data suggest that pharmacologically-relevant ciprofloxacin doses affect the expression of proteins involved in synaptic transmission (SNAP-25, Syn), GABAergic neurotransmission (GABRA5), and immune response (CD11b, IL-17R). Notably, these effects were observed in brain areas associated with emotional response, reward, learning and memory, hinting at a correlation between ciprofloxacin and MCAEs.

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P07-12

Impaired renal function is a confounding factor in the use of plasma citrulline as a marker of intestinal toxicity in rodents

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Drug-induced gastrointestinal (GI) toxicity is a concern in oncologic treatments. However, gut-specific biomarkers are not commonly used in non-clinical toxicity studies. The amino acid citrulline has been suggested as a biomarker of GI toxicity. Citrulline is converted to arginine in the kidney, thus effects on renal function may complicate interpretation as a GI biomarker. For example, in a mouse toxicity study with Compound A, a molecule with known intestinal toxicity, there were degenerative lesions in the GI tract and renal tubules with changes in renal function biomarkers, low plasma arginine levels, and dose-dependent increases in citrulline levels in plasma and jejunum. To further explore the utility of citrulline as a biomarker of GI toxicity, we investigated citrulline dynamics in mice and rats after administration of intestinal or renal toxicants. The GI toxicants doxorubicin and gefitinib were tested in mice. Doxorubicin was dosed intravenously at 0 (vehicle), 5 or 10 mg/kg, once daily for 2 days and mice were euthanized at 6, 24, 72, or 96h after the second dose. Gefitinib was dosed at 0 (vehicle), 8 or 250 mg/kg, orally, once daily for 10 days. Mice were euthanized at 4 timepoints postdose: 6h, 24h, 6 days, and 10 days. GI toxicity was assessed by histopathology, permeability to FD4, and citrulline levels in plasma and jejunum. Doxorubicin caused a dose- and time-dependent decrease in citrulline in the plasma and jejunum, that corresponded with the minimal to moderate degenerative findings identified by histopathology. Permeability to FD4 was less sensitive in detecting doxorubicin-induced damage, with a tendency towards increased values only at the high dose. Plasma citrulline levels were unaffected by gefitinib and corresponded to minimal villus blunting that was limited to animals in the high dose group at 6h postdose. Jejunal citrulline levels were reduced at 6 and 24h postdose in animals in the high dose group. Plasma FD4 showed a trend towards increased values at all timepoints. Citrulline levels were also evaluated in a rat model of cisplatin-induced renal injury. Rats were euthanized on days 1, 3, 5, 8, 10 ,12 ,15 ,20 & 28 after receiving a single intravenous bolus dose of cisplatin at 0 (vehicle) or 5 mg/kg. Cisplatin induced necrosis of renal tubules and changes in renal function biomarkers from day 3 onwards and increased and decreased serum citrulline and expression of Ass1 in renal proximal tubules, respectively, from day 5 onwards. In conclusion, our data indicate that extraintestinal factors can interfere with the association between citrulline and enterocyte mass in rodents. These confounding factors need further exploration before applying citrulline as a biomarker of GI toxicity in nonclinical studies.

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P07-13
Bisphenol A levels in head hair of children of Crete with health issues

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Purpose: Bisphenols are endocrine disruptors used in daily life, highly linked with pathologies such as obesity, diabetes and other endocrine diseases. They are characterized as environmental pollutants and have been detected in plastics, food and personal care products. Children constitute one of the most vulnerable groups to bisphenols exposure. The aim of our study is to assess the chronic exposure of children to bisphenols via hair analysis. Also, the study aims to correlate the degree of exposure with health problems that have already been manifested in the child study population (obesity, diabetes and endocrine problems as hypothyroidism, growth hormone deficiency, precocious puberty).

Materials & Method: One hundred children, who have visited the University Hospital of Heraklion and have already been diagnosed with endocrine diseases, participated in this study. The participants were divided equally into four groups; control, diabetes, obesity and endocrinology group. Total length head hair samples were collected and washed. The proximal to the head segment of the hair (0–12 cm) was extracted by using methanol for 4 hours in an ultrasonic bath. Analysis was carried out by liquid chromatography-mass spectrometry (LC-MS).

Results & discussion: Bisphenol A was detected in 56%, 88%, 76% and 56% of the hair samples for the control, diabetes, endocrinology and obesity group, respectively. The diabetes group provided the higher concentration levels of bisphenol A (mean 243.9 pg/mg, range 17.5–720.6 pg/mg) compared to the rest groups. Specifically, the mean concentration value for control group was 116.8 pg/mg (12.7–380.0 pg/mg), for the endocrinology group was 109.3 pg/mg (29.8–372.5 pg/mg) and for the obesity group was 95.0 pg/mg (10.7–295.8 pg/mg).

Conclusion: Differences in the detection frequencies and the mean detected values were observed between the investigated groups of children for bisphenol A.

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P07-14
Exposure of children of Crete to phthalates and its association with diabetes

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Purpose: Phthalates, often called plasticizers, are used in consumer products like plastic toys, medications, food packaging and personal care products. There seems to be a link between exposure to phthalates and the incidence of metabolic dysfunctions. The aim of our study was to assess the chronic exposure of children to phthalates via hair analysis of phthalates metabolites and specifically mono methyl phthalate (MMP), mono isobutyl phthalate (MiBP), mono butyl phthalate (MBP) and phthalic acid mono 2-ethyl hexyl ester (MEHP).

Materials & Method: Fifty children participated in this study; 25 of them consisted the control group and 25 of them have visited the University Hospital of Heraklion and have already been diagnosed with T1DM. Hair samples (0–12 cm) were cut as close to the scalp as possible which corresponds to a period of 12 months of exposure. The extraction process included sequential incubations in methanol that repeated twice (4 hours in total) into the unltrasonic. Analysis was executed by liquid chromatography – mass spectrometry (LC-MS).

Results & discussion: High detection rates were observed for the phthalate metabolites MiBP, MBP and MEHP. MEHP was detected at a rate of over 92% in all the examined populations without particular differences between the groups in contrast to MMP which was the metabolite with the lowest detection rate. Specifically, the% detection frequencies of MMP, MiBP, MBP and MEHP was 12%, 80%, 56% and 92%, respectively, for the control group compared to 52%, 20%, 60%, and 100% for diabetes group. MEHP was the metabolite with the largest contribution to the total population burden with mean concentrations ranging from 224.5 pg/mg (diabetes group) to 192.0 pg/mg (control group). For MiBP and MBP the mean concentration values were from 83.9 pg/mg and 69.7 pg/mg for diabetes group, and 61.3 pg/mg and 51.7 pg/mg for control group, respectively. The corresponding mean values of MMP was 34.3 pg/mg for control group and 108.7 pg/mg for diabetes group.

Conclusion: Differences in the detection frequencies and the mean detected values were observed between the investigated groups of children for MMP, MiBP and MBP. The concentrations of MEHP in hair were higher than the other examined metabolites.

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P07-15
Proposal of a non invasive approach for sleep alterations assessment in night shift workers

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Night shift work (NSW) is essential in industries such as harbor activities or healthcare, ensuring uninterrupted operations. Prolonged NSW has been associated with sleep disorders and work-related stress, increasing the likelihood of errors and accidents. It is also linked to short-term illnesses like metabolic disorders and chronic diseases such as cancer [1]. Melatonin, crucial for synchronizing internal processes, is rhythmically produced by the pineal gland, regulating the day-night cycle [2]. Despite recognizing the importance of assessing workers' sleep, there is a lack of rapid and reliable biomonitoring methods. The measurement of salivary biomarkers offers non-invasive and real-time features. Melatonin salivary levels have been shown to accurately reflect blood levels, presenting a promising avenue in health assessment [3]. This study aims to elucidate potential sleep alterations associated with NSW across diverse worker populations. We analyzed harbor workers and nurses engaged in night shifts. Concurrently with health and work history, we administered the Pittsburgh Sleep Quality Index (PSQI) questionnaire to evaluate sleep quality [4]. Additionally, morning and evening salivary samples were collected for melatonin level assessment [5]. Among harbor workers, lower morning melatonin production was observed in night shift workers compared to their day shift counterparts. Additionally, 70% of harbor worker subjects exhibited PSQI scores below 5, indicating good sleep quality, while 30%

reported mild sleep disturbances. A significant negative correlation between PSQI scores and melatonin levels was observed. Similarly, among nurses, a larger proportion exhibited diminished morning melatonin levels, although with no correlation found with PSQI results. As in deck workers, also among nurses, there was a predominance of good sleep quality (76%), but without gender-based disparities. In conclusion, melatonin measurement effectively discriminates between day and night shift workers, highlighting a significant time-shift in melatonin synthesis associated with NSW. Integrating PSQI with melatonin measurement may enhance health assessments among workers. Overall, the proposed multi-assessment strategy holds potential in identifying workers susceptible to sleep alterations, thereby facilitating the development of targeted preventive strategies. Importantly, this comprehensive approach will support occupational physicians in strengthening health promotion endeavors, leveraging salivary assessment for prompt and reliable biological monitoring in workers. However, further studies are warranted to validate these pilot observations. Moreover, administration of additional questionnaires may help in providing comprehensive insights into sleeping habits and promote sleep hygiene among night shift workers.

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P07-16
Exposure level of alternative Per- and polyfluoroalkyl compounds in Koreans

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Most countries have regulating and managing the use of persistent organic pollutants(POPs) as outlined in the Stockholm Convention. To replace POPs such as PFOA and PFOS, which are known to have carcinogenic and hepatotoxic risks, new substances are being developed or patented. But due to the lack of information on the risks, exposure amount and product contamination level of the new substances, accurate risk assessment is not being done.

Korea government has been continuously monitoring and conducting risk assessments on substances that pose a risk to humans to use various products, and has promulgated the “Risk Assessment Act for Products for Human Use” for the first time in the world. Before evaluating risk of alternative materials, the actual exposure of Korean citizens to hazardous substances is monitoring the content of substitute substances such as 9Cl-PF3ONS, which is replacing PFAS.

We reviewed 20 PFAS and developed simultaneous analysis method of PFAS in serum. Simultaneous analysis was performed using Liquid Chromatography and Triple Quadrupole Mass Spectrometer. The developed method was fully validated and showed good results with respect to selectivity, linearity (r2>0.998), limit of detection (0.02–0.14 ng/mL), limit of quantification (0.07–0.43 ng/mL), recovery (87.48–119.32%), and reproducibility (%RSD<18.07). All parameters satisfied standards suggested by Ministry of Food and Drug Safety guidelines. As a result of analyzing the serum of about 800 people aged 10 years or older in Korea using this analysis method, arithmetic mean(AM) was 29.35 ng/mL, the lowest content was 3.85 ng/mL, and the highest content was 102.45 ng/mL for 20 PFAS. There were differences in exposure levels depending on gender, age, and region, and in 95% of the high exposure groups, all 20 substances were detected at high levels.

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P07-17
Recommendation of health-based biological guidance value for aluminium and its inorganic compounds for workers and the general population

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Human biomonitoring is a useful tool for assessing exposure to chemicals, and the resulting individual and collective health risks, in both occupational and environmental settings. It allows targeting of preventive measures on specific populations or tasks, then to assess their effectiveness. For the interpretation of biomonitoring results, health-based reference values are needed.

For aluminium exposure biomonitoring, this element is generally measured in blood (whole blood, serum or plasma) and/or urine. As blood measurements lack of sensitivity for individual biomonitoring in people with normal renal function, urinary aluminium is preferred as a biomarker of exposure both in the workplace and for the general population. In workers, urine sampling is recommended at the end of shift and end of the working week

Literature analysis showed that the critical effects for aluminium toxicity in humans were neurological and consisted in cognitive impairment (especially in memory and concentration tasks). The available data were estimated sufficient for the recommendation of health-based biological limit values aiming at preventing neurotoxic effects for both workers and general population. A total of 21 cross-sectional studies and 4 longitudinal studies reporting human biomonitoring data and cognitive effects following occupational exposure to aluminium were analysed. Two longitudinal studies were considered for the identification of a point of departure for the elaboration of a biological limit value for workers and a toxicological reference value for the general population.

A general population guidance value corresponding to the 95th percentile of background levels measured in a general population of French adults was also recommended. It allows the identification of overexposure before health-based limit values are attained.

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P07-18
Acute phase proteins in Göttingen minipigs: comparison between haptoglobin, Pig-MAPP and CRP in the context of a vaccine study

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In the context of increased difficulties related to monkeys' supplying and ethical concerns, the use of the Minipig as a model in immunotoxicology is a promising alternative to the Non-Human Primate (NHP). The physiological and immune similarities with humans are more and more documented in the literature. The recent IHI initiative that aims at expanding translational knowledge in Mini Pig is one additional example of this trend. The case study presented here is a toxicology study conducted with one prophylactic vaccine at CRL Lyon where inflammation was monitored (among others) with Acute Phase Proteins (APP).

APP are indeed used to monitor normal or abnormal inflammatory responses depending on the intensity, maintenance, and reversibility of the response together with additional clinical-pathology signs. APP are easily measurable in blood samples by ELISA. An appropriate combination of different markers allows to track innate reaction. The kinetic profile and activation spectrum of these different proteins is also discussed to rank the relevance of these different biomarkers. Humoral response documented by quantitative ELISA is considered as a primary proof of administration and allows to rely potential toxicity effects.

Theses assays, all together, provide critical information to explore underlying mechanisms linked to inflammation and demonstrate the existence of relevant comparable biomarkers to NHP allowing to draw robust conclusion on toxicity in Gottingen Mini pig.

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P07-19
Levels and temporal trend of phthalates in the Greenlandic adults during 2000–2019

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Background and aim: Phthalates are widely used as plasticizers in daily life products. Human exposure to phthalates occurs through food, dust, water, personal care products and medical devices. Phthalates have endocrine disruption potential and are reproductive and developmental toxicants. Urinary phthalate metabolites are sensitive biomarkers for assessing human exposure to phthalates. Until now, there are no reports on urinary concentrations and temporal trends of phthalates in Greenlanders.

This study aims to evaluate the exposure and temporal trend of phthalates in adult Greenlanders.

Methods: The study includes 602 adults across Greenland recruited during 2000–2019. The demographic and lifestyle data were collected through questionnaires. The urinary concentrations of eleven metabolites of phthalates and phthalate substitutes such as di-(iso-nonyl)-cyclohexane-1,2-dicarboxylate (DiNCH) were measured using solid phase extraction prior to ultra-high pressure liquid chromatography-tandem mass spectrometry in negative electro-spray mode. The urinary concentrations were standardized by the urinary creatinine concentration. The comparisons of phthalate exposure among regions and sex were evaluated by one-way ANOVA and independent Sample t-test. Multiple linear regression models were used to assess the influence of demographic and lifestyle factors on urinary phthalate concentrations and the temporal time trend of phthalate levels.

Results: The metabolites of the regulated phthalates and non-regulated phthalate substitute (DiNCH) were detected in spot urine samples of the participants.

The urinary concentrations of phthalate metabolites differed among the Greenlandic regions. The participants from east region had higher levels of regulated phthalates while lower levels of non-regulated phthalate substitute (DiNCH).

Higher urinary concentrations of regulated phthalate metabolites were found in the older participants and female participants as well as the participants with lower education and high marine food intake. Time trend analyses showed that exposure to regulated phthalates elicited a remarkable reduction during 2000–2019 among Greenlandic adults.

For the non-regulated phthalate substitute (DiNCH), the influence of sex, education, and marine food intake was not obvious. Interestingly, younger and current smoking participants had lower DiNCH urine metabolites concentrations. The urinary level of DiNCH significantly increased during 2000–2019.

Conclusion: The decreased time trend of regulated phthalates indicates the effectiveness of regulation. However, the detection of the regulated phthalates in the urine samples in Greenlandic adults in 2019 suggest the Greenlanders still are exposed to phthalates which have been restricted since 2015. Remarkably, the increasing time trend of the non-regulated phthalate substitute DiNCH address the importance of regulation and evaluation of the health effect of DiNCH in the Greenlandic population.

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P07-20
Human biomonitoring guidance values derived for nickel and its compounds

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Human biomonitoring guidance values (HBM-GVs) can be used to interpret human biomonitoring (HBM) data in terms of health, enabling the use of biomonitoring in assessment and management of chemical risks to humans. Within PARC (Partnership for the Assessment Risks from Chemicals) project HBM-GVs are derived for environmentally and occupationally relevant chemicals following the strategy laid down within earlier EU project, HBM4EU (www.hbm4eu.eu).

HBM-GV for general population and for workers are proposed for Nickel and its compounds. General population is mainly exposed to nickel via oral route whereas workers are typically exposed through inhalation. Nickel compounds can be divided into soluble and sparingly soluble. Soluble nickel compounds are more readily absorbed and excreted than sparingly soluble nickel compounds. For general population HBM-GV was derived from tolerable daily intake (TDI) value 13 µg/kg bw/day recommended by EFSA 2020. This value was based on reproductive toxicity as there was no evidence for carcinogenicity through oral exposure. Both urinary mass balance approach and physiologically based pharmacokinetic (PBPK) modelling was used to estimate corresponding urinary concentrations for external intake levels. The proposed HBM-GV for general population was 3.0 µg/l in urine.

HBM-GV for workers was based on occupational exposure limit (OEL) value 0.03 mg/m3 for inhalable fraction recommended by RAC 2018. This value was based on nasal carcinogenicity. Available information supports mode-of-action based threshold for nickel compound carcinogenicity Established correlations between air levels and urinary

nickel levels were used to derive HBM-GV. Derivation was done separately for soluble and sparingly soluble nickel compounds Derived HBM-GV for occupational exposure to soluble nickel compounds was 12 µg/l. The proposed value is considered to protect also from reproductive toxicity effects. Due to limited bioavailability and urinary excretion, no HBM-GV for sparingly soluble nickel were recommended.

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P07-21
Chloropicrin induces transcriptional changes in mouse cornea

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Chloropicrin (CP; CCl_3NO_2, trichloronitromethane), a choking agent, causes acute and systemic effects. Historically deployed as a chemical warfare agent (CWA), CP is used as a pesticide today; thus, enabling its easy acquisition and potential use in terrorism in addition to its occupational and environmental exposures. CP have been reported to cause immediate ocular irritation and intense pain with lacrimation, edema, and temporary blindness. Our previous preclinical murine study indicated that exposure to CP causes corneal ulceration, edema, opacity, neovascularization, hyphema and hydrops. Further corneal assessments showed cell death, edema, inflammatory, and fibrotic changes; however, the molecular mechanism of corneal injury induced by CP remains unclear. In the present work, we employed bulk RNA-Seq to understand the molecular mechanism of CP- induced corneal injury. Corneas were harvested from the left eye of Balb/C mouse (6–8 weeks old, male, n=3/group) exposed to 10% CP for 1 min (~0.7652ppb) at 6 hours (group 1) and 24 hours post-exposure (group 2). Exposure was done using a vapor cap exposure system and the right eye served as a control. mRNA was isolated from the corneas using phenol-chloroform extraction method and mRNA concentration and quality control were assessed using Agilent 2100. NovoGene conducted sequencing on their illumina NovaSeq PE150 platform. Differential gene expression was performed using DESeq2 with standard cutoff for p-value ≤ 0.01 and absolute $|\log 2\text{-Fold Change}| \geq 1$. Transcriptomic analysis showed significant differences between CP exposed versus control group. Compared to the control group, totals of 1876 and 2376 genes were differentially expressed in post exposure groups 1 (6 hours) and group 2 (24 hours) respectively. Quantitative PCR (qPCR) analysis was performed on selected genes, from differentially expressed list, to validate their mRNA levels as differentially expressed. qPCR results showed the overexpression of Il-6 and Cxcl1 in exposed cornea as compared to control, which confirmed the results of RNA-seq analysis. Functional analysis was done using EnrichR. It showed that CP exposure resulted in significant enrichment (p-value ≤ 0.01) of pathways related to ferroptosis, inflammatory response, oxidative stress, wound healing, and apoptosis. These findings suggest that pathways related to both oxidative stress and inflammation play crucial roles in corneal injury induced by CP. Our study provides new insights into the mechanism of CP and identifies important signaling pathways in CP toxicity with a potential to develop targeted countermeasures for CP-induced ocular injuries while serving as a reference for studies on other CWAs.

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P07-22
Dermal and pulmonary injury induced by acute cutaneous nitrogen mustard exposure

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Chemical warfare agents (CWA) were widely used during World War One (WW1) and remain a serious threat to human health. Sulfur mustard (SM), arguably the most well-known CWA, is a vesicating or blister agent. In addition to dermal blisters, SM causes severe ocular and respiratory injury, as well as immune suppression and systemic injury. Some injuries can persist for decades after exposure, leading to inflammatory diseases. SM and its analog nitrogen mustard (NM, also used as a chemotherapeutic agent) remain potential chemical threats. Despite the SM's destructive ability to cause multi-organ injury being widely known, its mechanism of action remains poorly understood and we are lacking any SM-specific therapies as a result. This study is aimed at characterizing the dermal and pulmonary toxic effects of NM cutaneous exposure in C57BL/6 mice. This study will lead to the development of a novel model of acute vesicant cutaneous exposure that can be used to evaluate dermal as well as systemic effects like pulmonary injury.

Male C57BL/6 mice were topically exposed to 1.0 mg of NM dissolved in 100 uL of acetone. Mice were sacrificed at 1-, 3-, and 8-days post-exposure. Skin and lung tissue were collected and snap-frozen or fixed and sectioned for analyses.

NM-exposed mice showed dermal wounding, edema, erythema, and experienced significant weight loss by day-1 post exposure, which continued until hitting a humane endpoint and forcing the termination of the study at day-8 post exposure. Their skin began to display micro-vesication, inflammatory changes, and scabbing and eschar formation by day-1 post-exposure that worsened significantly by day-8 post exposure. Dermal NM exposed mice also showed lung injury with bronchiolitis, alveolitis, hyperemia, hemorrhage, interstitial edema, and infiltration by monocytic cells, by day-1 post exposure. Dermal and pulmonary mast cell degranulation and inflammatory markers including IL-6, IL-1b, and TNF-α were significantly elevated in the skin and lung samples of cutaneous NM-exposed mice.

Overall, our analyses have yielded several novel findings including that cutaneous exposure to NM causes both skin and pulmonary injury. There are reported animal models of pulmonary injury from vesicant inhalation exposures, this study reports a mouse model of dermal and pulmonary injury from its dermal exposure. Since both skin and lung tissues show NM-induced mast cell degranulation and an inflammatory response, there could be parallel mechanisms of injury in both the injured tissues. Further analyses are currently being carried out to explore other systemic effects of cutaneous NM exposure and molecular signatures to identify medical targets to counter injuries from vesicant cutaneous exposure.

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P07-24

Analysis of genes expressed differently in alveolar macrophages of smokers, non-smokers and individuals with and without lipopolysaccharide exposure

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Cigarette smoking is a type of exposure that is closely associated with various lung diseases and deaths related to these diseases worldwide. Alveolar macrophages, which undertake the first defense against foreign substances such as bacterial toxins that we breathe into our body, are our immune cells located in the space between the airway lumen and the alveolar space. In this study, gene expression changes in alveolar macrophages as a result of smoking and bacterial toxin lipopolysaccharide (LPS) exposure were analyzed. Gene expression datasets were provided from Pubmed Gene Expression Omnibus, FunRich was used to draw Venn diagrams. Protein Atlas was used to reveal the expression findings of the genes found. Datasets GSE8823, GSE2125, and GSE40885, selected from the Pubmed Gene Expression Omnibus, contain gene expression data of alveolar macrophages from smokers, nonsmokers, and lipopolysaccharide-exposed and unexposed individuals. Gene expression changes with a p value less than 0.05 and a logFC value greater than 1.5 or less than -1.5 were used in all analyses. First, up- and down-regulated genes were identified between non-smokers and smokers in GSE2125 and GSE8823 and between control and LPS groups from the GSE40885 dataset. When the genes downregulated in non-smokers compared to smokers and controls compared to LPS-exposed groups were intersected, only 1 gene, *FCN1*, was found at the intersection of the 3 groups. No genes were found at the intersection of upregulated genes. On the other hand, double intersections of the groups yielded 4 genes. *TNFSF8, LOC102723899///CLDN12* and *MMP7* are 3 other genes that were commonly downregulated in the GSE8823 and GSE40885 datasets; *PLA2G7, MREG, CCR5,* GSE40885, and GSE2125 were found to be common downregulated genes, and finally *ATP6V0D2, SPP1,* and *CYP1B1* were found to be commonly downregulated genes in GSE8823 and GSE2125. According to Protein Atlas data, *FCN1* and *CYP1B1* have been reported to be expressed in classical, non-classical and intermediate monocytes, indicating their function in immune activation. *PLA2G7* is also expressed in classical and intermediate monocytes. Other genes found have been reported to exhibit low immune cell specificity and *MMP7* is an unfavorable prognostic marker in lung cancer. Collectively, the results indicate that alveolar macrophages from controls compared with LPS-exposed individuals and alveolar macrophages from nonsmokers compared with smokers do not exhibit high numbers of common upregulated and downregulated genes. Commonly found genes such as *FSCN1* and *CYP1B1* have been found to exhibit immune cell specificity, indicating that smoking and exposure to bacteria can commonly induce differential expression of a limited number of genes in alveolar macrophages. Uncovering new genes may help explain different or common mechanisms and markers in tobacco smoking and exposure to bacterial agents.

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P08 | Developmental toxicology

P08-01

T-cell-dependent Antibody Responses and Immunopathology: A Comparative Study in Juvenile Cynomolgus Monkeys

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Objectives: The objectives of this study were two-fold: to compare T-cell-dependent antibody responses (TDAR) to keyhole limpet hemocyanin (KLH) in juvenile and mature nonhuman primates (NHPs) and to compare findings of a retrospective analysis of organ microscopic evaluation data in infants of different origins. It has become apparent, based on NHP infant availability, that a better grasp of historical background data sets from different origins is crucial in utilizing this rare resource. This is to assure that there are adequate options to support research assessing the safety of the range of possible therapies for rare diseases that is growing at a rapid pace, especially in therapeutic products such as gene-, cell- and oligonucleotide-based therapies. Given the high degree of genetic similarities between NHPs and humans, studies for these therapies are often performed in juvenile animals (~9- to 17-month-old) in order to better predict their safety and efficacy in the human pediatric population.

Methods: The TDAR *in vivo* functional assay, with use of KLH as the immunogen, was completed in Mainland Asia (MA, Cambodia [CA] and China [CH]) and Indonesian (IN) origin animals with analysis for IgG and IgM levels using ELISA. Analyses were conducted by measuring serial dilutions of each sample.

Results: Following primary and secondary KLH challenges, robust immune responses were generally evidenced by antibody production in all juvenile animals, with no notable differences between males and females, and between different origins. A comparison with data from mature animals indicated the main difference to be in the incidence of response. In juveniles, mean IgG vales (µg/mL) following primary challenge were 2.2 (CH), 2.5 (CA) and 3.1 (IN) on Day 7; 29.4 (CH), 24 (CA), and 15.5 (IN) on Day 14; 45.2 (CH), 38 (CA), and 22.9 (IN) on Day 21. Mean IgM values (µg/mL) following primary challenge were 95.2 (CH), 85.1 (CA), and 69.2 (IN) on Day 7; 88.1 (CH), 72.5 (CA), and 48.2 (IN) on Day 14; 42.6 (CH), 37.4 (CA), and 26.4 (IN) on Day 21. Background microscopic findings were limited to an increased incidence of mononuclear cell infiltration (7% in CB, 15% in CN, 20% in IN), extramedullary hematopoiesis (8% in CB, 23% in CN, 27% in IN), increase lymphocytes in lymphoid follicles (0% in CB, 15% in CN, 18% in IN), hepatocellular vacuolation (11% in CB, 10% in CN, 2% in IN), mineralization of adrenals (5% in CB, 13% in CN, 10% in IN), and occasional ectopic thymus (0% in CB, 9% in CN, 15% in IN).

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P08-02

Effects of perinatal exposure to nanoparticles on lung development and function

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Purpose: Nanoparticles (NP) are organic, inorganic, or composite materials with 3 dimensions between 1 and 100 nm[1]. Due to their physico-chemical properties, they can be found in many everyday products (cosmetics, food additives, cleaning products, etc.)[2] and thus, we are increasingly exposed to them. This raises questions about their potential effects on health, particularly in the perinatal period, when the developing organism is more vulnerable to environmental stresses. In mouse models, it has been shown that NP administered to pregnant or lactating mice can reach the fetus, crossing the placental barrier via the bloodstream, or the offspring after translocation in the breast-milk[3,4,5]. However, the long-term consequences of such exposure are still poorly studied. Our goal is to better understand the perinatal toxicity of NP on lung development, by focusing on two types of NP widely used in industry: silver (Ag) and titanium dioxide (TiO_2).

Methods: Pregnant and/or lactating C57BL/6J mice were exposed to different particles sizes: 10 nm (Ag10) and 20 nm (Ag20) for Ag and 10 nm (Ti10), 18 nm (Ti18) and 21 nm (P25®) for TiO_2 by non-surgical intra-tracheal instillation[6] (100 µg of NP) once a week, during the 3 weeks of gestation and/or lactation. The pulmonary phenotype of the offspring was analyzed at P60 (n=8–10 per group). The lung morphology was analyzed by the Mean Linear Intercept (MLI) to quantify inter-alveolar distance and the pulmonary function was measured by two different techniques: whole-body plethysmography (VivoFlow®) that measures respiratory times and the FlexiVent® system that evaluates lung mechanical properties.

Results: Although the morphometry analysis revealed no change in lung MLI after maternal exposure to NP, abnormalities in respiratory parameters measured at P60 by plethysmography were found after exposure to Ti10 with an increase in expiratory time (150.8±24.62 msec vs. 112.6±30, 47 ml/cmH2O) and total time (212±35.65 msec vs. 163.4±40.08 msec), as well as a decrease in respiratory rate (368.6±72.48 bpm vs. 465.9±80.68 bpm) as compared with control. Changes in mechanical properties have been highlighted with the FlexiVent® system. Exposure to P25® induced a significant increase in elastance which represents pulmonary stiffness (39.65±4.093 ml/cmH2O vs. 36.09±2.844 ml/cmH2O) associated with a decrease in compliance which is the lung ability to change its volume in response to a change of pressure (0.025±0.004 ml/cmH2O vs. 0.028±0.001 ml/cmH2O) as compared with control. No abnormalities were found after exposure to Ag10 and Ag20 whatever the assessed parameter.

Conclusion: Altogether, our results show that gestational exposure to TiO_2 leads to abnormalities in lung function parameters: respiratory cycles for Ti10 and pulmonary mechanics for P25. Further experiments will be needed to better understand the mechanisms underlying these effects.

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P08-03
Comprehensive digital documentation and computer-assisted evaluation of the skeleton of Alizarin Red and Alcian Blue-stained rat foetuses

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The classical teratology evaluation of the skeleton of Alizarin Red and Alcian Blue (ARAB)-stained foetuses is a time-consuming process, which requires careful teratology expert evaluation. Even if documented by individual photos, a peer review of the findings is a not less tedious process. To establish a straightforward method, we developed a system enabling the complete digital representation, documentation and evaluation of the skeleton ARAB-stained rat foetuses. The method consists of the video imaging of axially rotated, ARAB-stained, rat PND 20 foetuses in a custom-made device. A purpose-made program allows the quick and easy review and annotation by an expert and provides data which are suitable for 3D reconstruction (optical projection tomography) and can be the basis of subsequent automatic segmentation and morphometric evaluation of the bones and ossification centres. The method we have developed accelerates the evaluation of the skeleton of ARAB-stained rat foetuses and provides a comprehensive digital documentation of the findings and is the basis of a quantitative morphometric analysis of embryonic skeletal development.

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P08-05
The Role and mechanism of O-GlcNAcylation in abnormalities of oocyte maturation and early embryonic development caused by PFOS exposure

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Objectives: The perfluorooctane sulfonate (PFOS) concentration gradients were established based on human internal exposure levels, to investigate the effects of *in vitro* and *in vivo* exposure of PFOS on ovarian function, Oogenesis and Embryogenesis in mice.

Methods: The concentration gradient was set based on PFOS exposure does in human (10µg/L, 0.02µM), to construct an exposure model of C57BL/6J mice with the conversion factor of the measurements between human and mice (0.001, 0.01, and 1 mg/kg/day), and observing the effects on mouse embryogenesis through *In vitro* exposure (0.02, 0.2, 20µM). The hormones related to oogenesis and the rate of oocyte germinal vesicle breakdown (GVBD), polar body extrusion (PBE), fertilization were measured. The Proteomics and O-Glycoproteomics analyses were performed on mice ovary exposed to 1 mg/kg/day PFOS, incorporating real-time quantitative PCR (RT-qPCR) and Western Blot to investigate the differential O-GlcNAcylation of proteins and sites.

Result: Exposure to PFOS *in vivo* decreases the number of primary follicles, delays the process of GVBD, and reduces the PBE rate. Meanwhile, the result of *in vitro* fertilization has shown that the ratio of 2-cells and 4-cells decreased in the 1mg/kg/day exposure group, and ELISA demonstrated that the level of Estradiol (E2) and progesterone (P4) decreased significantly in the 1mg/kg/day group. Combined with the results of proteomics, it was shown that PFOS exposure led to abnormal steroid anabolic processes and ovarian hormone synthesis pathways. The results of RT-qPCR and Western Blot have shown that the expression level of Estrogen Receptor β (ERβ) was decreased significantly, indicating that PFOS exposure can Interfere the hormone synthesis of granulosa cells. The O-Glycoproteomics analyses demonstrated that there was a significant increase in the O-linked N-acetylglucosaminylation (O-GlcNAc) level in the 1mg/kg/day group and the control group.

Conclusion: PFOS exposure may interfere with the O-GlcNAcylation level in the ovary, resulting in abnormal ovarian function, oogenesis, and embryogenesis in mice, suggesting that PFOS is toxic to the female reproductive system.

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P08-06
An oral developmental toxicity study of pinoxaden technical in rabbits

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Aim: this study was conducted to determine the teratogenic potential of the pinoxaden pesticide in pregnant female rabbits. Several authorities evaluated Pinoxaden. The Joint Meeting on Pesticide Residues (JMPR) concluded that a NOAEL of 30 mg/kg/bw/d for maternal and embryo/fetal toxicity in a rabbit developmental toxicity study. However, during the Pesticide Peer Review Expert meeting, EFSA considering a low incidence of diaphragm malformations in one developmental toxicity study in rabbits, a NOAEL of 10 mg/kg/bw/d was suggested, along with a proposed classification for developmental effects (Cat 2 R63* "Possible risk of harm to the unborn child", or H361d: Suspected of damaging the unborn child). This discrepancy highlights the need for further research to ascertain the accurate threshold for adverse effects and ensure precise risk assessment.

Materials and methods: the test item was orally administered as an aqueous emulsion by gavage daily from gestation days (GDs) 6–28 to two groups of animals, each composed of 21 females. The doses administered were 10 and 30 mg/kg/bw/d. Control animals, consisting of 21 females, received an equivalent volume of solvent: distilled water with an emulsifier. Following the administration period, body weight changes and clinical observations were assessed. On GD 28, all females who survived the scheduled necropsy were euthanized by CO2 asphyxiation. The dam was examined macroscopically. Ovaries and uterus were examined to determine the numbers of corpora lutea, live, dead, and resorbed fetuses/embryos, placenta and fetal body weight, and the presence of internal abnormalities. Skeletal and soft tissue examinations were determined. The study was based on OECD TG 414 (Prenatal Developmental Toxicity Study).

Results: One female at 30 mg/kg/bw/d was euthanized in extremis on GD 27. Clinical observations revealed erected fur, and body weight loss during the period prior to premature birth. The premature birth was most likely caused by the poor general condition of the animals and was therefore considered to be an indirect effect of pinoxaden. One female at 30 mg/kg/bw/day had entirely dead litters except for one live male pup. Since the incidence of postimplantation loss within the remaining dams in this group that survived to necropsy was not increased, this suggests that the toxic effect was most likely on the dam, rather than the concepts. Visceral and skeletal examinations revealed several minor skeletal variants, e.g. incomplete ossification or dislocation of sternebrae and incomplete lobulation of the lungs. These findings were incidental and not dose-response; thus, they were considered spontaneous findings unrelated to the test item administration.

Conclusions: Under the conditions of the study, the pinoxaden produced maternal toxicity at the highest dose tested. Thus, NOAEL for maternal toxicity was determined to be 10 mg/kg/bw/d. NOAEL for developmental toxicity – 30 mg/kg/bw/d.

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P08-07
Prenatal developmental toxicity evaluation of higher olefins in Sprague-Dawley Rats

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Higher olefins are used primarily as intermediates in the production of other chemicals, such as polymers, fatty acids, plasticizer alcohols, surfactants, lubricants, amine oxides and detergent alcohols. The potential pre-natal developmental toxicity of five higher olefins (i.e. hex-1-ene, Nonene, branched (3-methyloct-1-ene), Octadecene, Octadec-1-ene (octadecene isomers, UVCB), and Hydrocarbons, C12-30, olefin-rich, ethylene polymn. by-product (mixed olefins isomers, UVCB)) were evaluated in prenatal development toxicity studies (OECD TG 414 (2001)) conducted in Sprague-Dawley rats. In each study, these five higher olefins were administered by gavage at dose levels of 0, 100, 300 and 1000 mg/kg bw/day from Day 3 to Day 19 of gestation. Maternal food consumption, body weights, and clinical signs were monitored throughout gestation. The rats were sacrificed on Day 20 of gestation and examined for standard parameters of reproductive performance (number of corpora lutea, number of implantations, pre- and post-implantation loss, number of live- and dead fetuses, sex-ratio and the weight of the reproductive organs). The fetuses were weighed and examined for external, visceral, and skeletal variations and malformations. The results from these studies showed that none of the higher olefins treated groups showed maternal and embryo–fetal toxicity. Although occasional and incidental skeletal and visceral malformations were observed in hex-1-ene and octadecene, these findings were found to be spontaneous, unrelated to the treatment and did not indicate any disturbance of fetal development. In conclusion, the No-Observed-Adverse-Effect Level (NOAEL) for all tested higher olefins was determined to be 1000 mg/kg bw/day, the highest dose level administered, for both maternal and developmental toxicity.

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P08-08
Transcriptional biomarker signatures distinguish teratogenic and non-teratogenic compounds in targeted RNA sequencing of pluripotent stem cell derived neuroectoderm

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To this date, *in vivo* model systems such as the extended one-generation reproductive toxicity study represent the gold standard in the assessment of developmental toxicity. Yet, animal testing remains controversial, labour- and cost-intensive, and overall has limited human relevance. Directed differentiation of induced pluripotent stem cells (iPSC) *in vitro* has been shown to successfully mimic early phases of embryonic development and therefore is considered a promising future resource for developmental toxicity testing. The UKN1 assay can be applied to assess teratogenic effects on early stages of embryonic neurodevelopment by measuring compound induced deregulation of gene expression during neuroectodermal differentiation of iPSC. Previously, applying the UKN1 assay and microarray analysis revealed common signatures of transcriptome deregulation among more than 20 different teratogens. Based on this data, we established a panel of ~200 transcriptional biomarkers representative of teratogen induced gene expression deregulation. Here, we applied our biomarker panel combined with targeted RNA sequencing to an independent set of compounds, reliably separating teratogens from non-teratogens. Finally, our results suggest that the strategy of a data driven biomarker selection in a stem cell based *in vitro* model system represents a valuable tool for efficient and accurate identification of developmental toxicants.

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P08-09
Evaluating teratogenic potential with newest NAMs: refining hazard with exposure, a case study with valproic acid?

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Addressing reproductive toxicity, including teratogenicity with New Approach Methodologies is a challenge, given the mechanistic complexity of these effects. L'Oréal has developed a decision tree for identifying teratogens based on a training set of 46 non-teratogenic and 39 teratogenic chemicals. A high sensitivity above 96% was achieved based on a "2 out of 3" approach, when combining the *in silico* DART model from the OECD QSAR Toolbox, an induced pluripotent stem cell assay (*in vitro* devTOX quickPredict assay) and Zebrafish embryos Test (ZET).

Risk Assessment relies on combining hazard data with exposure estimates. Including exposure estimates relies on dedicated methods and models. Thus, use of PBK pregnancy models and development of an *in vitro* placental barrier model are being explored.

Valproic acid (VPA), an antiepileptic drug with teratogenicity as a well-known side effect, was used as a case study, to evaluate our decision tree. To investigate if the adult therapeutic plasmatic concentration (Cmax) could be protective for fetal and pregnant exposure, bioactivity exposure ratios -BERs- were calculated as the ratio of $POD_{Bioactivity}$ (using EC50 malformation value in ZET and the stem cell-based assay potential concentration for developmental toxicity to human exposure levels).

- To refine the internal human concentration, the pregnancy PBK model in GastroPlus software was used. The predicted versus observed maternal serum concentrations were within a factor of 2, indicating that the model reasonably captured VPA's PK in pregnancy. Simulated profiles of VPA in maternal and foetal fluids were obtained at a specific gestation age, aiding in mechanistic understanding VPA's ADME processes and identifying key physiological parameters through sensitivity analysis. The model highlighted uncertainty of some predicted values in foetal fluids, likely due to scarcity of pregnant PK data for validation, and inherent complexities of physiological responses during pregnancy. Further refinement approaches using in vitro testing were identified.
- Access of chemicals to the human embryo relies either on active transport or diffusion across placental barrier. To improve the human relevance, a placental barrier model is being developed to mimic and predict chemical transfer, based on co-culture of trophoblastic (JEG-3) and endothelial (HUVEC) cells. Using such model aims to optimize concentration ranges to be tested *in vitro* according to foetal exposure, and thus to refine risk assessment interpretation. Further evaluation of VPA transport through the placental barrier is on-going. This model will also be used to feed the PBK pregnancy model, to generate human mother and foetal concentrations for BER refinement.

This case study is to show the potential of such tiered approach in investigating the teratogenicity of VPA in a Next Generation Risk Assessment context.

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P08-10
Advancing the use of New Approach Methodologies for assessing teratogenicity using a tiered approach: metabolism consideration to improve the human relevance

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Since the 2013 European animal testing ban, the requirement for new alternative methodologies (NAMs) has become essential especially in the cosmetics sector. To that end, research fields, particularly into reproductive toxicity and teratogenicity is a high priority.

Given the mechanistic complexity of these endpoints, we have developed a decision tree for assessing ingredients based on a training set of 85 test chemicals which comprised 46 compounds which were classified as non-teratogenic and 39 which were classified as teratogenic. We demonstrated a high sensitivity with a combination of three different NAMs: the *in-silico* DART model from the OECD QSAR Toolbox, the devTox assay and the ZET (Zebrafish Embryo Testing). The sensitivity of this approach is above 96% and the mean specificity is above 72%, indicating it is protective of human health.

However, considering this tiered approach, sensitivity data for all three tests showed that false-negative results account for 4.1%, 13.8 and 22.2% of Teratogenic chemicals tested for the OECD QSAR Toolbox, devTox assay and ZET respectively.

To have a better understanding on the mechanisms underlying the process of teratogenesis with false negative chemicals using both dev-

Tox assay and ZET, few hypothesis were drawn and the role of metabolism in the teratogen process could not be ruled out. Indeed, the capacity to metabolically convert xenobiotics by zebrafish embryos is supposedly low and likewise, a lower predictivity of the devTox assay could be due to the lack of a metabolizing component because it is likely that iPS cells used in this assay do not have an inherently high metabolic capacity to metabolize the test chemicals. Previous studies have also shown that *in vitro* metabolic conversion (rat microsomes) prior to exposing fish embryos could improve the predictivity of the toxic potential of the compound.

First, a selection of parent molecules and their associated teratogenic metabolites was carried out on different molecules that gave negative results on fish embryos and the cell model. The guiding materials which were selected for this study are two well-known teratogens and their metabolites used as fungicides (benomyl and carbendazim) and two drugs (Diphenytoin and 5-(4-Hydroxyphenyl)-5-phenylhydantoin). The two parent teratogenic molecules showed no malformation on the Zebrafish embryos and Phenytoin was also negative in the devTox assay.

To test this hypothesis, both parent compounds and metabolites will be tested by using iPSCs based devTox assay, the zebrafish embryo testing (ZET) and the QSAR DART Decision tree with the aim of knowing whether the toxicity is driven by the metabolite or not and to understand in more detail the mechanistic process leading to teratogenicity.

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P09 | Developmental neurotoxicology

P09-01
Dimorphic effects of Chlorpirifos in immortalized hypothalamic murine cell line (GT1-7) by co-exposure with sexual hormones

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Chlorpyrifos (CPF) is an organophosphorus pesticide banned in EU but still widely used in some countries. Human exposure occurs mainly through diet and some sub-populations are more vulnerable, such as children and pregnant women. CPF induces several neurodevelopmental and reproductive adverse effects, but little is known on central dysregulation of the Hypothalamic-Pituitary-Gonadal-Axis (HPG axis). Based on previous results demonstrating sexually dimorphic effects of CPF in the hypothalamus of exposed mice, we investigated the mode of action of CPF by an *in vitro* approach using the murine hypothalamic GT1-7 cell line of GnRH neurons, central regulators of the HPG axis. Cells were treated with CPF at human relevant concentrations (1 nM–100 μM) in absence and presence of physiological concentrations of 17β-estradiol (E2) (100 pM) or testosterone (T) (10 nM), then assessing cells viability and proliferation, both in 2D and 3D conditions. Spheroid morphology was also assessed at different time points. At not cytotoxic doses (1, 10, 50 e 100 nM) we analysed GnRH secretion by ELISA, the gene expression of a panel of neuroendocrine markers (GnRH; the estrogen receptors, ERα and ERβ; the androgen receptor, AR; the oxytocin precursor Neurophysin I, OXT; the oxytocin receptor, OXTR; the gene encoding the enzyme aromatase, CYP19A1) by real time PCR, and cell morphology by transmission electron microscopy (TEM).

CPF induced a significant dose-dependent reduction of cell metabolic activity, with a stronger effect in presence of E2, and a significant decrease in cell proliferation in presence of testosterone. By contrast, spheroid areas increased at CPF highest doses in presence of testosterone. GnRH secretion was reduced in CPF+E2 treated cells at all tested concentrations and in CPF+T cells only at the highest concentration. This effect was confirmed also at gene expression level. The CPF+E2 combined exposure induced ERα and OXTR expression whereas CPF+T induced ERβ, aromatase and OXT gene expression; AR expression was not significantly affected in either condition. Moreover, TEM analysis evidenced protective effects of E2 and T on CPF-induced mitochondrial damage.

Overall, exposure of hypothalamic GT1-7 cells to CPF exerted dimorphic effects in presence of the two sexual hormones, confirming *in vivo* results and supporting the reliability of the *in vitro* approach. Further investigation on the mode of action of CPF in the two hormonal conditions is in progress by proteomics analysis.

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P09-02
A global new alternative methodological approach to complement teratogenicity and developmental neurotoxicity studies with thyroid disruption analysis in zebrafish embryos for different chemicals

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Chemicals pose an increasing risk to human and environmental health. Significant causes for concern are Teratogenicity, Neurotoxicity, and Endocrine-Disrupting Chemicals (EDCs). Using the zebrafish larvae as a New Alternative Methodology (NAM), we showcase a high-content strategy to distinguish the embryotoxic potential of reference chemicals as well as their thyroid disruptor potential and developmental neurotoxicity. Embryos of the transgenic fish line Tg(*tg*:mCherry) were exposed to a set of chemicals -Potassium perchlorate, Benzophenone-2, Propylthiouracil, Phenobarbital, and Acetaminophen- to evaluate the fluorescence of the reporter in the thyroid gland. By fluorescence measurements of the reporter for the thyroglobulin (tg), the compounds Potassium perchlorate, Benzophenone-2, and Propylthiouracil were properly classified as having a goitrogenic outcome for concentrations <EC10 of systemic toxicity. To determine the potential downstream effects of the impaired endocrine system, T4 and T3 levels can be assessed by a newly developed LCMS technique for whole-embryo homogenates, which allowed for the detection of a T4 decline for Potassium perchlorate, Benzophenone-2, and Propylthiouracil-treated embryos from ca. 0.5 $\mu g_{T4}/L_{extract}$ to around 0.1 $\mu g_{T4}/L_{extract}$. Furthermore, gene expression analyses of thyroid genes (*tshβ, tpo*, and/or *tg*) were assessed using RT-qPCR. A dose-dependent induction of tg mRNA for Potassium perchlorate, Benzophenone-2, and Propylthiouracil-treated zebrafish embryos was identified. However, this induction could not rescue the low T4 level-phenotype as pointed out by the LCMS analyses. This could potentially be related to one of the chemicals' frequent modes of action to inhibit the thyroid peroxidase, which might have suppressed the iodide oxidation needed for functional T4. The results were complemented with the developmental toxicity (malformations; mortality) and developmental neurotoxicity (DNT, with the Light/Dark transition Assay) assays in zebrafish embryos to emphasize the applicability of thyroid disrupting assay, especially for EDCs, as its indirect effect related to them.

Despite Acetaminophen not having a goitrogenic effect, we were able to detect its teratogenic effect, while neither the thyroid disruption effects of Phenobarbital was detected, but it was possible to see its hyperactive behavior in zebrafish embryos under the tested conditions.

Benzophenone-2 was classified as toxic but likely not teratogenic and thyroid inhibitor under the testing conditions. While Potassium perchlorate and Propylthiouracil were classified as not toxic for zebrafish embryos, they were detected as thyroid inhibitors. Therefore, those results reinforce the importance of studying the potential endocrine-disrupting effect of chemicals as complementary studies to teratogenicity and developmental neurotoxicity.

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P09-03
Prenatal particle exposure and ageing of the CNS-a study in young and aged mice cohorts

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Workers in occupations with continuous air pollution exposure, such as transportation, construction, mining, and manufacturing, are at significant risk of developing respiratory and cardiovascular diseases as well as neurological disorders. Furthermore, in many countries, women have a high employment rate, and a significant proportion of them are exposed to environmental air pollution at their workplace. Women continue to work well into their pregnancy, resulting in a potentially high number of particle-exposed pregnant women; maternal exposure to air pollution particles has been associated with neurodevelopmental disorders in offspring.

With existing studies predominantly focusing on the adverse effects of particle exposure in young offspring, our understanding of the long-term health effects remains limited. This study seeks to explore the potential link between prenatal particle exposure, ageing, and neurodegeneration. Ageing constitutes the largest risk factor for neurodegeneration. With the rapidly increasing ageing population worldwide, investigation of the long-term impact of processes affecting the central nervous system is vital, considering that it is estimated that the prevalence of neurodegenerative conditions will triple by 2050.

Our hypothesis posits that prenatal exposure to air pollution particles either accelerates or alters the normal ageing process, resulting in the emergence of neurodegenerative traits in the offspring. To investigate this hypothesis, we will expose pregnant mice four times throughout gestation via the airways to 268 μg of carbon black nanoparticles or vehicle. We will assess maternal pulmonary inflammation at postnatal days 25 and 26. The offspring will be examined for behavioural, anatomical, and physiological indicators of neurodegeneration at 3, 5, 10 and 15 months of age. Assessments include cognitive and memory functions, sociability, motor skills, neuronal function, protein misfolding and aggregation, excitotoxicity, and neuronal, mitochondrial and microglial dysfunctions.

We aim to investigate how prenatal exposure influences key aspects of neurodegenerative diseases, identify factors that heighten susceptibility later in life and guide the development and selection of disease models to understand the impact of prenatal exposure.

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P09-04
Assessment of developmental neurotoxicity of imidacloprid on hippocampal neurogenesis and cerebellum in rat offspring

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Purpose: Developmental exposure to imidacloprid (IMI), a neonicotinoid insecticide, has recently been suggested to affect mammalian brains due to oxidative stress induction. In the present study, we examined the developmental exposure effects of IMI on hippocampal neurogenesis and cerebellar development, as highly sensitive endpoints of developmental neurotoxicity (DNT), in rats. We further examined the chemopreventive effects of α-glycosyl isoquercitrin (AGIQ) as an antioxidant on IMI-induced DNT.

Methods: Experiment I: Dams were exposed to IMI (83, 250, and 750 ppm in diet) from gestation day 6 until weaning. Experiment II: Dams were administered AGIQ at 0.3% in drinking water until weaning in addition to IMI at 750 ppm in diet. After weaning, offspring were administered AGIQ until adulthood.

Results and Discussion: Experiment I: At weaning, IMI at 750 ppm decreased hippocampal acetylcholinesterase (AchE) activity. At this dose, IMI decreased the numbers of DCX^+ cells, $TUBB3^+$ cells, $PCNA^+$ proliferating cells, and c-FOS^+ or p-$ERK1/2^+$ granule cells in the subgranular zone (SGZ) and/or granule cell layer (GCL), and $RELN^+$ interneurons in the hilus of the hippocampal dentate gyrus (DG), suggesting suppressed proliferation of neural progenitor cells (NPCs) to cause decreases in late-stage NPCs and postmitotic immature granule cells and suppressed synaptic plasticity by suppressing reelin signaling. IMI at 750 ppm also increased the numbers of $GFAP^+$ astrocytes, $CD68^+$ or $Iba1^+$ microglia in the DG hilus and upregulated oxidative stress and inflammation-related genes. In adulthood, IMI decreased $GFAP^+$ type-1 neural stem cells and $NeuN^+$ postmitotic granule cells in the SGZ/GCL at ≥ 250 ppm, increased $TUNEL^+$ SGZ cells and downregulated *Pcna* and *Bcl2l1* in the DG at 750 ppm, indicating progressive disruption of neurogenesis. Besides persistent decrease in AchE activity, elevated malondialdehyde level at 750 ppm in the DG suggests enduring impacts on hippocampal nicotinic receptor signaling and worsened oxidative stress. In the cerebellum, IMI at 750 ppm decreased $CALB1^+$ Purkinje cells, impaired related behavioral endpoints, disrupted cholinergic system and induced neuroinflammation and oxidative stress on weaning. In adulthood, IMI irreversibly decreased $CALB1^+$ Purkinje cells and disrupted behaviors.

Experiment II: At weaning, AGIQ reversed IMI-induced disruptive hippocampal neurogenesis involving $RELN^+$ interneurons, improved Y-maze behavior, and upregulated *Ntrk2*, suggesting restoration of

neurogenesis by recovering reelin and enhancing BDNF/TrkB signaling. In adulthood, AGIQ upregulated *Bdnf* and *Ntrk2*, rescuing neurogenesis and countering oxidative stress and immune imbalance by modulating microglia and anti-oxidant system.

Conclusion: These results suggest that IMI causes sustained disruption of hippocampal neurogenesis and cerebellar development by induction of oxidative stress, and AGIQ may be effective for chemoprevention of IMI-induced DNT.

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P09-05

Glyphosate exposure *in utero* induced social behavior alteration and neuronal cell death, which could be rescued with postnatal butyrate administration

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Developmental neurotoxicity (DNT) induced by chemical exposure has become a severe problem in modern society. In recent years, the DNT of Glyphosate (GP), one of the main ingredients of the world's most frequently used herbicides, attracted attention. Some reports have observed neurotoxicity, behavioral abnormality, and alteration of organ physiology of humans and animals who were exposed to GP; however, we know little about the pathology and physiology of the DNT of GP. Previously, we have shown that acute exposure of GP to pregnant rats on gestation day 15 (G15) led to Purkinje cell death and microglia increase in the developing cerebellum of the offspring in a dose-dependent manner two weeks after birth. In this period, GP-exposed pups showed abnormal movement, and we observed that glutamate transporter expression in astrocytes was enhanced. In this report, we investigated the social behavioral disorder of GP-exposed offspring. Additionally, we attempted how to recover neuronal development and social behavior.

We administered GP 250 mg/kg the mother's weight p.o. on gestation day 15 (G15). Some pups with or without GP exposure in utero were administered butyrate of 400 mg/kg pup weight /day from P3 to P10 p.o. In postnatal week 8 (PW8), we observed the simple and social behavior of the offspring. The developing cerebellum was observed on postnatal day 14 (P14).

GP 250 mg/kg-exposed offspring significantly avoided to stay the center circle of the cage and showed less contact with strange rats than vehicle rats. Additionally, we observed frequent grooming of GP-exposed offspring beside strange rats, which was similar to anxiety behavior. In contrast, postnatal butyrate-administrated GP-exposed pups showed little different behavior from control pups. Butyrate administration could recover the alteration of the developing cerebellar cortex. Our investigation clarified that prenatal GP exposure would induce disorders of simple and social behavior related to cerebellar neuronal alteration. Furthermore, GP-exposed alteration could recover with postnatal butyrate administration. Because butyrate has an effect of HDAC inhibitors, we suggest that prenatal GP exposure would induce epigenetic change in developing neurons and glial cells, and this change could be reversible during the early period after birth.

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P09-06

The endocrine disruptor17-α-ethinyl estradiol affects the glutamatergic maturationof rat primary hippocampal neurons *in vitro*: post-synaptic molecular dynamics, morphological and functional effects

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17-α-ethinyl estradiol (EE) is a semi-synthetic estrogen-like hormone contained in most combined contraceptives and drugs with therapeutic indications such as hormone replacement therapy. It binds to the cytosolic and nuclear estrogen receptor-α and -β (ERα, ERβ) and membrane receptors such as the G protein-coupled estrogen receptor (GPER) mediating both rapid signaling and transcriptional regulation. Thus, interfering with estrogen pathways, it is classified as an endocrine-disrupting chemical (EDC). In Europe and the USA, approximately sixty million women use oral contraceptives, and it is estimated that nearly two million unintended pregnancies occur each year due to medication errors, potentially exposing the embryo to EE during the first weeks of development. Contraceptives are often taken for several weeks before the unintended pregnancy is detected. Considering its widespread use, EE is found in soil and water and it is classified as an environmental contaminant, thus also oral exposure via contaminated water has to be taken into account. During pregnancy, EE can cross both the placental and the blood-brain barrier (BBB) reaching the hippocampus, a glutamatergic area expressing hormone receptors. It is known that hormones can influence synaptogenesis and that glutamatergic synapses' development is regulated by the GluN2B/GluN2A switch of the NMDA receptor subunits in a precise time window. This study aims to investigate the EE effect on the expression and distribution of glutamatergic receptors in rat primary hippocampal neurons and the consequences on spine morphology and calcium transients. Neurons are treated with EE following different exposure schemes: from day *in vitro* (DIV) 1 to 18 to replicate an exposure covering the whole maturation period; (ii) at 7 DIV for 24 hours to replicate a short exposure covering the critical time window relevant for the GluN2B/GluN2A switch; (iii) from DIV 7 to 18, to replicate an exposure starting around the key GluN2B/GluN2A switch event but lasting until maturation. NMDA and AMPA receptor subunits in the homogenate and at the postsynaptic site are analyzed. Results obtained suggest that EE alters the developmental program of the glutamatergic system. The effect depends on the exposure time window and involves altered expression and molecular dynamics of receptor trafficking to the post-synaptic spine. Moreover, analysis by confocal microscopy in GFP-transfected neurons showed a decrease in mature mushroom-shaped spines. This morphological alteration is reflected from a functional point of view detected with live imaging experiments on post-synaptic calcium transients. EE-treated neurons did not show differences in spontaneous calcium transient compared to control but are characterized by non-sensitivity to bicuculline, showing altered bicuculline-induced post-synaptic calcium transients.

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P09-07
Hazard identification and risk assessment based on developmental neurotoxicity *in vitro* data – natural compound case study

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The Organization for Economic Cooperation and Development (OECD) has issued initial recommendations for the evaluation of data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery (IVB). This battery consists of 17 assays measuring 8 neurodevelopmental key events, including neural progenitor cell (NPC) proliferation and apoptosis, migration of different cell types, neuronal and oligodendrocyte differentiation, neurite outgrowth, neuronal maturation and synaptogenesis, and neural network formation. A compound is deemed "*in vitro* DNT positive" if it exhibits specific activity in any of these assays. Initial results reveal a high positive hit rate across the currently tested compound set including several agrochemical compounds. However, guidance on how to incorporate "*in vitro* DNT positive" conclusions into human hazard and risk assessments within existing regulatory frameworks is not available. One open question is if a safe margin of exposure for human risk assessment, considering the difference between *in vitro* point of departure and estimated human exposure can be established. To address this gap, the DNT IVB compound test set was expanded to include natural compounds with human exposure levels similar to or higher than agrochemicals and without known human DNT concerns. A subset of the DNT IVB assays, the human neural progenitor cell assays 1-5 (NPC1-5), and the human neural network formation (hNNF) assay, were utilized for this extension. Eight natural compounds, including epigallocatechin gallate (EGCG), folic acid, vanillin, limonene, vitamin E, curcumin, genistein, and caffeine, were tested in both the NPC1-5 and hNNF assays up to the solubility/cytotoxicity limit. Three compounds, EGCG, genistein and curcumin, demonstrated DNT specific effects across multiple endpoints while caffeine demonstrated a DNT specific effect only in the hNNF assay. Four compounds did not show any activity in the tested concentration range. The data collected was used to (i) assess current performance characteristics of individual assays, (ii) compare *in vitro* activity concentrations with human internal exposure levels to investigate if a safe margin of exposure can be established, and (iii) discuss potential data interpretations and their implications for the hazard and risk assessment based on DNT-IVB testing data. With this study we aim to support the integration of data from the DNT IVB for future human hazard and risk assessment of agrochemicals.

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P09-08
Further exploring the automated touchscreen-based mCANTAB device: testing for motor precision and employing a self-ordered spatial search paradigm

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In the early stages of developing drugs that affect the central nervous system, regulatory authorities often request an examination to understand whether the new drug might affect cognitive function or development. When the drug target requires the use of cynomolgus monkeys as the pharmacologically relevant animal model, the learning and memory assessment are included in safety studies.

We have conducted a study that evaluated the effect of scopolamine, as a reference compound, on simple discrimination learning. Our study found that acetylcholine receptor blockade disrupts stimulus reward association causing learning impairment, as expected. In this study, we reported two additional investigations performed in this study with scopolamine as the reference compound. First, we investigate the precision of touch responses as a potential influence on discrimination performance by evaluating the effect of target size on the performance of animals. Second, we assess whether the self-ordered spatial search paradigm (SOSS task), can be trained under these experimental conditions in parallel to the main discrimination tasks. The study included a group of eight naïve male Cynomolgus monkeys that were trained using an automated device (Monkey CANTAB intellistation).

After initial touch training, animals were trained to a high level of stable performance on discrimination tasks and a basic level of SOSS tasks, however, the period of training was defined by success in discrimination tasks, with the SOSS paradigm trained as feasible within this period. Subsequently, animals were divided into two groups, receiving either scopolamine or Vehicle, followed by additional cognitive testing. Then groups were crossed over and animals were tested again.

Our results showed that scopolamine-induced learning impairment among subjects on discrimination tasks was not due to impaired motor precision, because the animals performed well on touch targets equal to or smaller than the discrimination targets. Second, it was feasible to train most of the animals successfully on basic levels of the SOSS task in parallel to discrimination tasks, with performance on SOSS being more variable than discrimination performance. No significant effect of the drug treatment was found on the SOSS performance correspondingly.

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P09-09
In vitro evaluation of the possible neurotoxicity, neuroprotection, and MAO-B inhibitory effects of pyrrole-based hydrazones

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Parkinson's disease is a huge burden in modern medicinal practice. A serious drawback of current antiparkinsonian therapy is its symptomatic nature. This directed our investigations in the search for new more potent derivatives, affecting not only the loss of dopaminergic neurons but also the oxidative damage of neuronal cells.

Purpose: *In vitro* neurotoxicity and neuroprotective analysis on a group of N-pyrrolyl hydrazide-hydrazones were performed. The neu-

rotoxicity of the target derivatives was determined on a subcellular level in isolated rat brain synaptosomes, mitochondria, and microsomes. The neuroprotective effects of the evaluated hydrazones were measured in three models of induced oxidative stress: 6-hydroxidopamine (6-OHDA), *tert*-butyl hydroperoxide (*t*-BuOOH) and non-enzyme (Fe2+/AA)-induced lipid peroxidation.

Methods: The rat brain synaptosomes and mitochondria were received by multiple centrifugations, using Percoll gradient. The rat brain microsomes – by using differential centrifugation.

Results: The results identified the ethyl 5-(4-bromophenyl)-1-(3-hydrazinyl-3-oxopropyl)-2- methyl-1H-pyrrole-3-carboxylate (**12**) as the most promising compound with the lowest neurotoxicity and highest neuroprotection on all evaluated parameters and inhibiting the human recombinant MAOB (*h*MAOB) enzyme by 50%, comparable with the activity of the reference, Selegiline.

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P09-10

The synthetic cannabinoid ADB-FUBINACA disrupts mitochondrial biogenesis of NG108-15 neuroblastoma cells during neuronal differentiation at human-relevant concentrations

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The use of ADB-FUBINACA (ADB), a potent synthetic cannabinoid (SC), by young adults (including women of child-bearing age or those who are pregnant/lactating) raises substantial concerns, as it carries a high risk of triggering neurodevelopmental disorders in their offspring. Here, we hypothesize that this SC may disrupt cellular mechanisms regulating mitochondrial dynamics during neurodifferentiation, using NG108-15 neuroblastoma cells as a neuronal model.

Neurodifferentiation of NG108-15 cells was induced in serum-starved (1% fetal bovine serum) cell culture medium supplemented with 10μM retinoic acid and 30μM forskolin and a cholinergic phenotype attained after 72h. ADB was added at the start of differentiation, at human-relevant concentrations, ranging from 1pM to 1μM. A vehicle control (0.1% DMSO) was also tested. The expression levels of mitochondrial markers, including mitochondrial mass ([e.g., voltage-gated anion channel (VDAC)], biogenesis (e.g., PGC-1α, NRF1), mitophagy (e.g., Parkin, Pink1), fusion (e.g., OPA1, MNF2), and fission (e.g., DRP1), were evaluated 24 and 72h after initiating the differentiation, by Western blot in total cell protein extracts.

VDAC expression levels, an indirect marker of mitochondrial mass, increased in differentiating cells (control) between 24 and 72h during differentiation. However, such an increase was not sustained in cells exposed to 1nM and 1μM ADB. Indeed, at 72h, VDAC levels in the presence of ADB were approximately 40–55% lower compared to the vehicle control, suggesting disturbances in mitochondrial turnover (i.e. biogenesis or mitophagy). Interestingly, an increase in PGC-1α levels was noted (for 1nM ADB at 24h, and for both 1nM and 1μM at 72h), suggesting the dysregulation of the biogenesis pathway and potential compensatory mechanism. Neverthless, NRF1 levels remained unaltered. Notably, no significant alterations were observed in Parkin and Pink1 levels, indicating that ADB did not affect mitophagy. Concurrently, exposure to ADB led to an increase in DRP1 and a decrease in OPA1 levels by approximately 60–70% at 72 h, indicating a disruption in the fusion/fission balance in favor of mitochondrial fission. Our findings highlight the ADB-triggered disturbance of mitochondrial dynamics during NG108-15 cell differentiation. These effects may potentialy compromise mitochondrial physiology (e.g. membrane potential) and function efficiency (e.g. ATP production), crucial to neurodifferentiation. Nevertheless, further in-depth research is needed to fully elucidate the underlying mechanisms.

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P09-11

Historical control data on pathology and neuropathology collected in OECD TG 443 or OECD TG 426 studies

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In context of neurodevelopmental toxicity assessment more and more Extended-One-Generation toxicity studies (EOGRTS – OECD TG 443) with DNT cohort or Developmental Neurotoxicity (OECD TG 426) studies are requested. The OECD Guidelines require brain weight and neuropathology evaluations at two timepoints: at weaning (postnatal day: PND 22) and in young adults at PND 70/77. The neuropathology examinations comprise histopathological investigation of major brain regions (e.g. olfactory bulbs, cerebral cortex, hippocampus, basal ganglia, thalamus, hypothalamus, midbrain, pons, medulla oblongata and cerebellum) and morphometric evaluation (e.g. linear or areal measurements). As the dynamic growth process of body and brain is largest between PND 0 and 21 ("brain growth spurt"), also the individual body weights of the animals might impact the brain neuropathological parameters (Garman *et al.*, 2016).

Here, control data of several studies (EOGRTS or DNT) are shown. The studies were conducted between the years 2013 and 2024 and comprised different administration routes. All studies were conducted according to OECD Guidelines and ran under GLP. More explicitly offspring body weight data at PND 22 and PND 70/77 are summarized. In correlation to that brain weight data will be shown, as well as the length and width of brain and morphometric measurements will be analyzed.

Descriptive statistics and coefficients of variation for these endpoints will be presented. Recommendations for interpretations will be given.In order to assess brain morphometric or brain weight changes as being treatment-related, concurrent and historical control data and variability of measurements should always be taken into account. Also, the observation of only single findings at one timepoint in one sex or in just one parameter – not corroborated by other findings (like e.g. neurobehavioural effects) should overall be considered to be of lower weight of evidence compared to observed patterns of effects.

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P09-12

Thyroid hormones concentrations in cerebellum, cortex and whole-brain rat samples at certain life stages

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The development of the brain from fetal stage to young adulthood can be disturbed by affecting thyroid hormone (TH) homeostasis. A correlation between maternal thyroid function during pregnancy and the offspring's gray matter volume and IQ have been described[1].Typically, disturbances of the TH homeostasis are detected by measuring TH concentration in plasma. The target organ of developmental neurotoxicity is, however, the offspring's brain. Measuring TH concentrations in the target organ may be more relevant to developmental neuronal effects due to disturbed TH homeostasis than measuring TH concentrations in the maternal plasma[2, 3]. Considering standardized study sampling timepoints, we analyzed whole brain as well as cortex and cerebellum regarding their TH metabolite concentrations in adult and postnatal day 4 and 21 (PND4 and 21) rats using an on-line solid phase extraction liquid chromatography tandem mass spectrometer (on-line SPE-LC-MS/MS) method. T4 and T3 in plasma and T2 in brain reached their peak concentration on PND21 and the concentrations in adults were similar to values on PND4 in both matrices. The analyte rT3 on the contrary reached high brain concentrations on PND4 and showed similar concentration between PND21 and adult rats. In plasma rT3 and T2 did not change between the examined ages. Looking specifically into brain regions we detected lower T3 and T2 concentration on PND4 in cerebellum compared to cortex. Both regions yield similar T3 and T2 concentration on PND21 as well as in adult rats. rT3 behaved contrary to the other analytes namely similar concentration in both regions on PND4 and lower concentration in cerebellum than cortex on PND21 and in adult rats. On PND4, 21 and in adulthood respectively, T4 yield similar concentration between the two brain regions.

Currently we are aiming at generating a data base regarding TH concentrations in control rat brain which could help to improve the differentiation of dose-related effects from biological variation in the given matrix. Additionally, it is interesting to investigate for a possible correlation of THs in brain with total TH and free TH concentration from plasma samples at each age. In a final step, the aim is to develop a TH analysis using formalin fixed and/or formalin fixed paraffin embedded (FFPE) samples so that TH analysis could be done in retrospect. This could reduce animal numbers because no additional animals for brain TH analysis would be needed.

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P09-13

Methylmercury induces visual deficits of zebrafish embryos involving in dysregulation of gene expression in the retina

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Mercury is a well-known neurotoxicant for humans and wildlife. The World Health Organization has listed mercury as one of its top 10 chemicals of public health concern. Visual systems are sensitive to methylmercury (MeHg) exposure, and oculomotor disturbances and blindness are frequently present in patients with MeHg poisoning while lower level exposure is associated with losses in visual acuity and constriction of the visual fields [1]. However, we know little about how MeHg can influence the visual systems development and optomotor response. Mercury has been found in the retina and optic nerve of experimentally exposed mouse and fish [2,3]. Therefore, to see if MeHg exposure could affect the retinal development, we examined the retina and optomotor response of zebrafish embryos that had been exposed to MeHg, and analyzed the gene expression profiles using RNA-sequencing. Transgenic zebrafish *Tg(gad1b:mCherry)* embryos were exposed to 6–30 μg/L from 4 to 72 hpf. At 96 hpf, the number of the retinal cells expressing fluorescent protein was significantly reduced comparing with the control, even at concentrations as low as 10 μg/L. The defects in optomotor response and color preference of embryos at 6 dpf were observed. MeHg significantly disrupted expression of 1412 genes in the retina, including gene ontologies in terms of visual and sensory perception, glutathione metabolism, steroid hormone biosynthesis and necroptosis. In conclusion, MeHg-induced alterations in gene expression of the retina may disturb the retinal cells differentiation resulting in histological changes and cell death, and ultimately affect function of visual systems.

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P09-14

The synthetic cannabinoid AMB-FUBINACA promotes neuronal differentiation and astrocyte activation while compromising the maturation of the newly formed neurons in *in vitro* primary hippocampal cultures

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Synthetic cannabinoids (SCs) are a class of New Psychoactive Substances that mimic the effects of THC (Δ9-tetrahydrocannabidiol) with increased potency. Their use by young adults, including pregnant and

breastfeeding women, as well as women of childbearing age, poses significant concerns regarding potential neurodevelopmental disorders in their offspring. This study aimed to evaluate the impact of the widely abused SC AMB-FUBINACA on the differentiation and maturation of primary hippocampal cultures (PHC). Furthermore, we investigated whether this SC affects astrocyte activation, given the key role of astrocytes in promoting neuronal network formation. PHC isolated from Wistar rat embryos at embryonic days 18–19 were exposed to AMB-FUBINACA at human-relevant, non-cytotoxic concentrations (1pM–1μM) after 24h in culture and every 4 days *in vitro* (DIV) up to 14 DIV. A solvent control (0.02% DMSO) was also tested. Neuronal differentiation and maturation were assessed by immunocytochemistry at 3, 7 and 14 DIV by determining the percentage of cells labeled with Tuj-1 (for neuron-specific beta-tubulin III; early differentiating neurons) and MAP2 (microtubule-associated protein 2; mature neurons), relatively to the total number of cells. Astrocyte number and activation were determined as the percentage of cells positive for glial fibrillary acidic protein (GFAP) labeling and by the relative fluorescence intensity of GFAP per cell, respectively.

AMB-FUBINACA increased the percentage of Tuj-1-positive cells after 14 DIV by about 17% at 1nM ($p<0.05$) compared to the solvent control, indicating a stimulation of neuronal differentiation. Conversely, at 14 DIV, MAP-2-positive cell labeling decreased by 26% for 1nM and about 23% for 1μM ($p<0.01$), compared to the solvent control. This SC also decreased the number of GFAP-positive cells after 14 DIV by about 16% at 1nM ($p<0.01$), compared to the solvent control. However, intriguingly, it appeared to enhance astrocyte activation by approximately 2-fold at 1 μM at 14 DIV.

In summary, our findings indicate that AMB-FUBINACA promotes the differentiation of neurons in PHC, while simultaneously hindering the maturation of the newly formed neurons. Notably, this SC-induced neurodifferentiation aligns with early astrocyte activation. Given the pivotal role of astrocyte activation in neurodifferentiation, further investigation is warranted to ascertain whether AMB-FUBINACA's impact on astrocyte function correlates with its effects on neurogenesis.

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P09-15
Utilizing neuronal differentiation reporter mice for *in vivo* detection of developmental neurotoxicity

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Current *in vivo* developmental neurotoxicity (DNT) tests are not routinely conducted for chemical risk assessment due to their time, resource intensiveness, and high animal usage. Therefore, there is a need for New Approach Methodologies that can detect and assess the DNT potential of chemicals in a simpler, more quantitative, and objective manner. To address this need, we generated transgenic mice expressing reporter genes (luciferase and lacZ) under the control of the rat synapsin 1 promoter (Syn-Rep mice) and evaluated their utility as a DNT detection tool.

Brain luciferase expression levels in Syn-Rep mice showed a significant increase from just before birth to after birth, peaked early in the postnatal period, sharply decreased thereafter, and remained low after weaning. This temporal expression pattern mirrors the well-established changes in synapse numbers during mammalian brain development.

To further assess the responsiveness of Syn-Rep mice to DNT induction, we administered valproic acid (VPA), a known DNT-inducing chemical, to pregnant mice and evaluated its impact on reporter gene expression in the developing brains of Syn-Rep pups. *In vivo* luminescence in the brains of VPA-exposed pups was significantly lower than in controls from postnatal days 4 to 13. Additionally, luciferase activity in the prefrontal cortices of 8-week-old VPA-exposed offspring was significantly reduced compared to controls, reflecting the decreased number of neurons in the prefrontal cortex.

These findings suggest that Syn-Rep mice represent promising tools for the streamlined detection of chemical-induced DNT in the developing mammalian brain.

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P09-16
Functional alterations of neuronal signalling in human dopaminergic neurons and hiPSC-derived astrocytes by a subgroup of neonicotinoid pesticides

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The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is a tightly regulated process in neurons. $[Ca^{2+}]_i$ has a pivotal role in several important functions in neurons like neurotransmitter release, synaptic transmission, learning and consolidation of memory, as well as cell differentiation. The direct coupling between depolarization and $[Ca^{2+}]_i$ allows the measurement of molecular initiation events (MIE) of adverse outcome pathways (AOP) through Ca^{2+} imaging. We developed a single-cell Ca^{2+} imaging assay as an addition to the existing in-vitro test battery (Blum *et al.* 2022) to be able to test for disturbed neuronal signalling. Transient effects of toxicants on neurotransmitter signalling may be relatively harmless in adults, but they may permanently affect brain connectivity when occurring during sensitive periods of development. The single-cell Ca^{2+} imaging assay was able to identify compounds that disturb neuronal signalling in humans that showed up as negatives in other tests of the in-vitro battery like some members of the compound class of neonicotinoids. The single-cell Ca2+ imaging assay is applicable to different neuronal cell types like dopaminergic LUHMES cells, and astrocytes. Furthermore, we provide first evidence for a developmental neurotoxic effect of nicotine by disturbing glutamate signalling. In conclusion we present an addition to the in-vitro battery as a ready to use in-vitro assay to asses chronic and acute toxicant-induced disturbed human neuronal signalling.

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P09-17
Developmental neurotoxicity (DNT) of contaminants of emerging concern (CECs) in the zebrafish (*Danio rerio*)

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Introduction: Contaminants of emerging concern (CECs) are a large group of environmental micropollutants that can cause developmental toxicity, which can be oxidative stress (OS) mediated and lead to cellular damage, including in the nervous system. Microglia (MG) are a distinct population of cells with developmental and immunological functions in the brain. The aim of this project is to use high throughput testing (HTP) for developmental neurotoxicity (DNT) and identify the oxidative stress and MG response to CECs.

Materials and Methods: Zebrafish (Zf) (*Danio rerio*) embryos of the ABwt strain were exposed from 1 day post fertilization (dpf) to environmental concentrations of CECs (carbamazepine 0.012–245 mg/L, aspartame 100–5000 mg/L, fipronil 0.0085–85 µg/L and PFASs 0.1–1000 x human blood [1]). HTP testing, light and fluorescence imaging was performed to determine exposure ranges and developmental effects (hatching, mortality and head and eye deformities), including the effects on the behavior (EthoVision XT, Noldus, Wageningen, Netherlands) and reactive oxygen species (ROS) formation at 72 hpf by use of H2DCFDA probe (Cytation 3, Gen 5™, BioTek, Winooski, Vermont, US). Fluorescence activated cell sorting (FACS) in transgenic mpeg1:eGFP Zf, which express GFP+ under the control of the macrophage-expressed gene promoter in microglia will be used to quantify potential changes in MG cell function.

Results and conclusion: Aspartame and carbamazepine caused 100% mortality at 2500 mg/L and 245 mg/L, respectively, and 50% mortality for fipronil at 8.5 µg/L. Lower total hatching occurred at 1000 mg/L aspartame, 0.245 mg/L carbamazepine and fipronil 8.5 µg/L. Behavioral changes such as decreased swimming distance and velocity were observed only in concentrations which are higher than the commonly found environmental levels (500 mg/L aspartame, 24.5 mg/L carbamazepine and 85 µg/L fipronil). No changes in eye or head size were observed after exposure to 100 mg/L, 0.012 mg/L and 8.5 µ/L aspartame, carbamazepine and fipronil, respectively, compared to control, determined as sub-morphological concentrations. ROS was increased in Zf exposed to >500 mg/L aspartame and to >0.012 mg/L fipronil, while no effect was observed for carbamazepine compared to control. The results of the PFAS exposure are under analysis. The CECs exposure effects will further be compared to the microglia responses in transgenic Zf. By implementing the HTP assay for oxidative stress parameter and microglia response testing, we aim to develop a new approach method (NAM) for filling data gaps in DNT testing. This project will potentially contribute to identifying modes of action (MoA) of selected CECs and the development of Adverse Outcome Pathways (AOPs).

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P09-18
Comprehensive search for genes that vary across three developmental neurotoxicants

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Many studies have indicated a relationship between brain effects of environmental toxicants and increased risk of neurodevelopmental disorders such as autism spectrum disorder (ASD), attention deficit/hyperactivity disorder (ADHD), and schizophrenia. Current developmental neurotoxicity studies require the sacrifice of many animals, time, labor, and cost, which limits the ability to assess the developmental neurotoxicity of chemical substances, and there is a need for alternatives to simple and high-throughput testing methods. However, few genes have been useful as biomarkers for *in vitro* developmental neurotoxicity screening. In this study, we aimed to identify genes with variable expression that are common to three developmental neurotoxicants, methylmercury (MeHg), tributyltin (TBT), and acrylamide (AA), which are thought to have different toxicity mechanisms, by conducting a comprehensive gene expression analysis, and to search for indicators that can be used to easily evaluate unknown developmental neurotoxicants. Primary cortical neuron culture: Cells prepared from the cerebral cortex of Slc:Wistar/ST 18-day-old rats were cultured in Neurobasal™ Plus medium. The cells were exposed to MeHg, TBT, and AA from day 1 after seeding, and glial cells were removed by CultureOne. RNA sequencing was performed by NextSeqTM 2000 using mRNA recovered on DIV10. The concentrations of 100 nM MeHg, 10 nM TBT, and 30 µM AA were determined for RNA sequencing experiments as concentrations that have little effect on cell viability, but may slightly affect neurite outgrowth. Genes with an expression variation ratio of 1.5 or higher and a p value of less than 0.1 were extracted as expression variation genes, and genes that were commonly varied by two of the three compounds were extracted, narrowing the list of candidate genes to 46 genes. It is expected that useful gene indices for screening developmental neurotoxicants will be found among these genes.

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P09-20
Optimization and application of a rosette formation assay to assess potential developmental neurotoxicity effects caused by valproic acid analoga

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A large gap in human safety assessment exists in the field of developmental neurotoxicity (DNT) due to a lack in chemical testing. To overcome this, the establishment of New Approach Methodologies (NAM) for DNT testing has been highly intensified and recommended in the past few years. However, especially for complex DNT endpoints a lot of NAM are still in an optimization process to reach a high readiness level. An example of a challenging key neurodevelopmental processes to address in a NAM is the formation of the neural tube. Even though several protocols for *in vitro* rosette formation exist, there is still a need for more high fit-for-purpose test methods that address this process.

Therefore, we aimed here to optimize the previously published rosette formation assay (RoFA). One drawback of the previous protocol was, that only single (or very few) concentrations of the test compounds could be tested. We adapted the protocol in such a way, that multi-concentration testing was feasible. A newly written quantification program allowed to measure >1000 rosette like structures per single well after 12 days of differentiation. Upon treatment with known DNT causing compounds we were able to observe drastic reduction of the amount of rosette-like structures at concentrations were no cell death occurred.

Furthermore, we utilized and challenged the new protocol in a real-life case study scenario in regards to valproic acid (VPA), a compound known to cause neural tube defects in humans. For this, we tested (together with other NAM) a set of 'data-rich' structural analoga of VPA. We found, that if combined with only one other NAM the new RoFA protocol gave already a good hazard prediction for these compounds. Consequently, we applied the two NAM to test the DNT hazard potential of an extended set of 'data-poor' VPA analoga where *in vivo* data was mostly lacking. Ultimately, our case study approach could serve as an example of how to build trust and apply NAM in the future for hazard assessment where no *in vivo* data is given.

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P09-21
Oligodendrocyte development as a crucial key event for developmental neurotoxicity

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There are multiple examples indicating the contribution of altered white matter in human neurodevelopmental disease. White matter mainly consists of myelin produced by oligodendrocytes. Reduced white matter can be seen due to a delay in oligodendrocyte formation or maturation as well as due to oligodendrocyte cytotoxicity.

The oligodendrocyte differentiation (NPC5) assay of the OECD/EFSA developmental neurotoxicity (DNT) *in vitro* battery (IVB) identified multiple modes-of-action leading to a reduced number of oligodendrocytes. Here we summarize these MoA and complement them with oligodendrocyte toxicity published in the current literature.

Data shows that chemicals that interfere e.g. with cholesterol metabolism, voltage gated sodium channels, or produce reactive oxygen species interfere with oligodendrocyte development. Moreover, the compound classes of flame retardants and biocides were identified to be oligodendrocytotoxic. Assembling these data around the key event oligodendrocyte `Reduced, number of oligodendrocytes`, leads to a valuable addition to the adverse outcome pathway network for DNT.

Identification of chemical domains contributing to impairment of oligodendrocytes/white matter during development is crucial for maintaining brain health in children.

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P10 | Reproductive toxicity

P10-01
A unique finding of stage specific and reversible sperm cell arrest in monkeys induced by small molecule SMN splicing agents

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Small molecules designed to interfere with mRNA splicing are a new group of agents suitable for treatment of diseases driven by splicing errors. One such disease is spinal muscular atrophy (SMA), in which the survival of the motor neuron gene 1 (SMN1) is mutated. A human-specific SMN2 rescue gene only generates an insufficient amount of an instable protein based on a splicing error, which deletes Exon 7 in the process. Risdiplam is a new kind of small molecule approved to treat this genetic disease. It was designed to modify the mRNA splicing process to lead to an inclusion of Exon 7 in the transcript and thereby to increase the level of functional SMN protein in SMA patients. In the course of the non-clinical development of risdiplam and its predecessor, RG7800, we noticed a sperm cell arrest in monkeys. An investigative study with RG7800 to study staging and reversibility of the sperm cell effect yielded clear evidence for a stage-specific arrest of spermatocytes in the pachytene stage of meiosis. This effect was fully reversible in monkeys following a sufficient recovery period of eight weeks following cessation of treatment with RG7800. In addition, none of the studies conducted yielded any evidence for damage to spermatogonia. As risdiplam and RG7800 are capable of affecting splicing of genes other than SMN2, further investigations surfaced FOX M1 (a cell cycle regulator) and MADD (a gene involved in apoptosis) as possibble candidates for mechanistic explanation. For both of these genes, Exon inclusion variants as generated by treatment with risdiplam or RG7800 generate proteins that stop the cell cycle and incude apoptosis. As FOX M1 is highly expressed in spermatocytes of the pachytene stage (allowing for meiotic cross over), this mechanistic explanation is congruent with the histopathological findings in the testis of monkeys. Further investigations into determination of splicing shifts in FOX M1 in monkey testis confirmed this hypothesis. According to our knowledge, this represents a rather unique finding of a stage-specific and reversible sperm cell arrest with a new class of small molecules. As SMN2 is not present in the monkey, this finding represents an off-target effect. Studies in human and monkey cells on the splicing of FOX M1 confirmed that risdiplam and RG7800 are capable of shifting the splicing of FOX M1 to a cell cycle arresting variant in both species. Thus, the monkey finding may be translatable to humans. Yet, no lasting damage in patients is to be expected and any sperm cell arrest is reversible. Further, the clinical dose of risdiplam in SMA patients is lower than the dose leading to sperm cell arrest in monkeys.

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P10-03

Polystyrene nanoplastics exposure during early life induces testis development disorder in male offspring through m6A methylation

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Nanoplastics (NPs) were of male reproductive toxicity, but the effects and mechanisms of early life exposure to nanoplastics on the reproductive function of male offspring are unclear. In this study, ICR mice were randomly divided into the control group (saline group) and the nanoplastics polystyrene (PS-NPs) group. Regarding the PS-NPs group, the mice were gavaged with 100 mg/kg bw PS-NPs with a diameter of 60 nm every two days during pregnancy and lactation. The toxicity of PS-NPs on the testis of the offspring at postnatal day (PND) 23, PND 30, and PND 70 and their mechanisms were investigated. The result showed that early life exposure to PS-NPs caused damage to the developing testis and increased testicular cell apoptosis. The structural analysis revealed that early life exposure to PS-NPs affected the number of primary spermatocytes and spermatocytes and the diameter of the seminiferous tubules of the testis at PND 23, PND 30, and PND 70. The early life exposure to PS-NPs also caused significant down-regulation of the methylation transferase WTAP and up-regulation of the reading protein YTHDF2, and resulted in up-regulation of m6A peak of 500 genes and down-regulation of m6A peak of 320 genes in the PND 23 testis. Combining the m6A RNA sequencing results and bioinformatics analysis, the Hippo signaling pathway, sphingolipid metabolism, and apoptosis were shown to be involved in testis development. The validation results showed that early life exposure to PS-NPs upregulated the expression of PAK1 in the Hippo signaling pathway, CerS6 in the sphingolipid metabolism pathway, and Caspase-7 in the apoptosis pathway at PND 23. Collectively, the results of this study suggest that early life exposure to PS-NPs may cause damage and apoptosis in the developing testis by altering the m6A modification of RNA through disruption of methylation modifying enzymes.

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P10-04

Polystyrene nanoplastics exposure during pregnancy and lactation induces testis development disorder in male offspring in mice

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Nanoplastics (NPs), a type of new environmental pollutant, have attracted considerable attention for their hazardous effects on humans. Furthermore, NPs have been shown to be able to cross the placental barrier into the foetus, causing transgenerational toxicity. However, whether early life exposure to NPs could cause reproductive toxicity in male offspring has been the subject of limited studies and its underlying mechanisms are unclear. Therefore, this study aimed to investigate the harmful effects and underlying mechanisms of polystyrene nanoplastics (PS-NPs) exposure during pregnancy and lactation on the testis development in male offspring. ICR mice were exposed to 0 and 100 mg/Kg bw PS-NPs (60 nm) by oral gavage every two days from the gestational day (GD) 1.5 to postnatal day (PND) 21. The results showed that exposure to PS-NPs caused impaired testis development in male offspring. At PND 23, the spermatogenic epithelium showed fewer cell layers and more vacuolated structures in the PS-NPs group. The number of primary spermatocytes and the diameter of the seminiferous tubules were significantly reduced in the PS-NPs group compared with the control group. At PND 30 and 70, the number of primary spermatocytes and spermatids also showed a significant decrease in the PS-NPs group compared to the control group. The results of RNA sequencing results indicated that there were 627 differentially expressed genes between the PS-NPs and control group at PND 23, with 312 genes significantly upregulated and 315 genes significantly downregulated. By combining RNA sequencing results and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, steroid hormone biosynthesis, metabolism of xenobiotics by cytochrome P450, pentose and glucuronate interconversions, and pantothenate and CoA biosynthesis were shown to be involved in testis development at PND 23. These results revealed that PS-NPs exposure during pregnancy and lactation induced testis development disorder in male offspring of mice, which may be regulated by the disrupted metabolic pathways, such as hormone biosynthesis and energy production.

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P10-05

Effects of antiviral drug favipiravir on human placental cells

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Introduction: Favipiravir (Avigan®) was approved for manufacturing in Japan in 2014 as an anti-influenza virus drug. However, due to its teratogenic effects observed in animal reproductive and developmental toxicity studies, favipiravir is contraindicated in pregnant women or those who may become pregnant.

The placenta plays a critical role in fetal survival and growth by providing essential nutrients and oxygen from the maternal circulation and eliminating unnecessary waste products and xenobiotics from the fetal circulation. In this study, we investigated the effects of antiviral drugs on human placental BeWo cells.

Methods: Human placental BeWo cells (CCL-98) were obtained from the American Type Culture Collection. BeWo cells were cultured in the presence of 0–2000 μM antiviral drug for 24 hours. Cell viability was determined by WST-8 assay (Dojindo). Cell plasma membrane integrity was assessed by measuring the release of lactate dehydrogenase (LDH). Live cells were stained with Calcein Violet-AM (BioLegend) and dead cells were stained with 7-aminoactinomycin D (BioLegend), and then observed under a fluorescence microscope (BZ-X800, Keyence). Uptake of favipiravir in BeWo cells was measured using a microplate fluorometer (Spark 10M, Tecan Group Ltd.) with excitation and emission wavelengths of 361 and 432 nm, respectively.

Results and Discussion: Human placental BeWo cell viability was significantly decreased by exposure to 15.625 μM remdesivir for 24 hours, whereas favipiravir had no effect. Extracellular LDH activity increased with 15.625 μM remdesivir, 125 μM GS-441524 (an active metabolite of remdesivir), and 62.5 μM ribavirin, indicating membrane damage in BeWo cells. In contrast, exposure to 2000 μM favipiravir did not affect LDH leakage. The ratio of dead to live cells, as measured by fluorescence microscopy, increased with 250 μM remdesivir but was unaffected by favipiravir. Favipiravir was taken up by BeWo cells in a time-dependent manner. 4-Nitrobenzylthioinosine, a known inhibitor of the nucleoside transporter ENT1/2, inhibited the cellular uptake of favipiravir by BeWo cells.

Conclusion: These results suggest that the antiviral drug favipiravir does not exhibit toxicity to human placental BeWo cells. Favipiravir can be transported across the placenta by the nucleoside transporter ENT1/2.

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P10-06
Establishment of placental organoids and application of metabolomic analysis to reproductive toxicity studies

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Objective: A three-dimensional (3D) culture system that closely mimics *in vivo* tissues and organs can reproduce the complex spatial patterns of differentiated tissues and is useful for the analysis of physiological functions. Until now, there have been no tissue models of the placenta to compare with the development of other organs. We established placental organoids (mini placentas) and investigated their utility as a model of reproductive toxicity.

Methods: We used placental tissue from abortions or births at our institution. We developed original methods to reconstitute cytotrophoblast and syncytiotrophoblast structures from a single multifunctional stem cell in Matrigel and confirmed reconstitution by immunohistochemistry. Thalidomide loading (0–200uM) was used as a model drug and a comprehensive metabolomic analysis was performed in a time-dependent manner. Variation was statistically analysed. (The study was approved by the ethics committee of our university).

Results: Placental organoids were identified by 3D immunohistology using antibodies against cytotrophoblast marker, ITGA6, and syncytiotrophoblast marker, syndecan. The results confirmed that the two-layered structure of trophoblast cells is inverted and reconstructed. Metabolomic analysis (multivariate analysis) of metabolites under thalidomide exposure showed identical clustering (n=3) in a time-dependent manner for each concentration. In addition, 32 primary metabolites were identified that were unique to mouse placental organoids and 8 primary metabolites were identified that were unique to human placental organoids. Thus, new insights into the effects of species differences were gained by examining primary metabolites produced by organoid tissues.

Conclusions: In this study, we succeeded in establishing human and mouse mini placentas as a 3D culture system. We also report for the first time that they keep their tissue functions by analysing their metabolites. It is expected that these mini placentas will allow reproductive toxicity studies to be performed at the tissue level rather than the cellular level.

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P10-07
Polystyrene micro – and nanoplastics induced spermatogenesis disorder via disturbance of mitochondrial dynamics in mice

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Abstract: Polystyrene micron and nano-plastic particles are widely present in the environment[1,2,3]. It was reported that plastic particles were detected in human blood, which indicated that these particles posed a potential threat to human health[4]. The study from animal showed that Polystyrene micro- and nanoplastics (PS-MPs/NPs) caused male reproductive toxicity in mice[5–8], while its mechanism is still unclear. So the present study was designed to investigate underlying mechanisms. Male Balb/c mice were randomized into 3 groups: the control, 1 μm PS-MPs and 70 nm PS-NPs group, and they were given PS-MPs/NPs by intratracheal instillation for 28 days. Results revealed that PS-MPs/NPs up-regulated the expressions of mitochondrial fission related factors (p-DRP1/DRP1, FIS1) and down-regulated the levels of mitochondrial fusion related factors (MFN1/2, OPA1), causing the disturbance of mitochondrial dynamics, which is consistent with the oxidative stress via increasing MDA and decreasing SOD level induced by PS-MPs/NPs. Additionally, PS-MPs/NPs activated mitochondrial apoptotic pathway (BAX, Cleaved-caspase9, Cleaved-caspase3), resulting in the apoptosis of spermatogenic cell. Meanwhile, PS-MPs/NPs raised levels of inflammatory – related factors (cGAS, STING, NLRP3, ASC, Caspase1 p20 and IL-1β), and increased the expressions of pyroptosis markers (GSDMD and GSDME). *In vitro*, we found that PS-NPs induced mtDNA mislocalization in cytoplasm of spermatogenic cell lines GC-2spd. Mdivi-1, an inhibitor of mitochondrial fission, reversed the above changes induced by PS-NPs. The present results suggested that PS-MPs/NPs caused male reproductive toxicity possibly through oxidative stress-induced the disturbance of mitochondrial dynamics, which activated mitochondrial apoptotic pathway, resulting in spermatogenic cell apoptosis; the disturbed mitochondrial dynamics also led to mtDNA mislocalization, which induced the activation of cGAS-STING pathway and inflammation, ultimately resulting in the pyroptosis of spermatogenic cells. This study might provide a new reference to the potential mechanism of male reproductive toxicity caused by PS-MPs/NPs.

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P10-08
Evaluation of real-life PFAS mixture toxicity and impact on 3D placenta spheroid model

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Background: Per- and polyfluoroalkyl substances (PFAS) are prevalent and long-persistent organic pollutants that have been associated with many adverse effects including adverse pregnancy outcomes. However, as most of these data are based on single chemical exposure, we aim to identify relevant PFAS mixture of concern to further study their toxicity and impact on trophoblast spheroids as a proxy of human placenta.

Methods: PFAS concentrations were assessed in 1st trimester placenta tissue obtained from elective terminations of pregnancy with liquid chromatography/ triple quadrupole mass spectrometry. Based on PFAS levels in the placenta, mixture of concern was designed to test in a placental 3D model. The trophoblast spheroids were obtained by culturing JEG3 and HTR8/SVneo cell lines in ultra-low attachment plates and consequently exposed to PFAS mixture for 48–96 hrs at 0.01–300 µM concentrations. Viability was assessed with multiparametric live-cell toxicity assay. Functional placenta properties e.g. invasion and human chorion gonadotropin (hCG) production were assessed with matrix invasion assay and ELISA.

Results: The following PFAS, perfluorononanoic acid (PFNA), perfluorooctane sulfonic acid (PFOS), perfluorobutanoic acid (PFBA), perfluorooctanoic acid (PFOA), perfluorohexane sulfonic acid (PFHxS), and perfluorodecanoic acid (PFDA), were detected at the highest levels out of 57 PFAS tested in 1st trimester placentas and included to prepare the PFAS mixture of concern. 48 hrs exposure to PFAS mixture affected the viability of JEG3 trophoblast spheroids only at 300 µM, while HTR8/SVneo viability was unaffected. The invasive properties of JEG3 trophoblast spheroids were inhibited already at 72 hrs due to exposure to the PFAS mixture at varying concentrations. Further, we observed decreased hCG production after 48 hrs exposure to the mixture of concern. Additionally, HTR8/SVneo trophoblast spheroids showed decreased invasion protrusions already at 48 hrs of exposure to the mentioned PFAS mixture.

Conclusion: Our study offers valuable insights into real-life PFAS concentrations in placenta tissue and the negative impact of PFAS mixture on placenta functionality by using representative human placenta models. Collectively, our results call for more stringent risk assessment of chemical mixtures that include pregnancy relevant end points.

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P10-09
The importance of weight of evidence for assessment of non-endocrine active substances: a case study for Diflufenican

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Diflufenican (DFF) is a herbicide registered globally since the mid-1980's and was submitted for renewal in the EU in 2016. During the Pesticide Peer Review Experts Meeting in March 2021, the attendees agreed that the DFF toxicology dataset was considered complete according to the EFSA/ECHA Endocrine Disruption (ED) Guidance for the thyroid (T) modality. It was also agreed by the attendees that, although there was no evidence of an estrogen, androgen or steroidogenic (EAS) mediated pattern of adversity in the toxicology database, given the overall age of the DFF dataset, EAS-modalities were not sufficiently investigated according to updated guidance. Therefore, a series of Level 2 *in vitro* and Level 3 *in vivo* studies, based on the OECD conceptual framework, were conducted. All Level 2 and Level 3 studies were negative for ED parameters, apart from a positive response in estradiol release in the H295R *in vitro* steroidogenesis assay. Based on the EFSA/ECHA ED Guidance, a Level 5 extended one-generation reproductive toxicity (EOGRT; OECD 443) study was then required due to the positive response in the steroidogenesis assay, regardless of the lack of evidence of ED effects in the DFF toxicity database.

The objective of the EOGRT was to evaluate the systemic and reproductive/fertility and developmental toxicity potential of DFF. Diflufenican was administered through the diet to male and female Wistar rats to four dose groups up to the limit dose and a concurrent control (target dose levels of 0, 30, 100, 300 and 1000 mg/kg bw/d). Animals were treated continuously starting from ten weeks prior to mating of the parental (P) generation until sacrifice of the F2 pups at weaning. No endocrine related parameters were impacted due to DFF treatment up to the limit dose of 1000 mg/kg bw/d.Prior to the conduct of the EOGRT, the DFF toxicity database contained a full complement of acute, sub-chronic and chronic studies as well as ToxCast/Tox21 high-throughput screening data. These studies overwhelmingly demonstrated a lack of endocrine effects and showed no evidence of potential endocrine activity, with the vast majority of effects observed across these studies being decreases in body weight and food consumption. Hence, the data generated in the EOGRT only confirmed what was previously demonstrated in the existing toxicology data package. According to the ED Guidance, positive or inconclusive results from the steroidogenesis assay require the next step to run a Level 5 study, regardless of the evaluation of the existing *in vivo* data. This does not align with the principles of reducing animal use and additional testing should be weighed considering the existing data package. Prior to testing, submission of a weight of evidence report on the potential for DFF to be an ED would have been more effective in properly concluding that DFF does not demonstrate ED properties, thereby negating the requirement for further evaluation and significantly reducing animal usage.

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P10-10
Acetamiprid exposure induces cytotoxic alterations in mouse Sertoli cells *in vitro*

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Neonicotinoids, a relatively new class of insecticides, are widely used in urban and agricultural settings. Exposure to many of them has been linked to a couple of chronic health diseases, such as neurological development issues, liver pathologies, and carcinogenesis as well as with the potential to induce adverse effects on reproductive health in

males. Acetamipird, (E)-N′-[(6-Chloro-3-pyridyl)methyl]-N2-Cyano-N′-methylacetamidine, is the new-generation insecticide belonging to the neonicotinoids family. It is widely used in agricultural, domestic and public health activities as a replacement for more hazardous pesticides like organophosphates etc. However, its safety has not been investigated sufficiently and, current evidence indicates a various negative effect on the reproductive system. The objective of the present *in vitro* study was to evaluate the potential effect of acetamiprid on mice Sertoli cells. The TM4 cell line was treated with experimental doses of acetamiprid starting from 10 to 500 μM for 48 hours. Subsequently, metabolic activity, lysosomal integrity, and the potential of acetamiprid to induce reactive oxygen species were examined. Gained experimental data from mitochondrial cytotoxicity assay revealed that metabolic activity was significantly ($p<0.01$; $p<0.0001$) inhibited in the range starting from 200 to 500 μM of acetamiprid after 48 hours of exposure. In the case of Neutral Red uptake, lysosomal integrity was significantly ($p<0.001$) disrupted at 300 μM; 350 μM and 500 μM. Nitroblue-tetrazolium assay confirmed the potential of acetamiprid to generate reactive oxygen species in exposed cells. Increased levels of superoxide ($p<0.05$; $p<0.001$; $p<0.0001$) were recorded at 250 μM; 300 μM; 350 μM and 500 μM of acetamiprid. A considerably more detailed and systematic research in neonicotinoid toxicology is definitely required for a better understanding of the risks associated with reproductive dysfunctions in males.

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P10-11
Effects of a mixture of PCB 138, 153 and 180 on early embryo development *in vitro*, using human relevant doses

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Purpose: PCBs are endocrine disrupters, meaning they can interfere with the hormone system, particularly affecting reproduction [1]. Oocyte quality, essential for embryo survival, is influenced by maternal factors and its direct surroundings, i.e. the follicular fluid [2]. *In vitro* embryo production (IVEP) allows studying adverse effects on oocyte maturation and embryo development. This study used the IVEP model to investigate the effects of three PCB congeners (138, 153 and 180) previously identified as potentially harmful to embryos in women undergoing *in vitro* fertilisation treatment [3].

Method: Two doses of PCB mixture were used, with concentrations of 50.4 ng/g of PCB 138, 84.0 ng/g of PCB 153 and 58.8 ng/g of PCB 180 in the high dose, which is within the range found in human follicular fluid [3]. The low dose had concentrations a hundred times lower. Dimethyl sulfoxide was used as solvent and control treatment.

Oocytes (n=940) aspirated from bovine follicles were randomized into groups. Exposure to PCB or vehicle occurred (22 hours) before fertilization and culture according to standard protocols. Fertilization was assessed by examining the first cell divisions (cleavage) after 44 hours. At day 7 and 8, blastocyst number and stage were evaluated.

Proportions of oocytes introduced to the trial reaching first cleavage, cleaved above 2 cells and developing into blastocysts were compared between treatments. The developmental stage of the blastocysts (blastocyst, expanding blastocyst, hatching blastocyst, hatched blastocyst) was assessed.

Logistic generalized models (R 4.3.1, Lme4 package) were used to investigate the effect of treatments on developmental parameters (proportion cleaved, cleaved above 2-cell stage, blastocysts at day 7 and 8) compared to the control. Replicate was added as a random effect. Cumulative mixed-effect models were used to investigate the effect of treatment on stage (ordinal package).

Results: PCB high dose had a lower cleavage rate (mean 63±8%) compared to the control (mean 73±9%, odds ratio (OR) 0.61, confidence interval (CI) 0.41–0.86, p=0.006)). This group also had a lower rate of above 2-cell cleavage (49±12%) compared to the control (62±11%, OR 0.54 (CI 0.38–0.77, p=0.0006)). No significant differences were found in the low dose. PCB treatment did not affect the proportion of blastocysts developed or stage of development. The average blastocyst rate at day 8 for the high dose, low dose and control were 13±6%, 13±9% and 16±9% respectively.

The lower rate of cleavage in the high dose group is interpreted as a delayed development, although they did reach blastocyst stage at day 7 or 8. Timing of events during the first cleavage is important for future development. These effects aligns with the results found in Björvang *et al.* 2022, but further research is needed, i.e. lipid constitution, which has been linked to embryo quality [4], or RNA sequence data.

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P10-12
The mixture of PFAS may affect female reproductive health by causing disruption of mitochondrial function in ovarian granulosa cells

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Purpose: Disruption of homeostasis in granulosa cells, the main functional cells in the ovary, is associated with impaired female fertility [1]. This can be caused by accumulation of exogenous chemicals in ovarian tissue and follicular fluid (FF). The analysis of the FF composition of patients undergoing *in vitro* fertilization revealed the presence of numerous exogenous chemical compounds such as perfluoroalkyl compounds (PFAS), which dissolve in water and are widely used in convenience goods [2]. Recent literature suggest that PFAS can act as mitochondrial disrupting chemicals by altering various aspects of mitochondrial function [3]. As mitochondria are complex organelle involved in many cellular processes, their impairment can indirectly lead to fertility issues [4].

Methods: The study, approved by the Ethical Committee of the Jagiellonian University (1072.6120.30.2023), included human granulosa cells isolated from the follicular fluid of 32 women undergoing IVF, along with the human nonluteinized cell line HGrC1 used as an *in vitro* model. Cells were treated with PFAS mixture (Mix x1) of 7 compounds (22.4 ng/ml PFOS, 14.5 ng/ml PFOA, 21.3 ng/ml PFHxS, 0.9 ng/ml PFDA, 0.6 ng/ml PFHpA, 0.4 ng/ml PFUnDA and 2 ng/ml PFNA) based on literature data [5] and Mix x10, in which concentrations of PFAS were 10-fold higher than in Mix x1. Cell viability was measured using PrestoBlue assay. Mitochondrial membrane potential (Δψ, MMP) was measured with JC-1 cell dye. Metabolic profile and ATP production from glycolysis and mitochondria simultaneously and was performed using Seahorse XF analyzer (Agilent).

Results: The response to environmental relevant PFAS mixture was considered nontoxic as there were no alterations detected in cell viability after 24 and 48 hours of treatment. We established a significant increase of Δψ (MMP) measured by JC-1 red/green ratio, both in HGrC1 cell line and primary granulosa cell culture after 1, 3, 6 and 24 hours of PFAS exposure. After 24h basal respiration of granulosa cells was reduced however we did not detect an acute response to PFAS mixture. In HGrC1 cells the energetic profile shifted towards glycolytic and comparable observation was observed in the primary cell culture.

Conclusions: Our results implement that PFAS act as mitochondrial disrupting chemicals which may contribute to mitochondrial imbalance in ovarian follicle and affect female reproductive health. This research provides an important insight how PFAS influence cell biology.

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P10-13
Special considerations in conducting an enhanced pre- and postnatal development (ePPND) study in cynomolgus monkeys of biotherapeutics

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Evaluation of developmental and reproductive toxicity (DART) is normally conducted in rodents and rabbits. However, it is often very challenging for biotherapeutics when nonhuman primates (NHPs) are the only relevant species. Currently, the cynomolgus monkey (*Macaca fascicularis*) is the predominant NHP species used for general toxicology and DART evaluation of biotherapeutics. In addition, the pattern of immunoglobulin G (IgG) placenta transfer during gestation is comparable between human and cynomolgus monkeys. Under these circumstances, an ePPND study in cynomolgus monkey is recommended for evaluation of embryofetal, pre- and postnatal developmental toxicity of biotherapeutics, instead of running one embryofetal development study and one pre- and postnatal developmental study. Evaluation of fertility in male and female animals is usually conducted in repeat-dose toxicity studies of at least 3 months duration.

In light to the progresses in scientific and regulatory background as revised in ICH M3(R2) and ICH S6(R1) guidelines, the ePPND study has evolved over the decade. This abstract focuses on the special considerations when conducting an ePPND study, including training of technical staffs, obtaining pregnant monkeys, establishment of background control data, and the most important one, animal welfare. In addition to standard housing and environmental enrichment, every effort should be taken to minimize stress on the monkeys throughout the study. Acclimation to technical procedures, positive reinforcement, and providing ample hiding spaces are strongly recommended to minimize stress. Close veterinary care on the dams and offsprings should also be available during the conduct of the study.

Due to the inherent expense and time commitment of an ePPND study, especially when considering the special considerations required for animal welfare and data integrity, meticulous planning and rigorous execution are paramount to ensure a successful outcome.

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P10-14
PFHxS exposure on male reproductive dysfunction

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Objective: Based on the perfluorohexane-1-sulphonic acid (PFHxS) exposed male rat model, we analyzed the effect of PFHxS exposure on male sperm function, further clarified the mechanism of PFHxS exposure in spermatogenesis, and explored the possible reasons for the imbalance of testicular immune microenvironment and the changes of testicular metabolic spectrum caused by PFHxS exposure.

Methods: Construct an exposure model of C57BL/6J mice, to determine male mice reproductive toxicity by computer-assisted sperm analysis (CASA) and fertility evaluation. The sperm morphology and ultrastructure were observed by scanning electron microscope and transmission electron microscope, and the effect of PFHxS exposure on male sperm function was determined by ATP energy detection; The testis was observed by pathological section scanning and TUNEL apoptosis test, and the morphology and function of blood-testis-barrier (BTB) was observed by transmission electron microscopy, immunofluorescence (IF) and Western blot.

Result: Based on the male mice exposed to PFHxS, the results of CASA showed that PFHxS exposure led to a decrease in sperm number and sperm motility, and the transmission electron microscope have shown that some sperm mitochondrial sheaths were structural abnormalities. Further detection of ATP content in mouse sperm showed that the ATP content of epididymal tail sperm in the exposure group decreased significantly, suggesting that PFHxS exposure could lead to Spermatogen-

esis impaired. The pathological section scanning of the testis was observed, and combined with TUNEL, the result have shown that testicular structure and function were damaged. And the results of RT-qPCR and Western Blot have shown that the expression level of Claudin 11, N-cadherin, and Connexin 43 decreased significantly. By counting the Sertoli cell and spermatogenic cells at each stage of the testicular section, it was clear that PFHxS exposure damaged the testicular BTB structure of male rats in the 5mg/kg/day dose group, resulting in BTB dysfunction, affecting the number and function of Sertoli cells, and influencing spermatogenesis.

Conclusion: Exposure to PFHxS damages the terminal loop of male mouse sperm, leading to sperm tail deformity and disruption of mitochondrial sheath structure, resulting in impaired ATP synthesis and affecting sperm energy metabolism; Simultaneously disrupting the structure and function of the blood testis barrier, inducing cell autophagy, affecting energy metabolism, and ultimately interfering with male reproductive system.

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P10-15
Pioneering intravenous infusion in the mouse for a Developmental and Reproductive Toxicity (DART) Program

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A Developmental and Reproductive Toxicity (DART) program, including fertility and early embryonic development (FEED), preliminary and main embryo-fetal development (pEFD and EFD), and pre- and postnatal development (PPND) studies, was performed for a novel antibiotic drug candidate. The mouse was selected as the rodent species. Intravenous (IV) infusion was used as it is the clinical route of administration. Mice were given 3x2 hours daily IV infusions.

Separate male and female FEED studies were required as IV infusions preclude dosing both sexes during cohabitation. Mice were implanted with a Vascular Access Button (VAB) at least 10 days before dosing. Animals were then allocated to 4 treatment groups receiving either the control or test article (TA) by IV infusion for 14 days before cohabitation and during mating for both studies, and through to Gestation Day (GD) 6 for the female study. Study parameters and endpoints as outlined in the ICH S5(R3) guidelines were evaluated, including reproductive performance with 1:1 pairing for a maximum of 14 days. The VAB allowed continuous IV infusion dosing during cohabitation, without disconnecting the infusion line and without disturbing normal mating behaviour.

For EFD and PPND studies in pregnant females, another vascular access method was investigated to shorten the acclimatization period between the catheter implantation and the initiation of dosing. This allowed time-mated females to be implanted on GD1 and dosing to start on GD6. In a pilot PPND study, two vascular access systems were compared: a standard fixed line-maintained patent with saline from GD1, and a Vascular Access Harness (VAH) allowing IV infusion of TA from GD6. The VAH system was selected because it resulted in a higher pregnancy rate, 82% vs. 29% for the fixed line. For the main EFD and PPND studies, 128 and 100 time-mated mice, respectively, were implanted with a VAH on GD1 and infused from GD6 up to GD15 for the EFD study and up to weaning for the PPND study. The overall pregnancy rate was, however, lower than expected (59%), probably impacted by the surgical implantation procedure on GD1. This VAH system allowed continuous IV infusion dosing, without disconnecting the infusion line and without disturbing normal pre-, peri- and post-natal behaviour.

For the first time we successfully completed a full DART program applying IV infusion as the route of administration in the mouse.

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P10-16
The role of gut microbiota in reproductive toxicity caused by fluoride and arsenic exposure

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Background: The co-contamination of fluorine and arsenic in drinking water poses a health burden. While numerous studies have shown that fluoride or arsenic exposure may damage the reproductive system, the specific pathogenesis and its effects on the reproductive system at different developmental stages are still unclear.

Methods: We established an animal model of male reproductive system injury caused by alone and combined exposure of fluoride and arsenic in rats. In addition, we also established the fecal microbiota transplantation (FMT) intervention in rats. On the one hand, the damage to the male reproductive system and changes in intestinal flora were monitored during adolescence and adulthood. On the other hand, we comprehensively investigated the key regulatory role of gut microbiota in male reproductive system injury under the exposure model and FMT model.

Results: In exposure experiment during adolescent to adult, our results showed that fluoride and arsenic reduced the reproductive organ coefficient, caused pathological damage of testicular and epididymis, and decreased the level of hormone and testosterone We also found fluoride and arsenic exposure regulated the degree of autophagy in the testis tissue. Furthermore, fluoride and arsenic disrupted the abundance and diversity of gut microbiota, and changed the relative abundance of gut microbiota at phylum and genus level. Among them, there were 4 phylum and 20 genera significantly related to the reproductive injury indexes. In bidirectional FMT experiment, FMT aggravated or alleviated the reproduction toxicity injury, significantly altered gut microbiota at family and genus level. Further correlation analysis of differential genera found that there were 18 genera significantly correlated with testosterone and sperm count.

Conclusions: Our results speculate that gut microbiota may participate in the male reproductive toxicity through modulating the occurrence of testicular autophagy and altering testosterone levels.

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P10-17
Metabolomic Insights: LC-MS profiling of human placental tissue from SSRI-exposed pregnancies

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Antenatal depression, a prevalent complication of pregnancy, poses significant risks for both mother and the developing fetus when left untreated. Consequently, expecting mothers globally are often prescribed antidepressants, particularly selective serotonin reuptake inhibitors (SSRIs). Reported by us and others, SSRIs have the potential to alter placental metabolic functions by affecting placental transporters and enzymes. However, thorough placental metabolic profiles of pregnant individuals using SSRIs are lacking. Therefore, we performed a comprehensive determination of the placental metabolome by non-targeted liquid chromatography-mass spectrometry (LC-MS) metabolomics approach, which allows elucidating metabolic pathway regulation and diverse exposures.

A total of 48 placental samples from individuals using SSRI medication throughout the pregnancy (n=24) and non-depressive controls without antidepressant medication (n=24) were included in the study. The LC-MS analysis employed hydrophilic interaction chromatography for more hydrophilic compounds and reversed-phase chromatography for more lipophilic compounds. Both methods were coupled to a high-accuracy, high-resolution mass spectrometer. Additionally, data were acquired using both positive and negative electrospray ionization modes. The method allows global characterization of the metabolites present in the sample without prior bias towards any specific molecules. Open-source software MS-DIAL was used for peak picking and feature alignment, as well as for identification of molecules, and notame R-package was used for data preprocessing and statistical analysis.

The initial results show a decreased level of oxidized glutathione (FDR-corrected q-value <0.001), phosphocreatine ($q=0.03$) and an increased level of reduced glutathione ($q=0.003$), cysteinglycine ($q<0.001$), monoacylglycerols (1-monostearin and 1-monopalmitin, both $q<0.001$), and serylleucine ($q=0.01$) in SSRI users. To conclude, the profiling of the placental metabolome of SSRI users revealed alterations in pathways related to oxidative stress, ATP homeostasis, and lipid metabolism.

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P10-18
Bayesian benchmark dose modelling of hypothalamic–pituitary–ovarian axis endpoints to assess DEHP exposure effects in female mice

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Purpose: Endocrine-disrupting chemicals (EDCs) affect female reproduction, yet efficient regulation is hindered by a lack of regulatory-relevant information. For example, it is unclear which female reproductive endpoints are most sensitive to EDCs. The current work assessed the relative sensitivity of various hypothalamic–pituitary–ovarian (HPO) endpoints in female mice. In this study, di(2-ethylhexyl) phthalate (DEHP) was studied as a prototypical EDC model compound. We identified dose-response studies in the literature involving DEHP in adult mice and modelled the HPO endpoints using the Bayesian benchmark dose (BBMD) approach.

Methods: Studies included sexually mature female mice exposed to DEHP, or a mixture containing 20.8% DEHP, for 30 days, at doses ranging from 0.02 to 200 mg/kg bw/day. The modelled HPO endpoints encompassed ovarian follicle counts and percentages (total, primordial, primary, antral, and preantral follicles), serum hormones (estradiol and progesterone) and estrous cyclicity (measured as% days in estrus and metestrous/diestrous phases). BBMD was performed on www.benchmarkdose.org to determine the credible interval of the benchmark dose (BMD), specifically the benchmark dose lower limit (BMDL) and benchmark dose upper limit (BMDU). Model averaging was performed using equal prior weights. The benchmark response (BMR), reflecting the change from the unexposed control group, was set at 10% for all endpoint**s.**

Results: Dose-dependent changes were observed in all modelled endpoints, although with different levels of uncertainty for the credible interval. For the ovarian follicle counts and percentages, the BMDLs were 0–176.2 and 0–198.0 mg/kg bw/day, respectively. For the reproductive hormones, the BMDLs were 0–55.1 mg/kg bw/day. Finally, for the estrous cyclicity endpoints, the BMDLs were 0–236.9 mg/kg bw/day. The results show that there was dose-dependency, for DEHP and the mixture, in all modelled HPO endpoints, including serum hormones, ovarian follicles staging, and estrous cyclicity.

Discussion & Conclusions: Using a BMR of 10%, BBMD modelling suggested differences in the sensitivity of classical HPO endpoints to DEHP. The results suggest that DEHP and the phthalate mixture exposure have a significant impact on the estrous cycle, hormones, and follicles in female rats. The findings may have implications for human health, as DEHP is a common environmental contaminant that is found in many consumer products, and can potentially be a reproductive toxicant. In conclusion, BBMD modelling is valuable in comparing endpoints to identify critical effects in reproductive toxicity testing. It may also be suited for comparing the effects of different EDCs and their mixtures, as well as for comparing the sensitivity of reproductive endpoints for male and female mice.

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P10-19
A comparative study of the cytotoxic effects of cannabidiol and minor cannabinoids on placental trophoblast cells

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Cannabinoids are the main compounds produced by *Cannabis sativa*, a commonly used illicit drug among pregnant women, particularly for relieving nausea in the first trimester of gestation [1]. Placental extravillous trophoblasts cells (EVTs) invade the uterus, participating in the remodeling of maternal spiral arteries to reduce resistance to blood flow [2]. It is known that cannabinoids can cross the placental barrier, which may result in pregnancy complications, such as intrauterine growth restriction and miscarriage [3]. We already reported the possible impact of the major cannabinoids cannabidiol (CBD) and delta-9-tetrahydrocannabinol (THC) on placental development [4]. However, the effects of minor cannabinoids remain unknown. Thus, the aim of this work was to compare the effects of CBD with the minor cannabinoids cannabichromene (CBC), cannabidivarin (CBDV), cannabigerol (CBG) and cannabinol (CBN) on HTR-8/SVneo cells, a representative model of EVTs. All cannabinoids induced a dose-dependent decrease on cell viability at 24 and 48 h of incubation, which was confirmed by MTT assay. Lactate dehydrogenase (LDH) release was observed for CBC and CBN at 5 and 10 μM, while for CBD, CBDV and

CBG it occurred only at 10 μM. The dependence of the cannabinoid receptors CB1 and CB2 and of the transient receptor potential vanilloid 1 (TRPV1) for the cell viability loss observed at 48 h of treatment was also evaluated, using the antagonists AM281, AM630 and capsazepine, respectively. CBN effect was CB1-dependent, CBC and CBG were CB2 and TRPV1-dependent, while CBD and CBDV effects were independent of receptors activation. Moreover, CBD and CBG were able to cause mitochondrial depolarization and to generate reactive oxygen/nitrogen species, as well as CBDV, which were assessed using the $DiOC_6$ and the DCDHF-DA fluorescent probes, respectively. Nevertheless, only CBD activated the apoptosis-related effector caspases-3/-7, an effect that was promoted by autophagy, as confirmed through observation of orange-stained acidic vacuoles (fluorescence microscopy) and increase of p62 gene expression (qPCR). On the other hand, the minor cannabinoids CBDV and CBG increased the mRNA levels of the endoplasmic reticulum (ER) stress markers BiPand spliced-XBP1, evaluated through qPCR. Hence, the results suggest that cannabinoid exposure during pregnancy may differently impair the normal trophoblast remodeling through alterations on key biochemical processes for placental development, such as cell death and ER stress, compromising pregnancy success.

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P10-20
Comparative gonadotoxic activity study of two generic pesticides epoxyconazole on female rats

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Aim: This study evaluated gonadotoxic activity after exposure to two generic pesticides, namely epoxiconazole (Epox), in female rats. The test substances, epoxiconazole technical, were obtained from different manufacturers, with the purity of the active ingredient measured at 98.7% (Epox-1) and 97.3% (Epox-2). Animal studies were conducted following the requirements and provisions of the Commission for the Ethics of Medical and Biological Research of L.I. Medved Research Center of Preventive Toxicology, Food and Chemical Safety, Ministry of Health, Ukraine and the European Convention for Protection of Animals used for Experimental and Other Research Purposes.

Materials and methods: The test substances were orally administered as an aqueous emulsion by gavage daily for nine weeks until the mating period to two groups of animals, each composed of 20 females. The doses administered were 0.5 and 2.0 mg/kg body weight. Control animals, consisting of 20 females, received an equivalent volume of solvent: distilled water with an emulsifier. Following the exposure period, functional indicators of gonadal state and animals' reproductive ability were assessed. The estrous cycle, as well as the duration and frequency of each stage, were studied. The reproductive function status was evaluated on the 20th day of pregnancy in experimental females impregnated by intact (untreated) males. At the same time, the number of corpora lutea in the ovaries, the number of live, dead, and resorbed fetuses and embryos, fetal body weight, total litter weight, and the presence of gross developmental anomalies were recorded. Mating, conception, fertility, and pregnancy indexes were determined, considering the duration of the pre-coital interval.

Results: Results revealed that female rats exposed to the test substance Epox-1 exhibited reproductive toxicity at a dose of 2.0 mg/kg. This dose group of females observed a significant increase in the number of preimplantation losses, a decrease in the number of live fetuses per female, and a decrease in the total weight of the offspring. The test substance Epox-2, at both studied doses, did not adversely affect the reproductive function of female Wistar Han rats.

Conclusions: The results suggest that epoxiconazole has the potential to impact female reproductive function adversely. Moreover, discrepancies in the intensity of toxicity observed after exposure to Epox-1 may be ascribed to its higher purity compared to Epox-2 in this context. This highlights the importance of evaluating generic pesticides containing differing levels of impurities.

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P10-21
Spinosad – mode of action and human relevance assessment of dystocia in rats

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Spinosad technical, a natural product insecticide derived from fermentation, associated with treatment-related adverse pregnancy outcomes which manifested as dystocia in the rat. A robust mode of action (MOA) programme was initiated to determine the MOA for dystocia in rats and relevance of this hazard to humans using the (WHO)/International Programme on Chemical Safety (IPCS) framework. A number of dose-related key events have been identified that characterise the rat MOA for Spinosad-induced dystocia. Dystocia was characterised by prolonged parturition which was associated with peri-partum maternal death and other peri-partum effects. Using *in vivo* and ex vivo contractility experiments, it was concluded that parturition became protracted due to inhibition of uterine muscle contraction, arising due to a pharmacological/receptor-mediated inhibition of action potential generation in uterine smooth muscle cells (myometrial cells). By using competition binding experiments with receptor ligands, it is hypothesized that the Spinosad receptor mediating uterine effects may be Translocator Protein (TSPO). The initial dynamic molecular initiating event of Spinosad binding to TSPO requires uterine exposure to Spinosad above a certain tissue concentration threshold. With pharmacokinetic studies, uterine exposure to Spinosad has been unequivocally demonstrated in the pregnant rat after Spinosad oral administration.

It was concluded that non-dose proportional (supralinear) increases in uterine tissue concentrations between the rat NOAEL and the LOAEL for dystocia at parturition caused exceedance of the contractility inhibition thresholds and consequently dystocia at the higher dose level. This MOA is not relevant for humans due to measured quantitative toxicokinetic differences in uterine tissue exposure to Spinosyns (Spinosad and metabolites) between rats and humans. By using a variety of *in vitro* comparative pharmacokinetic determinations the following was demonstrated: A) Spinosyns have higher hepatic clearance (oxidative metabolism) in humans; B) GSH conjugation (a key process for Spinosyns excretion via the bile) is likely saturated in rats where GSH depletion is expected earlier/at lower dose levels; C) humans are expected to have a lower uterine partitioning of Spinosyn based on generic PBPK modelling; and D) compound-specific PBPK (R-based) modelling for two Spinosyns up to the rat dystocia LOAEL levels showed that uterine concentrations in humans are expected to be approximately one order of magnitude lower compared to rats. The rat Spinosad adverse effect of dystocia will not be triggered in humans as tissue concentrations remain below the effect threshold for the dynamic (receptor-mediated) molecular initiating effect. Since, the rat mode of action is not plausible in humans, Spinosad does not pose a reproductive hazard to humans.

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P10-22
Analysing women's knowledge in hormonal contraception for reproductive health: insights from a Romanian study

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Background: Birth control pills (BCP) along with emergency contraception are among the most frequently used methods for pregnancy prevention. They are associated with cautions of use such as variable distribution depending on body weight or unsuitability for women with severe hepatic dysfunctions for levonorgestrel. On the other hand, in Romania 10–14-year-old girls give birth 8.5 times more than the EU average and girls aged 15–29 give birth 3.4 more than the EU average.

Aim: The aim of our study was to assess the knowledge and the behaviour of Romanian women regarding hormone oral contraception (OC) and highlight the gaps and burdens in this field.

Materials and methods: The cross-sectional observational study was carried out on the basis of a questionnaire with 24 questions that tracked the collection of socio-demographic and anthropometric data, the source of information regarding contraception, the type and frequency of use of emergency contraceptives, the choice and effectiveness of the used contraceptive methods, their adverse effects, the frequency of clinical and gynaecological investigations, the number of pregnancies and spontaneous or induced abortions, chronic conditions. The questionnaire was available online and addressed to fertile women living in Romania.

Results: The questionnaire received 517 valid fillings from women living in Romania. 21.47% of the subjects were overweighed and 6% obese. Most subjects were aged 26–35 years (36.37%); groups aged 18–25 and 36–45 represented 26.3% and 27.66%, respectively. 86.27% were urban residents. Almost half of the respondents (44.69%) had postgraduate studies (master's degree, residency, PhD or other specializations). Most women were employed (78.34%), while 18.38% were students.

The greatest proportion of normal weight BCP users corresponded to long-time consumers (more than 6 years). The youngest women (aged 18–25) most frequently did not use BCP at all or for few months, whilst women 36–45 were most often using BCP for 4–5 or 5–6 years. In the 26–35 years old group, the duration of BCP consumption was most probably to be of few months or more than 6 years. Women aged 46–55 most were most often using BCP for 5–6 years or more.

No woman aged 18–25 became pregnant while using BCP. More than 50% of the women experiencing one pregnancy under BCP administration were aged 36–45. All women having more than one pregnancy under OC were aged 36–45. Most women aged 46–55 experienced one pregnancy while using BCP.

Regarding the factors influencing the frequency of gynaecological check-ups, education was the most significant, followed by age. The combined influence of residence and education was also relevant. Only 15% of highly educated women were not using OC.

Conclusions: Anthropometric parameters are relevant for OC customization. Mainly education, but also age and residence influence women behaviour towards reproductive system health and pregnancy control.

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P10-23
Historical background data for extended One-Generation Reproductive Toxicity Study conducted using Wistar rats

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The extended one-generation reproduction toxicity study is scientifically prudent, and technically intricate study design. It is intended at evaluation the putative effects in the form of multiple endpoints for the test items. The endpoints cover adverse observations in respect of reproduction and development, including effects on the developing nervous and immune systems. The procedures for conducting the Extended One-Generation Reproductive Toxicity Study using rats are outlined in OECD Guideline 443. The EPA, ECHA, EFSA, and other regulatory bodies have well accepted the data generated from these studies. Several chemicals under REACH and TSCA are being evaluated to establish risks associated with exposure.

JRF has conducted several EOGRT studies, which have been reviewed by the authorities and are well accepted. The studies were performed using the Wistar strain of rats, which has been proven to be a sensitive strain for reproductive and developmental toxicity testing as well as endocrine disruption.One of the major pillars of strength for our studies lies in the extensive Historical Control data (HCD), which is available at JRF. The data is using Han Wistar rats, which is a widely recognized as a rodent model in toxicology studies. There is an extensive wealth of well-documented historical data on its genetic and physiological background of this strain at JRF. We present the Histor-

ical Control Data for the parent male rats, which includes fertility, sperm parameters, thyroid hormones, organ weights, clinical pathology, and histopathology in this publication/presentation/poster.

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P10-24
The impact of zinc oxide nanoparticles on various biochemical markers in seminal plasma

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Zinc has an indispensable role in spermatozoa physiology and is a part of many enzymatic pathways. Zinc nanoparticles can show unique characteristics that are not similar to their chemical equivalent in a larger dimension. This study focused on the impact of solutions containing ZnO nanoparticles at different concentrations on selected parameters of seminal plasma. Ejaculate was collected from adult reproductively efficient Holstein bulls (SBS, Lužianky, SK) using an artificial vagina in this study. The collected ejaculate was diluted in ratio 1:50 with solutions containing zinc oxide nanoparticles (saline+ZnO NP) (40 nm ≤100 nm aver. part. TEM size) at concentrations of 15.62, 31.25, 62.5, 125, 250, 500, 1000 and 2000 µg/ml, pure saline served as control. The ejaculate treated was incubated for 3h at 37°C with regular gentle mixing. Subsequently, the solutions were centrifuged (3000 rpm, 15 min) to separate the spermatozoa from saline with NP containing seminal plasma which was subjected to analysis for levels of glutathione peroxidase (GPx), superoxide dismutase (SOD), total antioxidant status (TAS), albumin (BSA), uric acid (UA) and alanine aminotransferase (ALT) using commercially purchased kits and a fully automated RX Monaco clinical chemistry analyzer (Randox, Crumlin, UK). A significantly higher ($P<0.05$) SOD activity at 125 µg/ml ZnO NPs compared to control was observed. However, GPx activity remained unchanged across all groups, and no significant differences compared to control were observed. The overall antioxidant status also showed no significant differences compared to the control. A significant increase in albumin concentration ($P<0.05$, $P<0.001$) was observed at 62.5 and 125 µg/ml ZnO NPs compared to control. On the other hand, the highest ZnO NP concentration of 2000 µg/ml initiated a significant decrease ($P<0.001$) in albumin level. Lower ZnO NP concentrations (15.62, 31.25, 62.5, 125, 250 and 500 µg/ml) cause no significant changes in uric acid levels. However, 2000 µg/ml ZnO NPs caused a significant increase ($P<0.001$) in uric acid compared to control. ZnO nanoparticles also affected ALT, a significant increase ($P<0.05$) at 125 µg/ml ZnO compared to control was detected. Our results suggest that the addition of ZnO NPs can affect the diluted semen samples. The addition of 125 µg/ml ZnO nanoparticles was the most beneficial which increased the activity of SOD, also had a beneficial effect on the levels of albumin and ALT, thus contributing to better protection of spermatozoa against the harmful effects of oxidative stress. On the other hand, high concentrations of 2000 µg/ml were toxic since they caused a significant decrease in albumin and an increase in uric acid as high concentrations of UA are associated with impaired ejaculate quality.

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P10-26
Effect of oral administration of Quercetin on reproductive damage caused by acrylamide exposure in rats

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Acrylamide (AA) is a toxic chemical that is formed during heat treatment of carbohydrate rich foods. AA has been found to have harmful effects on reproductive health. Exposure to AA can lead to reproductive toxicity, including testicular epithelial tissue degeneration, reduced tissue weight, and number of follicles, and increase sperm morphological abnormalities. These results emphasise the need for effective interventions to mitigate reproductive toxicity caused by exposure to AA. Quercetin (QU), a natural flavonoid, has been shown to have antioxidant and anti-inflammatory properties. Male and female albino rats were orally administered AA (40 mg/kg for 10 days) and then given treatment of QU (10, 20, 30 and 40 mg/kg) orally for 3 consecutive days to determine the protective effect of QU on AA. Exposure to AA significantly increased lipid peroxidation levels and reduced GSH, SOD, CAT and ATPase activity in the ovary. In addition, the organ weight index decreased and the levels of triglycerides, cholesterol, glycogen, and fructose were altered. Sperm analysis showed a significant reduction in the number of epididymal sperm, mobility and survival, while an increase in abnormal sperm was observed. Testosterone, FSH, LH, progesterone, and estradiol levels were also altered and increased DNA damage was also observed. However, QE therapy restored tissue and serological indexes back to normal levels. It also counteracts the DNA damage and sperm parameters toward normal. Overall, the results of this study suggest that Quercetin has protective effects against the reproductive toxicity induced by Acrylamide in rats.

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P11 | Metabolic toxicology

P11-01
BPS and BPF by acting as mitochondrial disrupting chemicals cause changes in energy metabolism in human ovarian granulosa cells

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The new generation of bisphenols (BPs) is represented by bisphenol S (BPS) and F (BPF) as they are the main substitutes for bisphenol A. Both BPS and BPF have been found in indoor dust, as well as in daily-use and personal care products [1,2]. This has resulted in their detection in human body fluids such as blood, urine, and follicular fluid [3,4,5]. In previous studies, we have established that BPs disrupt steroidogenesis in human ovarian granulosa cells (GCs) [6]. The process of steroid secretion is highly dependent on mitochondrial function. Mitochondria are multifunctional cellular organelles involved in several aspects of cell biology. Their main function is the production of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS), but they also regulate other cellular processes [7]. Knowing that

BPs can affect steroidogenesis, the aim of the study was to determine their effect on other mitochondrial parameters associated with cell energy metabolism.

To investigate the effects of BPS and BPF we used an *in vitro* model of human nonluteinized GCs line (HGrC1) and GCs isolated from follicular fluid from women undergoing *in vitro* fertilization (IVF). The study included 32 women undergoing IVF and was approved by The Ethical Committee of the Jagiellonian University (1072.6120.30.2023). The cells were treated with 10nM of each bisphenol for 6h prior to the JC-1 staining, and real-time Mito Stress Test (with acute injection) performed using Seahorse XF analyzer (Agilent).

We first established that BPs decrease the mitochondrial membrane potential, causing mitochondrial depolarization, as we observed a decline in the JC-1 red/green fluorescence intensity ratio. We then determined the effect of BPs on energy metabolism. After injection of each BPs, GCs showed a statistically significant acute response compared to control cells respiration under basal test conditions. Importantly, we observed changes in ATP production rate after BPs treatment – BPS decreased and BPF increased mitochondrial respiration. These results translated into changes in extracellular acidification rate (ECAR) and proton leak, where BPS decreased and BPF increased both parameters.

To conclude, BPS and BPF can act as mitochondrial disrupting chemicals but in the opposite manner. Both compounds disrupt cell energy metabolism and act as stressors activating cell cytoprotective mechanisms.

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P11-02
Pharmacokinetics of cannabidiol and its metabolites in plasma and tissues upon repeated oral dosing

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Previous studies have identified cannabidiol (CBD) as having several potential therapeutic effects such as anti-bacterial, anti-inflammatory, immunomodulatory, analgesic, and anti-psychotic. However, the pharmacokinetics of CBD and its metabolites, their accumulation over time, the steady state concentrations, and tissue levels upon repeated single oral dosing are largely unknown. Male Sprague Dawley rats were given a dose of CBD (26.6 mg/kg or equivalent of 300 mg in 70 kg human) in sesame oil by oral gavage once daily for 21 days. Blood samples were collected pre-dose at intermittent days during the 21-day period and in a series of timepoints following the first and last oral dose for PK analysis. Liver, lung, adipose tissue and mesenteric lymph nodes were collected following the last blood draw and extracted with 2 volumes of 5 mM ammonium formate using the TissueLyser II. Quantification of CBD, 7-COOH-CBD, 7-OH-CBD, and 6-OH-CBD in plasma and tissue extracts were by liquid chromatography with tandem mass spectrometry. Pharmacokinetics profiles were evaluated by non-compartmental analysis using Phoenix WinNonLin, version 8.3. The 21-day repeated dosing resulted in approximately 2- to 3-fold increase in plasma CBD exposure (area under the curve, AUC, 4650 h.ng/mL on day 1 vs 11844 h.ng/mL on day 21 and max plasma concentration, C_{max}, 577 ng/mL on day 1 vs 1231 ng/mL on day 21) and longer CBD half-life (5.9 h on day 1 vs 41.9 h on day 21) compared to single dosing. Repeated dosing also resulted in an approximately 2–3-fold increase in plasma 7-COOH-CBD and 6-OH-CBD exposure (AUC and C_{max}) compared to single dosing. The exposure level (AUC and C_{max}) of 7-OH-CBD was 1.2- to 1.5-fold difference between repeated and single dosing. A steady state in plasma CBD concentration was reached on day 14 of repeated dosing. While CBD and 7-OH-CBD were more abundant in selected tissues, 7-COOH-CBD and 6-OH-CBD were very low or below the limits of quantification in the examined tissues. Overall, repeated oral dosing of CBD resulted in differential accumulation and changes in plasma and tissue CBD and metabolite levels as compared to single dosing. These findings are likely to meaningfully inform on upcoming oral dosing regimens in preclinical research on the CBD effects in biological system and provide data for the extrapolation of CBD pharmacokinetics in humans.

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P11-03
Exposure to phthalates impair hepatic lipid metabolism and mitochondrial function

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Introduction: Phthalates are a group of mass-produced chemicals ubiquitously present in our environment. They are used in thousands of consumer products as plasticizers and solubilizing agents, e.g. in packaging materials, coatings and cosmetics. Human exposure to phthalates has been associated with adverse metabolic effects, such as glucose tolerance, obesity and non-alcoholic fatty liver disease (NAFLD). In experimental cell and animal models, exposures to certain phthalates such as di(2-ethylhexyl) phthalate (DEHP) and its primary metabolite mono(2-ethylhexyl) phthalate (MEHP) have caused alterations in lipid and glucose metabolism, leading for example to altered lipid deposition and increased lipid accumulation in cells. Phthalates have been suggested to affect the functions of several nuclear receptors, e.g. peroxisome proliferation-activated receptors (PPARs), which are the master regulators of lipid metabolism in metabolic tissues. To date, the cellular mechanisms leading to so-called metabolic disruption are largely unknown.

Materials and methods: Here, we have studied the cellular targets and functional effects of chosen phthalates, including DEHP, MEHP,

and their new replacement compounds di-isononyl phthalate (DINP) and its primary metabolite mono-isononyl phthalate (MINP) in human hepatic cell models. Specifically, we studied species-specific nuclear receptor activation and mitochondrial bioenergetic functions using the Seahorse XF analyzer. To shed light on wider systemic metabolic changes occurring as a response to phthalate exposure, we also analyzed lipids and metabolic parameters from C57BL/6J mouse tissue samples using histological staining and untargeted metabolomics approach.

Results: In cells, all the tested phthalates activated several nuclear receptors involved in metabolic regulation. Several parameters of mitochondrial bioenergetics were altered dose-dependently or with U-shaped dose response. The effects between parent compounds and their metabolites varied, indicating the importance of considering human metabolism in chemical testing. In the livers of C57BL/6J, the expression and distribution of several classes of lipids was altered by phthalate exposure.

Conclusion: Exposure to phthalates caused alterations in lipid metabolism and mitochondrial function *in vitro* and *in vivo*. These data shed light on cellular targets and functional effects of phthalates. More research is needed to study detailed processes behind adverse metabolic effects induced by common environmental contaminants.

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P11-04

In-depth xenobiotic metabolism characterization of human *in vitro* liver models for toxicology

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The liver is a main metabolic hub in the body, being responsible for central metabolic pathways, as well as xenobiotic metabolism. Having cellular systems able to mimic human hepatic metabolism is a priority in studying bioavailability and potential toxicity of drugs. Despite the existence of several hepatic cell models, many lack essential metabolic activities. We have established two novel human hepatocyte models: the human-induced pluripotent stem cells (hiPSc) derived into hepatocytes (HLCs, Boon et al 2020) and the metabolically matured HepG2 (mHepG2, Boon et al 2020), that we aim to characterize for their xenobiotic metabolism. Of relevance for toxicology, the cytochrome P450 (CYP) superfamily is responsible for the metabolism of most pharmaceutical drugs. In cases of toxicity through bioactivation, CYPs are often involved. This study aims to characterize the xenobiotic metabolism machinery of the hepatic liver models HepG2, mHepG2, HLCs and HepaRG by using transcriptomics, proteomics, and metabolomics. Moreover, multi-omics integration approaches were used to integrate three expression levels and compare in-vitro model's xenobiotic machinery. Our analyses have revealed that mHepG2 and HLCs at late stages of the differentiation significantly increase their expression, protein amount and metabolic activity of both phase I and phase II enzymes compared to HepG2 and earlier stages of HLCs differentiation respectively. Nevertheless, multi-omics integration reveals that transcript, protein and metabolite levels do not always correlate among the studied in-vitro models. A better metabolic characterization of *in vitro* models is an essential milestone for their potential use in drug development programs.

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P11-07

***In vitro* effects of polystyrene microplastics, alone or in combination with environmental pollutants, on viability and lipid content of a human hepatocarcinoma cell line**

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The pervasive water and soil pollution by microplastics (MPs) poses uncertain impacts on human health. Beyond their direct effects, research and discussions have focused on their role as vectors for other environmental pollutants into organisms. The concurrent presence of MPs and environmental pollutants may constitute potential hazards to both human health and the ecosystems. Therefore, to contribute to the understanding of this complex phenomenon, we assessed *in vitro*, on a human hepatocarcinoma cell line (HepG2), the acute toxicity and impact on lipid content of commercial 500 nm diameter polystyrene (PS) MPs. Additionally, we investigated their effects in the presence of commonly found environmental contaminants, such as bisphenol A (BPA) and cadmium (Cd), representing significant organic and heavy metal pollutants, respectively.

Physicochemical characterization of MPs were conducted using dynamic light scattering and electron microscopy, and we evaluated if the presence of Cd or BPA modifies the appearance or aggregation state of the MPs. MPs internalization was assessed by confocal microscopy.

After 24h exposure to MPs w/o each environmental pollutants, cell viability was evaluated by CellTiter-Glo® Luminescent Cell Viability Assay.

The lipid content, both basal and induced (by exposing cells to 24-hour incubation in serum-free MEM containing a 0.5 mM mixture of free fatty acids), was assessed by AdipoRed/NucBlue staining following a 24-hour exposure to MPs with and without various concentration of each of the pollutants. AdipoRed assay quantifies intracellular triglycerides, while the DNA content is estimated by NucBlue staining.

In our experimental model, after 24 hours of contact, HepG2 cells internalize to some extent the 500 nm PS MPs. Despite their internalization, they do not affect viability of HepG2 up to concentrations of 1000 μg/ml.

The toxicity of each tested contaminants was not modified by the presence of MPs and the Bliss independence model confirms that there is no synergistic effect. The MPs do not alter the basal lipid content nor the induced one, either alone or in conjunction with BPA. Conversely, when cells are exposed to MPs and Cd at concentrations of 1–5 microM, the induced lipid content significantly decreases after only 24 hours of incubation. However, this association has no effect on basal lipids. Therefore, our ongoing studies are focusing on lipid transport mechanisms that appear to be altered by the association between non-cytotoxic concentrations of Cd and PS MPs. If this were the case, we could presume that the association of PS MPs with an environmental contaminant alters the impact of MPs on human cells.

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P12 | Liver toxicology

P12-01
Moderate intake of beer improves nonalcoholic fatty liver disease (NAFLD) in a high fat diet (HFD)-induced mouse model

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Purpose: Both beer and some of its components, particularly polyphenols and iso-alpha-acids, have proven to be able to attenuate hepatic lipid accumulation or perturbed blood parameters in different rodent models through different putative mechanisms [1,2,3]. The current study was carried out within the NUTRATGE project (https://nutrage.it/) and aimed to evaluate the anti-steatotic capacity of beer in an HFD-induced NAFLD mouse model.

Methods: The beer was characterized for bioactive molecules content and individual phenolic compounds using UHPLC-ESI-MS/MS. In the *in vivo* study, forty-eight six-weeks-old male mice (C56BL/6) were randomly divided into four groups and supplemented daily during 10 weeks as follows: 1) normal diet (CTR); 2) a CTR diet and 0.14 ml/day beer (CTR+Beer); 3) a HFD (HFD); 4) a HFD and 0.14 ml/day beer (HFD+Beer). Prior to sacrifice, the weight of each animal was recorded, and blood was collected. We quantified liver lipids, performed histopathological evaluation using hematoxylin and eosin staining, and analyzed biomarkers of oxidative stress. Additionally, analysis of gene expression and DNA methylation of hepatic tissue was performed by RNA-Seq and Reduced Representation Bisulfite Sequencing.

Results: The beer displayed a good content in total phenols (25.01±1.27 mg GAE/100 ml), flavonoids (3.17±0.17 mg CE/100 ml) and flavonols (3.07±0.23 mg QE/100 ml). Among the single phenolic compounds, isoquercetin emerged as the predominant polyphenol (14.68±2.68 mg/100 ml). Compared to CTR, HFD group showed significantly higher levels of AST, ALT, TC, LDL-C, glucose, body weight and liver lipids, indicating the presence of steatosis, confirmed also by histological analysis. In HFD+beer group all the parameters returned to levels similar to those of CTR. All groups exhibited comparable levels of both protein carbonylation and lipid peroxidation in the liver, suggesting that our model represents an early stage of NAFLD with no oxidative stress. Analysis of transcriptomic and CpG methylation profile showed a clear separation between CTR and HFD groups. Beer consumption only partially affected gene expression whereas specifically changed the DNA methylation profile. RNA-Seq revealed 162 differentially expressed genes (DEGs) between CTR and HFD, whose biological function was related to cellular inflammatory processes and regulation of lipid metabolism. Beer consumption ameliorated the HDF effect (CTR*vs*HFD+beer, DEGs=43) showing alteration in the inflammatory response but not in the lipid homeostasis. RRBS profile identified 562 (CTR*vs*HFD), 429 (CTR*vs*HFD+beer), 469 (CTR*vs*CTR+beer) and 860 (HFD*vs*HFD+beer) differentially methylated cytosines (DMCs). DMCs target genes related to acyl glycerol and lipid biosynthetic process for CTR*vs*HFD+beer and insulin signaling for CTR*vs*CTR+beer comparisons. In summary, beer was capable to improve NAFLD likely due to the ability of polyphenols to modulate lipid metabolism.

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P12-02
effects of high-fat diet and streptozotocin-induced diabetes on CYP2E1 protein expression in rat liver

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The starting point of our study was the demonstration in various studies that CYP2E1 enzyme expression is affected by diabetes. As a toxicological concern, CYP2E1 is of interest because it metabolizes and activates a wide range of toxicologically significant compounds, including ethanol, carbon tetrachloride, acetaminophen, benzene, and halothane. Additionally, procarcinogens such as nitrosamines and azo compounds are among the substrates of CYP2E1 [1]. The metabolism of these compounds by CYP2E1 generates toxic intermediates and excessive levels of reactive oxygen species. As a consequence of its ability to produce reactive oxygen species at high levels, CYP2E1 has been linked to a wide range of pathological conditions, including diabetes, non-alcoholic steatohepatitis, and cancer [2]. All this information indicates that CYP2E1 is an important microsomal source of oxidative stress and lipid peroxidation [3]. For all of these reasons, our study examined the expression changes of CYP2E1 in liver tissues from Spraque-Dawley rats with type 2 diabetes caused by a high-fat diet combined with streptozotocin. On the other hand, we also highlight, for the first time, the effect of dapagliflozin, which is used to treat type 2 diabetes, on CYP2E1 expression. In our study, 32 maleSprague-Dawley rats were randomly divided into four groups: control, high-fat diet and streptozotocin-induced diabetes, dapagliflozin treated control, and dapagliflozin treated diabetes. In the microsomes obtained from the livers of these rats, the protein expression levels of CYP2E1 were determined by western blot. In our study, hepatic CYP2E1 expression level increased in control rats compared to the other three groups, but this increase is not statistically significant This result contrasts with previous studies reporting that hepatic CYP2E1 expression enhanced in diabetes [4]. Further research with a larger sample size is needed to clarify these conflicting results. From the result, hepatic CYP2E1 protein expression levels in the diabetic group treated with dapagliflozin were increased compared with in diabetic group. Although there was not statistically significant difference between two groups, this finding might indicate that increased CYP2E1 expression with the use of dapagliflozin under diabetic conditions may significantly affect impact

therapeutic efficacy or toxicity of medicines and other chemicals metabolized by CYP2E.

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P12-03
Hepatotoxicity induced by a mixture of PAHs: role of extracellular vesicles

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Purpose: Nowadays, metabolic-associated steatotic liver diseases (MASLD) evidenced by the presence of lipid droplets in more than 5% of hepatocytes, is the most common chronic liver diseases in the world occurring in 30% of adults, globally. Polycyclic aromatic hydrocarbons (PAHs) are abundant environmental toxicants released by anthropic gas emissions, whose main exposure is food ingestion. PAHs are currently considered as possible contributing factors in the pathological progression from benign hepatic steatosis to harmful steatohepatitis, consisting of hepatocyte death and inflammation besides steatosis. In addition, exposure of non-steatotic rat hepatocytes to some PAHs alone such as benzo(a)pyrene, pyrene and dibenzo(a,h)anthracene, has been previously shown by our team to increase the release of extracellular vesicles (EVs), i.e. membrane-surrounded nanovesicles, along with alterations of their content. When delivered to recipient non-steatotic rat hepatocytes, this modified EV content has also been proven to induce cell death. Therefore, the aim of this work is to study the role of EVs in the progression of hepatic steatosis to steatohepatitis due to PAHs.

Methods: Steatotic WIF-B9 hepatocytes were treated by a 100 nM mixture of 18 PAHs. The composition of the mixture was determined in order to be as close as possible to the composition in PAHs found in contaminated food. A prior steatosis was obtained by supplementing hepatocytes with 100 µM palmitic acid and 450 µM oleic acid. EVs were isolated from culture medium by differential ultracentrifugation.

Result:. Exposure of steatotic hepatocytes to the PAH mixture for 5 hours induced a necroptotic cell death along with inflammation, thus suggesting a steatohepatitis-like stage. Interestingly, an exposure of hepatocytes for up to 72 hours triggered apoptosis, another type of cell death, while the effect on necroptosis was no longer detected. In addition, inhibitors of PAH metabolism, whether dependent on nuclear factors AhR or CAR, both involved in PAH metabolism, prevented any increase in necroptosis or apoptosis. As described previously for non-steatotic hepatocytes, exposure of steatotic hepatocytes to PAHs also increases EV release, but with different changes in protein content. Inhibition of early necroptosis by inhibiting RIPK1 or MLKL, two key players in the molecular program of necroptosis, prevented the EV release and late apoptosis at 72 hours. Using inhibitors of EV formation such as dynasore and GW4869, necroptosis due to PAHs increased while apoptosis decreased. In addition, EVs released by steatotic hepatocytes after 5 hours of PAH treatment, were isolated and added to the culture medium of untreated steatotic hepatocytes. An increase in apoptosis was then detected without any effect on necroptosis.

Conclusion: PAH mixture triggered several types of cell death as it is described in MASLD. EVs could be held responsible.

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P12-04
Assessment of cannabidiol from 4 different sources on hepatic toxicity and metabolism *in vitro*

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Cannabidiol (CBD) has generally been recognized as being well tolerated, yet under certain conditions its metabolic activity and hepatotoxicity may be of concern. We are leveraging the HepaRG liver model to assess the influence of CBD origin and quality on the CBD-related metabolism and hepatotoxicity. Here, we compared the effects of CBD from 4 different sources (CBD 1–4) on the viability and functionality of the liver cells using a 14-day repeated exposure. The CBDs differed in their purity and manufacturing process (2 synthetics with >99% purity, 1 plant extract isolate with 99.3% purity, 1 distillate with 84.3% purity) offering a better understanding of how their different properties may impact liver safety.

The cells were exposed to one of the 4 CBDs every 2 or 3 days for 14 days after which their ATP content was measured. The conditioned culture medium was collected on days 3, 8, and 15 to measure the secreted albumin and the lactate dehydrogenase (LDH) content. In addition to the hepatotoxicity assessment, the impact of the 4 CBDs on the activities of 6 cytochrome P450 (CYP) enzymes (1A2, 2A6, 2C9, 2C19, 2D6, and 3A4) was assessed. Finally, the metabolization was studied by collecting the medium of HepaRG cells exposed to the 4 CBDs and analyzing the detectable metabolites using a non-targeted approach.

Cytotoxicity: All CBDs had a similar EC_{50} comprised between 27.14 and 30.61µM. For cells exposed to doses of CBD 1–4 above the EC_{50}, a spike in LDH was measured 48h after the first treatment followed by low levels on days 8 and 15 of treatment. At doses above the EC_{50}, the albumin secretion was reduced by at least 50% of the solvent control. Interestingly, cells treated with subtoxic doses of any 4 CBD also secreted 20 to 40% less albumin than the solvent-treated cells.

Pharmacodynamics and metabolic profiles: Following a 14-day repeated exposure, the activity of 6 major CYPs was found to be reduced by at least 5% (for the lowest dose) and up to 95% (for the maximal subtoxic dose). Using the medium from the same cells, the formation of CBD metabolites and their proportion were evaluated on days 3, 8, and 15. A metabolite with glucuronide conjugate represented at least 70% of the total metabolites detected (for any CBD, and any time point). Only 10 to 12 other metabolites (out of 39 in total) were detected with an abundance above 1% of the total metabolites (in all CBDs).

In conclusion, the 4 CBDs tested in the present study, despite their different qualities, had similar EC_{50} with a comparable effect on albumin secretion. Metabolite formation and impact on xenobiotic metabolism were also comparable with no significant CBD quality-related differences observed. Therefore, this study shows that despite different origins, purity, and manufacturing processes, the effects seen in this study are likely attributable to CBD, rather than Impurities.

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P12-05
Species-specific liver microtissues: a set of micro-physiological systems to study translational hepatotoxicity in safety assessment

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Background and Purpose: Animal studies are used to assess the hepatotoxicity of therapeutic drug candidates. However, this approach is not always able to reliably predict hepatotoxicity in human, leading to the discontinuation of the development of drug candidates in the late stage of the safety assessment process. [1] Therefore, understanding species-specific differences in toxicities is crucial for a robust safety assessment. This study investigates the utility of species-specific liver microtissues for assessing hepatotoxicity translatability across multiple preclinical animal species to humans.

Methods: Liver microtissues are 200 µm spheric co-cultures of species-specific primary parenchymal and non-parenchymal liver cells. [2] Here, Canis familiaris (Beagle), Rattus norvegicus (Sprague Dawley), Macaca fascicularis (Cynomolgus), and human liver microtissues were characterized for viability, morphology, albumin production and temporal stability. Subsequently, the cytotoxicity of two FDA-approved drugs, fialuridine and chlorpromazine, was assessed in human, rat, dog, and monkey liver microtissues. Transcriptomic signatures were analysed following fialuridine treatment.

Results: The results of this work show through the evaluation of viability, albumin production and morphology the four species-specific liver models to be stable over 7 days of culture. Chlorpromazine, which is not reported to be more hepatotoxic in a specific species *in vivo*, is cytotoxic at similar drug concentrations in all four liver microtissues. In contrast, fialuridine is statistically significantly more cytotoxic in human liver microtissues. Transcriptomic analysis in fialuridine-treated samples revealed distinct gene expression changes in human liver microtissues, particularly in genes related to DNA damage response, consistent with known mutagenic effects of fialuridine in human hepatocytes. [3]

Conclusions: Altogether, this work suggest that the four species-specific liver microtissues form a relevant set of micro-physiological systems suitable to study the translation of the hepatotoxicity, or the innocuity, of a compound from animal to human. Moreover, their scalability and reproducibility indicate the species-specific liver microtissues to be compatible with the industrial drug development process.

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P12-06
Establishment of a new transgenic HepaRG cell line expressing CYP2D6: new means for *in vitro* toxicity assessment of xenobiotics hydroxylated by CYP2D6

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Introduction: Primary Human Hepatocytes (PHH) are the best-known model to study the liver specific functions. However, due to the lack of availability in liver biopsies and high donor variability, experimental work on these primary liver cells is very limited. To overcome these difficulties, HepaRG cell line shows high hepatocyte differentiation capabilities combined with a metabolic profile close to PHH [1]. It is therefore an essential tool in the fields of pharmacology, biotransformation [2] or toxicology [3]. However, differentiated HepaRG cells do not express cytochrome P450 2D6 (CYP2D6) protein which is essential for the biotransformation of nearly 25% of drugs on the market. To overcome this CYP2D6 deficit, development and characterization of HepaRG cells expressing CYP2D6 was performed to enhance their toxicological assessment capabilities after lentiviral infection. Our objectives were first to characterize CYP2D6 expression in our genetically modified HepaRG compared with the parental HepaRG cell line, and secondly to validate our model using tramadol and perhexiline (PHX), two drugs metabolized through CYP2D6-dependent pathways.

Methods: Progenitor HepaRG cells were expanded and differentiated as previously reported [4]. Lentiviral transductions of progenitor HepaRG cells were performed at passage 10 using a transgene encoding both CYP2D6 and GFP to follow the efficacy of infection until passage 18. Transgenic *vs* parental differentiated HepaRG cells were compared for CYP2D6 expression by RT-qPCR, western-blot and confocal microscopy. In addition, eight CYP P450 CLint activities were analyzed by LC/MS-MS. Both parental and transgenic HepaRG cells were incubated with CYP2D6 specific drugs. Tramadol metabolites were quantified by LC/MS-MS and PHX toxicology studies were performed using LDH, ATP and Seahorse assays.

Results: Progenitor HepaRG cells transduced with lentivirus encoding GFP and human CYP2D6 cDNA stably express CYP2D6 enzyme at levels close to high PHH metabolizers for RNA and protein expressions and enzymatic activities. As expected, CYP2D6 protein is mostly located at the endoplasmic reticulum, the known functional CYP P450 location. In parental HepaRG cell line, tramadol is only metabolized in N-desmethyl tramadol by CYP3A4. In contrast, the CYP2D6 transgenic HepaRG cells metabolize tramadol in both, N- and O- desmethyl tramadol as found in PHH. After PHX treatments, higher IC_{50} is found in transgenic HepaRG cells and lower mitochondrial damages are observed compared to parental cells for the same PHX concentrations. Together, these data confirmed that the two cell lines are highly similar but CYP2D6 transgenic HepaRG cells are more suitable for toxicology studies of specific compounds metabolized by CYP2D6. We are currently investigating the toxicity of other molecules, more specifically plant-derived compounds that can cause major liver injury via CYP2D6 biotransformation.

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P12-07
Early detection of hepatotoxic compounds in drug development: leveraging the potential of human liver microtissues for predictive screening

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Background and Purpose: The development of therapeutic drugs is often hampered by hepatotoxicity. Detecting possible hepatotoxic compounds as early as possible during the development process helps channelling resource on therapeutic compounds. However, the current safety assessment still regularly fails to predict hepatotoxicity in human, leading to numerous drugs being stopped in late stage of development. Micro-physiological systems are *in vitro* systems focusing on the accurate modelling of physiological organ features, with the aim to have better predictive power than less physiological systems. Human liver microtissues, comprising spherical co-cultures of primary parenchymal and non-parenchymal liver cells, recapitulate essential liver features and have demonstrated better hepatotoxicity prediction accuracy compared to planar hepatocyte cultures [1]. This study evaluates the predictive power of 3D liver microtissues by benchmarking the cytotoxicity (cellular ATP IC50) of a large drug set of FDA-approved drugs, screened in human liver microtissues, against the *in vivo* drug-induced liver injury (DILI) annotation and drug label information of hepatotoxicity.

Methods: A large drug set of 152 FDA-approved small molecular drugs with balanced clinical hepatotoxicity and a broad representation of drug classes were screened in human liver microtissues for hepatotoxicity assessment. The relevance of the liver microtissue for hepatotoxicity assessment was evaluated by comparing the cytotoxicity (cellular ATP IC50) of the liver microtissues with the *in vivo* hepatotoxicity and total peak plasma concentration reported from the clinics.

Results and Conclusion: The cytotoxicity of the drugs correlated well with all three types of classification, “DILIconcern class”, “Liver injury description” and “Hepatotoxicity related warning”, reported in the FDA resource, the DILIrank dataset. This study disclosed that 86% of withdrawn drugs and 78% of drugs leading to fatal hepatotoxicity were accurately predicted as hepatotoxic, whereas 85.3% of the non-toxic drugs were accurately predicted as such. The correlation between the *in vitro* cytotoxicity and the hepatotoxicity of the tested drugs demonstrates the utility of human liver microtissues for the detection of hepatotoxic compounds early in the drug development process.

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P12-08
Establishment of a novel and simple drinking water liver injury model for hepatotoxicity research

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A liver injury model utilizing [TI1] compounds is extensively employed in hepatotoxicity research. Continuous administration of compounds mixed in drinking water is a convenient and attractive approach, particularly for assessing temporal changes. By examining the temporal changes across multiple models, both similarities and differences can be elucidated, enhancing comprehension of the manifestation and progression mechanisms of compound-induced hepatotoxicity. However, established and widely utilized compounds for drinking water-based liver injury models is restricted to few compounds such as Thioacetamide (TAA). Thus, this study aimed to explore the feasibility of expanding the repertoire by developing a novel liver injury model beyond TAA.

Initially, we identified compounds known to elevate ALT, a key marker of hepatotoxicity, through database searches and literature review. A total of 7 candidates were obtained through screening. Among them, 4,4’-methylene dianiline (MDA) was chosen due to its cost-effectiveness, water solubility, and oral hepatotoxicity induction capability. A 28-day study in mice revealed increased ALT levels at 7 (373±56.6 U/l), 14 (253±27.0 U/l), and 28 days (135±30.9 U/l), along with elevated fibrosis markers (Col1a1 and Acta2) at 14 and 28 days, [忠水2] confirming hepatic dysfunction induction. It is noteworthy that no fatalities occurred during the study period. To assess toxicopathological insights, deep learning-based anomaly detection on pathological images unveiled unique features characterized by necrotic foci compared to the TAA model. Additionally, multi-view data composed of blood biochemistry, immune cell trafficking, and RNA-seq data were collected during early toxicity manifestation to explore mechanistic differences. Initial administration led to significant ALT elevation and increased TBIL (6.0±1.23 mg/dL, day3) and TCHO (857±110 mg/dL, day3), indicating cholestatic liver injury. Immune cell analysis revealed elevated neutrophil levels in both the MDA and TAA models, along with distinct behavior of monocyte-derived macrophages between them. RNA-seq analysis underscored differences in gene expression profiles, particularly in the characteristic fibrinolytic system response in the MDA model, indicating a potential connection between liver function impairment and fibrinolysis in this liver injury model.

In summary, we successfully established a novel drinking water liver injury model utilizing MDA, which represents an achievement in expanding the scope of drinking water administration models. Its simplicity in implementation and unique toxicological profiles offer promising prospects for diverse studies on hepatotoxicity.

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P12-09
Dissecting the idiosyncratic drug-induced liver injury (iDILI)-initiating mechanism using the individual-centric model: the role of the innate immune response

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Introduction: Idiosyncratic drug-induced liver injury (iDILI) is a rare event that is difficult to detect and to dissect its mechanism at preclinical stage [1]. Although there is evidence that most idiosyncratic drug reactions are mediated by an adaptive immune response against

drug-modified proteins that are formed by a covalent binding of reactive metabolites and liver proteins, little is known about the early steps of iDILI-initiating mechanisms, i.e. the role of the innate immune response [2]. Macrophages and dendritic cells play an important role in initiating innate immune responses [3]. We have developed a cell line-based individual-centric spheroid model that contains autologous monocyte-derived macrophages and dendritic cells that can detect troglitazone (TGZ)-mediated idiosyncratic liver injury at therapeutic dose. We used this model to analyze the response of immune cells to TGZ for a better understanding of iDILI mechanism and to explore new strategies to mitigate iDILI occurrence.

Methods: Individual-centric spheroids were generated with the cell educating technology (patent PCT/EP2024/052109) using processed blood from healthy donors. The spheroids that contain educated hepatocytes, and stellate cells, were supplemented with educated autologous monocyte-derived macrophages, and dendritic cells (DCs). Individual-centric spheroids and monocyte-derived macrophages and DCs were treated with troglitazone with concentrations up to 100x C_{max}. The cell viability was measured using CellTiterGlo. The expression of pro- and anti-inflammatory cytokines were measured by qPCR.

Results: We showed that the shape and the number of monocyte-derived macrophages and DCs were individual dependent. We observed that the profile of cytokines expression by immune cells was donor specific. We found an individual dependent alteration of cytokines expression upon TGZ treatment. Interestingly, we noticed that TGZ suppressed IL-12a expression by immune cells and correlated to iDILI occurrence in a 44-year-old male. Our data suggest that IL-12 could contribute to the protective mechanism against TGZ-mediated iDILI.

Conclusion: We provide here evidence that our preclinical individual-centric model is valuable to de-risk iDILI occurrence and to dissect its initiating mechanism opening a perspective for new strategies to mitigate idiosyncratic drug reactions.

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P12-10
Mitochondrial DNA release mediated GAS-STING pathway activation involved in the mechanism of graphene oxide hepatitis injury

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Aims: Graphene oxide (GO) exhibits significant potential as biomedical materials, particularly concerning its impact on the important target organs – liver is an important topic in graphene biological effect research. As the primary immune cell population type in the liver, Kupffer cells (KCs) possess robust phagocytic and antigen-presenting capabilities, crucial for efficiently clearing exogenous particles and modulating immune responses. Considering the prevalent use of low-dose exposures in biomedical applications, this study systematically investigates the early inflammatory damage and immune response characteristics in the liver induced by GO, particularly focusing on liver macrophages. It aims to elucidate the potential mechanisms at the subcellular and molecular levels, thereby offering comprehensive insights for liver toxicity risk assessment and safe utilization of GO in biomedical applications.

Methods: Initially, we investigated the impact of low-dose graphene oxide (GO) on liver structure, function, and inflammation in mice at the organismal level. Subsequently, we delved into the effects of low-dose GO on immune functions, including phenotypic alterations, phagocytic activity, and cytokine secretion of liver macrophages at the cellular level. Finally, leveraging transcriptomic analysis, we probed into the underlying regulatory mechanisms upstream.

Results: The results revealed that exposure to 1 mg/kg of graphene oxide (GO) did not significantly alter the structure and function of the mouse liver. However, it did trigger the secretion of inflammatory cytokines and the activation of liver macrophages. Moreover, GO exposure notably induced M1-type polarization of Kupffer cells (KCs), leading to changes in multiple cytokines (increased TNF-α, IL-1β, and CXCL-10, decreased IL-10), and enhanced phagocytic activity. Further mechanistic investigations unveiled that graphene oxide might induce cytoplasmic release of mitochondrial DNA (mtDNA) by opening mitochondrial permeability transition pores, thereby activating and facilitating the cGAS-STING pathway, ultimately contributing to the pro-inflammatory immune response of liver macrophages induced by graphene.

Conclusion: These findings indicate that low-dose exposure to graphene oxide (GO) can induce liver inflammation and trigger the pro-inflammatory transformation of liver macrophages. The underlying molecular mechanism involves the cytoplasmic release of mitochondrial DNA and its mediation of cGAS-STING pathway activation.

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P12-11
Understanding the impact of a real-life mixture of poly- and perfluorinated compounds (PFAS) on the liver metabolism: insights from a dynamic 3D *in vitro* model

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Poly-and perfluorinated compounds (PFAS) are a diverse group of substances known for their exceptional stability and widespread applications, including use in textiles, medical devices, and firefighting foams. However, mounting evidence highlights their toxic potential, persistence, global distribution, and bioaccumulation. Notably, perfluorooctanoic acid (PFOA), perfluorooctanesulfonic acid (PFOS), and perfluorohexanesulfonic acid (PFHxS) have been classified as persistent organic pollutants (POPs) under the Stockholm Convention. Within the European Union, the entire class of PFAS is currently under consideration for a general restriction. PFAS can cause endocrine and metabolic disruptions primarily through various non-genotoxic mechanisms, including disrupting metabolic cooperation mediated via gap junction channels. These perturbations occur in liver cells (hepatocytes) and may contribute to developing chronic diseases, such as hepatic steatosis or metabolic dysfunction-associated fatty liver disease (MAFLD). These conditions, characterized by the overaccumulation of fatty acids, triglycerides, and cholesterol, may further predispose individuals to hepatocellular carcinoma (HCC).

In our current study, we aimed to unravel the intricate mechanistic pathways underlying the actions of selected PFAS. Specifically, we investigated the impact of a PFAS mixture comprising seven different PFAS compounds on the liver metabolome. The ratio of these com-

pounds in the mixture mirrored real-life scenarios encountered by occupationally exposed firefighters. To achieve our aim, we employed an advanced scaffold-free three-dimensional (3D) liver-derived HepG2 *in vitro* model cultivated dynamically over an extended period (5 weeks) within the ClinoStar™ system (CelVivo) and combined it with ^{1}H Nuclear Magnetic Resonance (NMR)-based metabolomics. After treatment, non-polar and polar metabolites from both media and spheroids were extracted using a multiple-solvent extraction method and analyzed using ^{1}H NMR. Measurements resulted in a metabolic fingerprint of 30–40 metabolites, including lipids, amino acids, carbohydrates, carboxylic acids, and nucleotides. Besides the NMR metabolomics, markers of the maturity and functionality of the liver, such as albumin production or connexin 43 and 32 expression, were assessed. Additionally, the toxicokinetic assessment was performed to determine the concentrations of PFAS in media and spheroids.

Overall, this workflow integrates cutting-edge techniques, including an advanced dynamic 3D liver *in vitro* model, a multiple-solvent extraction method for detecting polar and non-polar compounds, and NMR-based metabolomics. This comprehensive strategy enhances our understanding of PFAS-induced hepatotoxicity and provides valuable data to inform regulatory measures to safeguard public health.

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P12-12
Elucidation of molecular initiating events in PFAS – mediated cholestatic liver injury

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Introduction: Per- and poly-fluoroalkyl substances (PFAS) are abundantly used in a plethora of products with applications in daily life. As a result, PFAS are widely distributed in the environment, raising concerns regarding accumulation in humans. Recent data show impairment of bile acid metabolism by legacy PFAS, yet the potential of short-chain and alternative PFAS as well as the mechanistic basis of these cholestatic effects remain elusive.

Objective: The present study was set up to test the cholestatic potential of different legacy, short-chain and alternative PFAS using an adverse outcome pathway as a mechanistic compass. Focus was hereby put on the effects of PFAS on molecular initiating events.

Methods: Monolayer cultures of human hepatoma HepaRG cells were exposed to 8 types of PFAS in 3 concentrations. Fluorescently labeled probes were used to investigate effects on the bile acid transporters at the functional level. Effects at the transcriptional level were studied by means of quantitative reverse transcriptase polymerase chain reaction analysis. Bile canaliculi dynamics and hepatocellular changes were monitored *viain situ* immunostaining and phase-contrast imaging as well as through indirect analysis of rho-kinase activity.

Results: Similar to well-known cholestatic drugs, PFAS displayed inhibitory effects on different bile acid transporters, including organic anion transporting polypeptides and multidrug resistance-associated proteins, while membrane integrity remained unaffected. This was in line with the downregulation of their expression at the transcriptional level. Furthermore, some PFAS inflicted morphological disruption of bile canaliculi and alterations in rho-kinase activity. Overall, PFAS hepatotoxicity decreased with decreasing carbon chain length, but was not proportional to the extent of alteration of the molecular initiating event.

Conclusion: This study provides the first steps towards the elucidation of the mechanisms beyond the induction of cholestatic liver toxicity by PFAS.

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P12-13
Role of endoplasmic reticulum stress in sunitinib-induced hepatotoxicity

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Sunitinib, a multitargeted tyrosine kinase inhibitor, is used for the treatment of metastatic gastrointestinal stromal tumors, advanced metastatic renal cell carcinoma, and pancreatic neuroendocrine tumors [1]. However, sunitinib is associated with the development of severe hepatotoxicity that can lead to death in patients. Moreover, there is limited information regarding the underlying mechanism of sunitinib-induced liver toxicity. the underlying mechanism of sunitinib-induced liver toxicity is still unknown [2]. This study aimed to investigate the role of endoplasmic reticulum stress in sunitinib-induced hepatotoxicity on AML-12 cells. The cells were incubated with sunitinib for 24 hours, and the IC50 concentration of the drug was determined via MTT assay. Then, the cells were incubated with the drug at IC50 concentration and possible alterations caused by sunitinib in the expression levels of mRNA and proteins related to endoplasmic reticulum stress were determined. In addition, oxidative stress, cytosolic calcium levels, caspase-12 and caspase-3 activities in sunitinib-treated cells were measured by DCFH-DA [3], Fluo-4AM fluorescent dye [4], and ELISA kits [4,5], respectively. Sunitinib (IC50 = 2.1 μM) dramatically increased the mRNA and protein levels of PERK, CHOP, pro-caspase12, and ATF4 in AML-12 cells. The findings revealed that sunitinib exhibited comparable effects to tunicamycin, a positive control. Furthermore, exposure to sunitinib resulted in statistically significant induction in the amount of reactive oxygen species in the cells, cytosolic calcium level, and caspase-3 and caspase-12 activities compared to the control. Our results suggest that sunitinib may cause hepatotoxicity by inducing oxidative stress and subsequent endoplasmic reticulum stress. This is the first study to investigate the role of endoplasmic reticulum stress in sunitinib-induced hepatotoxicity. Further studies are underway in our laboratory to determine the entire mechanistic pathway of this adverse effect.

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P12-14

Male exposure to di-n-butyl phthalate induces persistent and transgenerational adverse effects on mouse liver transcriptome

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Dibutyl phthalate (DBP) is a ubiquitous environmental contaminant reported to be involved in hepatic tissue damage. However, the effect of DBP on the liver transcriptome and pathological processes is unclear. Here we used RNA-sequencing to investigate the hepatic transcriptional effects in male mice (F0) orally exposed for five weeks to two DBP doses, or vehicle only. To examine more persistent effects the liver was collected one week after the final dose. In addition, we collected data from liver tissue of the male offspinrg (F1) and grandoffspring (F2) of the F0 to investigate multi- and transgenerational effects on the liver transcriptome that could be induced by preconceptual male exposure to DBP. The transcriptome analysis of F0 male mice revealed 4 differentially expressed genes (DEGs) in the low dose group and 9 in the high dose group. Of these genes, Gm20431, which belongs to the ubiquitin-conjugating enzyme family with a lipid desaturase domain, associated with lipid metabolism, was increased in both DBP treatment groups. Another example is Tifa, a gene upregulated in conditions of acute stress, that was increased in the high DBP dose group only. The Gene Set Enrichment Analysis (GSEA) revealed 18 pathways significantly enriched in both DBP treatment groups. According to the DEGs and Reactome pathways, irregularities in protein synthesis, apoptosis, degradation of extracellular matrix and alterations in the energy/lipid metabolism are the main effects caused by DBP in the liver. DBP also caused lipid accumulation in both groups and increased ATP and glucokinase levels in the high dose group. Selective transcriptome alterations were further investigated at the protein level. The results indicate hepatic stress and injury caused by DPB exposure and suggest that DBP exposure could induce development of non-alcoholic fat liver disease. Furthermore, the F1 and F2 liver transcriptome analysis revealed multiple DEGs and pathways that indicated alterations in the metabolic system, showing that DBP does not only cause persistent adverse effects in the treated animals, but also induce multi- and transgenerational effects in the offspring and grandoffspring.

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P12-15

Eltrombopag: mechanism of clinical hepatotoxicity; mitigation strategies

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Eltrombopag (ELT; Promacta™, Revolade™ ; Novartis) is an oral drug for the treatment of platelet deficiency (thrombocytopenia). ELT is usually well tolerated, but it carries a 'Black Box' warning for potentially life-threatening hepatotoxicity, which, in some cases, necessitates dose reduction or even cessation. The mechanism of ELT hepatotoxicity is unknown; there is no evidence for an immune-mediated mechanism but rodent studies show that covalent binding occurs.

Azoreduction mediated by the gut microbiota is a major pathway of metabolism of orally ingested azo compounds, releasing aromatic amines as products (Josephy and Allen-Vercoe, *Food & Chem. Toxicol.* 2023; Pay *et al.*, *Food & Chem. Toxicol.* 2023). The structure of ELT incorporates an azo-pyrazolone functional group similar to that of the common yellow food dye, tartrazine. Reduction of the azo bond of ELT forms an amino-pyrazolone derivative, 4-amino-2-(3,4-dimethylphenyl)-2,4-dihydro-5-methyl-3H-pyrazol-3-one; ADPDMP; CAS 2222711-76-2). It appears to have escaped notice that simple loss of H2O from ADPDMP, without further metabolism, generates a para-imine methide, an electrophilic reactive species. We hypothesize that microbiome-mediated reduction of ELT to ADPDMP is the key bioactivation step leading to ELT hepatotoxicity.

This hypothesis is being tested in two ways. 1) The methyl substituents on the 3,4-dimethylphenyl ring of ELT are essential for para-imine methide formation. In the absence of those methyl groups, azoreduction would generate an aminopyrazolone similar to the metabolites of pyrazolone analgesic drugs such as aminopyrine, none of which is hepatotoxic. ELT analogues lacking the methyl substituents will,we predict, be therapeutically effective without the liability of hepatotoxicity. 2) Blocking ELT reduction in the gut should prevent formation of ADPDMP. The rationale for incorporation of the azo-pyrazolone functional group into ELT was the observation that reduction of tartrazine is considerably slower than the reduction of azo-naphthalene food dyes, such as Sunset Yellow. This difference could be exploited clinically: co-administration of non-toxic Sunset Yellow should suppress ELT reduction, by competitive inhibition, and thereby mitigate the drug's hepatotoxic side effects.

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P12-16

Detection of extracellular vesicles (EVs) in hepatotoxicity using CD9-EGFP reporter mouse

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Introduction: Recent findings have revealed that extracellular vesicles (EVs) are secreted from cells and circulate in the blood. EVs are classified as exosomes (40−100 nm), microvesicles (50−1,000 nm) or apoptotic bodies (500−2,000 nm). EVs contain mRNAs, microRNAs, and DNAs and have the ability to transfer them from cell to cell. Recently, especially in humans, the diagnostic accuracy of tumor cell type-specific EV-associated miRNAs as biomarkers has been found to be more than 90%. In addition, microRNAs contained in EVs in blood are being identified as specific biomarkers of chemical-induced inflammation and organ damage [1,2]. Although EVs are known to originate from various organs and circulate in the blood, the percentage of EVs originating from the liver is unknown. In this study, Cre recombinase-inducible human CD9-EGFP knock-in mice at the Rosa26 locus were generated and crossed with Alb-Cre transgenic mice, which express human CD9-EGFP specifically in the liver cells. Hepatotoxic substances, carbon tetrachloride and vehicle control, were then administered to induce liver damage. The objective of this study was to observe the dynamics of liver-derived EVs by detecting human CD9 in the bloodstream.

Methods: Male Cre-inducible human CD9-EGFP knock-in mice (flox) were crossed with female Alb-Cre mice to produce flox/Alb-Cre and WT/Alb-Cre mice. 10-week-old mice were treated with carbon tetrachloride (70 mg/kg) or vehicle control (corn oil) were administered as a single dose. Serum was collected 24 hours later and analyzed for blood biochemistry using AST and ALT. In addition, human CD9-positive EVs in serum were analyzed by EXOVIEW IMAGER using human CD9 antibody; EVs were separated from serum by ultracentrifugation and analyzed for particle number and size using Nanosight. Western blot analysis using human and mouse CD9 antibodies was also performed.

Results: Increases in AST and ALT were observed only in the carbon tetrachloride group. The percentage of human CD9 positive EVs increased tenfold in the carbon tetrachloride group. The increase in human CD9-positive EVs was also confirmed by Western blot analysis using human CD9 antibody.

Summary/Conclusion: Carbon tetrachloride-induced liver injury increased the percentage of liver-derived EVs in the bloodstream. This suggests that the presence of liver-derived EVs changes significantly in response to hepatic injury. Further studies are planned to determine when the increase in EVs occurs after carbon tetrachloride administration and whether similar results are obtained in other organ toxicities.

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P12-17
Differential effects of triazole pesticides on phase I CYP P450 enzymes in the human hepatocytes HepaSH™

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Triazole pesticides are widely used fungicides in modern agricultural practices. However, their ubiquitous use mostly in grapes, orchards, wheat, corn, and other crops raisesconcerns regarding the potential threat to human health. This study aims to characterize the effects of 9 of these fungicides: bromuconazole, difenoconazole, mefentrifluconazole, metconazole, penconazole, propiconazole, tebuconazole, tetraconazole and triticonazole, on the expression and/or activity of some cytochromes in HepaSH™ hepatocytes deriving from chimeric humanized liver mice. Expression of CYP1A2, CYP2B6 and CYP3A4, regulated by key nuclear receptors such as the constitutive androstane receptor (CAR), the aryl hydrocarbon receptor (AhR), the pregnane X receptor (PXR), and others can be considered as a marker of hepatocytefunctioning and performance. Any alteration of the expression of CYP1A2, CYP2B6 and CYP3A4 by triazole pesticides can be associated with a potential binding of these fungicides to nuclear receptors. A 3-to 250- fold induction of mRNA has been observed in HepaSH™ exposed to prototypical inducers of these three cytochromes which supports the use of this model in xenobiotic effect assessment. HepaSH™ cells were exposed to 10 µM of triazole pesticides for 48h prior to the assessment of CYP expression. mRNA expression of all three CYPs and protein expression of CYP3A4, but not CYP1A2, were induced by bromuconazole, metconazole, propiconazole, tebuconazole and tetraconazole. Differential effects of these pesticides on the activity of CYP2B6 and CYP3A4 were observed between cells pre-exposed and cells exposed simultaneouslyto these pesticides. CYP activities were induced following 48 hour-exposure to triazole pesticides but repressed in simultaneouslyexposed HepaSH™ cells. Bromuconazole, predicted as a moderate inducer of CYP3A4 and a strong inducer of CYP2B6 *in vivo* in humans was shown to be a strong inducer of CYP3A4 *in vitro*. Altogether, our data suggest that triazole pesticides may have a potential risk of interaction with some drugs or contaminants in populations highly exposed to these pesticides.

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P12-18
Investigating the role of nuclear receptors in valproic acid-induced liver steatosis

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Valproic acid (VPA) is a commonly prescribed anti-epileptic drug that has been associated with liver injury. As a branched short-chain fatty acid, VPA can impede mitochondrial β-oxidation, thereby contributing to mitochondrial dysfunction in hepatocytes. Various nuclear receptors (NRs) have been implicated in the development of steatosis by regulating fatty acid metabolism. Nevertheless, the relative significance of these or other NRs in VPA-induced steatosis remains unclear. This study aims to investigate the role of NRs in promoting liver steatosis upon VPA exposure.

To gain a better understanding of the importance of NRs in regulating VPA-induced gene expression, RNAseq data was generated using 3D microtissues (MTs) containing primary human hepatocytes (PHHs) from 10 donors supplemented with Kupffer cells. The MTs were subjected to a maximum dose of 38 µM VPA over a 72-hour period to mimic *in vivo* pharmacokinetics. Differentially expressed genes (DEGs) were identified after exposure to VPA for 8 h, 24 h and 72 h respectively. Subsequently, the gene list was utilized to predict the transcription factors by algorithm ChEA3. As a result, four NRs overlapped among the predicted NRs at each timepoint, namely NR1H4, HNF4A, NR2F6, and NR1I2.

To evaluate the relative significance of NRs, we established a lentiviral RNA interference approach to downregulate gene expression. Initially, this methodology was optimized in HepG2 cells before being implemented in PHHs. PXR has previously been demonstrated to contribute to VPA-induced lipid accumulation in HepG2 cells. In agreement with previous studies, downregulation of PXR did indeed inhibit lipid accumulation upon VPA exposure in HepG2 cells.

Furthermore, to further elucidate which NRs altered cell sensitivity to VPA administration upon downregulation, we assessed cell viability by knocking down each NR individually in HepG2 cells, followed by exposure to 5mM VPA for 48 h. The NRs are ranked from lowest to highest based on fold change, with the top-ranked NRs being NR2F6, NR4A3, NR1H2, and RARB. We were pleasantly surprised to find that NR2F6 also appeared in the list of predicted NRs identified from the RNAseq data. Additionally, the downregulation of all four NRs significantly reduced the IC50 values compared to the control group, with NR2F6 exhibiting the most remarkable decrease. This suggests that the low expression of NR2F6 increased the sensitivity of cells to VPA.

It has been reported that NR2F6 is upregulated in obese mice and patients with non-alcoholic fatty liver disease (NAFLD). Its downregulation inhibits fat production, suggesting a potential role as a crucial regulatory and pathogenic factor in NAFLD development. Consequently, we are interested in the involvement of NR2F6 in drug detoxification and metabolic functions. In summary, this study will advance our comprehension of the role of NRs in VPA-induced hepatotoxicity and steatosis.

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P12-19

Early detection of hepatotoxicity risks by investigating drug impact on mitochondrial activity in HepaSH™ cells

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Mitochondrial dysfunction plays a major part in the occurrence of drug-induced toxicities specially in liver. Indeed, some pharmaceuticals can cause direct damages to the function of respiratory chain in mitochondria, by impairing the electron transport chain and/or electrochemical proton gradient leading to diverse liver lesions. Thus, assessment of mitochondrial toxicity during preclinical safety studies is required to identify potentially dangerous drugs. To allow early-stage detection of drug-induced mitochondrial dysfunction, MiToxView® platform combine isolated liver mitochondria and relevant hepatic cell models such as HepaRG® differentiated cells with sensitive read-outs of mitochondrial function such as oxygen consumption. We have recently integrated in our process the HepaSH™ cells corresponding to a new model of experimental hepatocytes that overcome some limitations of primary human hepatocytes (PHH), such as inter-batch variability, limited availability and rapid functionality loss. In this study, we characterized 3 batches of HepaSH™ cells regarding their mitochondrial activity, especially respiratory chain function by measuring oxygen consumption rate (Seahorse analysis). We also investigated the feasibility of detecting drug-induced mitochondrial dysfunction on HepaSH™ by testing drugs known to trigger mitochondrial alteration in liver: acetaminophen, acetylsalicylic acid, two drugs leading to mitochondrial toxicity by reactive metabolites, and amiodarone, which acts by direct effect. Finally, we compared the sensitivity of the two cell models to such mitochondrial toxicants. Results indicate that the HepaSH™ cells are suitable to measure oxygen consumption using Seahorse technology, with hepatic-like respiratory profile. Furthermore HepaSH™ cells show high sensitivity to amiodarone at comparable concentrations than in HepaRG® cells, while they present low sensitivity to acetaminophen and acetylsalicylic acid, after 4h exposure to drugs. This new hepatocyte source should represent, besides the HepaRG® cell line, a reliable tool in place of PHH to early detect mitochondrial toxicity of parent drugs and help to select safer drugs for the patient benefit.

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P12-20

Exploring liver function effects of sulforaphane in rats: dose-dependent and sex-specific variations

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Sulforaphane (SFN), an isothiocyanate compound, is renowned for its significant health effects, particularly as an adjunct in chemotherapy. Despite its promising potential, comprehensive safety data regarding its synthesized form remain limited. Thus, this study sought to assess the hepatotoxic effects of SFN in rats following subacute exposure to three progressively escalating dose levels. The animals were divided into 8 groups, each consisting of 5 rats: 2 control groups (1 male and 1 female) and 6 treated groups (3 male (SFN 1 M, SFN 2 M, SFN 3 M) and 3 female (SFN 1 F, SFN 2 F, SFN 3 F)). The treated rats received subacute oral SFN administration at doses of 0.5, 2, and 5 mg/kg bw/day for 28 days. The investigation included the determination of biochemical parameters in blood and inflammatory and antioxidant mediators in liver tissue homogenates. In female rats, no statistically significant differences in liver function-related biochemical parameters was observed. Yet, there was a slight, but non-significant rise in aspartate aminotransferase (AST) activity in the SFN 1 F group compared to the control. Subsequent to this rise at the lowest dose, a decrease in activity was noted in the SFN 2 F and SFN 3 F groups, while a similar trend was noted in alanine aminotransferase (ALT) enzyme activity. On the other hand, a statistically significant increase in albumin quantity was observed in the SFN 2 M and SFN 3 M groups compared to the control. Analysis of heme oxygenase-1 (HO-1), nuclear factor erythroid 2-related factor 2 (Nrf2), and tumor necrosis factor alpha (TNFα) levels revealed notable changes. In female rats, a significant increase in HO-1 quantity was observed in the SFN 2 F and SFN 3 F group compared to the control. Additionally, SFN 3 F group exhibited significantly higher Nrf2 levels compared to the control. Both SFN 2 F and SFN 3 F groups showed a significant increase in TNFα compared to the control, with the highest dose group demonstrating the most significant change. In male rats, a statistically significant increase in HO-1 quantity was observed in the SFN 1 M group compared to the control. Furthermore, the SFN 3 M group displayed a statistically significant increase in TNFα quantity compared to the control. The rise in HO-1 and Nrf2 levels noted in this study suggests activated antioxidant mechanisms in response to inflammation, indicating an adaptive response to mitigate oxidative stress. In conclusion, the observed influence of SFN on liver enzyme activity, inflammatory markers, and antioxidant responses, indicating potential dose-dependent effects and sex-specific variations, underscores the necessity for further exploration of its safety profile.

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P12-21
GTL synthetic paraffin oil shows low liver and tissue retention compared to mineral oil – differences in MOSH interpretation

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EFSA has recently conducted a safety assessment of mineral oil residues in food. The liver in particular retains a narrow fraction of *mineral oil saturated hydrocarbons* – MOSH, which is also qualitatively found in experimental animals. It was concluded that this alkane fraction does not seem to pose an adverse effect in experimental animals, but given the limitations of the available chronic studies, a NOAEL=236 mg/kg bw was selected as reference point for MOSH. It was concluded that certain sub-types of MOSH, in particular polyring cyclo alkanes should be further investigated for their human accumulation potential (e.g. in the liver) [1].

We herein present the data that we submitted to EFSA for their MOSH residue assessment which led to the recognition that there are different types of oils in the market that may lead to different outcomes when evaluating hydrocarbon retention in the liver.

Two oils were fed to female SD rats at dietary doses of ~200 mg/kg bw per day. The oils were comparable in viscosity, carbon number distribution and purity (pharmacopeia grade). The main difference is that one oil was a conventional petroleum-derived mineral oil with high levels of polyring cyclo alkanes (naphthenic structures) while the second oil was a synthetic oil derived from natural gas (*gas to liquids – GTL oil*) virtually composed of only iso-alkanes. Thus, these oils were comparable in technical specifications but different in chemical composition.

Liver samples were taken at different exposure intervals including the recovery period where no oil was fed to the animals. Samples were analyzed with one and two-dimensional chromatography that allowed the assessment of the amount and type of hydrocarbons retained in the liver.

Our results indicate that mineral oil residues exhibit higher hepatic retention and slower excretion compared to GTL constituents. The structural differences of GTL iso-alkane constituents contributed to their faster hepatic elimination. It was shown that it is the poly ring cycloalkanes/ naphthenic sub-class in mineral oils – absent in GTL oils – which are particularly susceptible to hepatic retention. Furthermore, the retention patterns of alkane sub-classes in SD rat tissues, including the liver, are similar to those observed in humans [2]. Consequently, the low retention of GTL oil provides an alternative for food contact applications and veterinary vaccine adjuvants where MOSH residues are undesirable because of human oral exposure.

It is concluded that when considering "MOSH" retention a clear distinction should be made between alkane sub-classes and focus on the type of "MOSH" most relevant for human health assessment and hallmark of oral exposure, namely the poly ring cycloalkanes/ naphthenic "MOSH" sub-class [3].

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P12-22
Exploring the potential of sulforaphane to induce oxidative stress in the liver of rats

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In today's medical world, there is increasing attention towards the utilization of phytochemicals as adjunct therapy in treating various health conditions. One prominent phytochemical is sulforaphane (SFN), found in vegetables such as broccoli and kale. Research has shown that sulforaphane possesses antioxidative, anti-inflammatory, and anticancer properties, which may be beneficial in the prevention and treatment of certain diseases, including cancer, cardiovascular diseases, and neurodegenerative disorders. Nevertheless, its toxicological characteristics, especially of chemically synthesized one, are still not fully understood. Therefore, the aim of this study was to examine the potential of SFN to induce oxidative stress in the liver following subacute exposure (28 days) of rats. The male and female Wistar rats were randomly divided into eight groups (n=42): two control (n=6) and six treated (n=5). The control group received deionized water, while the treated groups were administered SFN at doses of 0.5 mg/kg bw/day (SFN1 group male and female), 2 mg/kg bw/day (SFN2 group male and female), and 5 mg/kg bw/day (SFN3 group male and female). After 28 days, the rats were euthanized, their livers were isolated, and homogenization of the tissue was performed. Parameters of oxidative stress were determined in the liver tissue homogenate: the activity of the enzyme superoxide dismutase (SOD), total sulfhydryl groups (SH), and ischemia-modified albumin (IMA) were assessed. In male rats, a statistically significant decrease in SOD activity was observed in the SFN1 group, while an even more significant decrease in enzyme activity was noted in the SFN3 group compared to the control group. Also, there was a significant reduction in the amount of IMA in the SFN1 group. In female rats, there were no statistically significant changes in the values of oxidative stress parameters in the liver. The significant decrease in SOD activity in male rats receiving both the lowest and highest doses of SFN compared to the control group could indicate a potential oxidative stress occurring in the liver. In conclusion, SFN demonstrated potential hepatotoxic effects in male rats by inducing oxidative stress, emphasizing the need for further investigation into SFN's safety profile, while also suggesting gender-specific differences in SFN toxicity

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P12-23

Comparative mitochondrial changes in Paracetamol-induced kidney and liver toxicity in mice

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Paracetamol overdose is known to cause severe hepatic injury. While its mechanism has been well elucidated, some details remain unclear. Although less common than hepatotoxicity, paracetamol overdose also induces kidney proximal tubular toxicity, with little known about its toxic mechanism. Our recent findings demonstrate that the toxicity mechanisms in these organs differ at the molecular level; specifically, c-Jun terminal kinase (JNK) is not phosphorylated and translocated to mitochondria in kidney proximal tubular cells, unlike in liver hepatocytes. In the present study, we comprehensively investigated the mitochondrial responses of the kidney to paracetamol overdose in a comparative manner with the liver in mice. Additionally, we used the mitochondria-targeted antioxidant MitoTempo at two different doses as a probe. Both organ damages were evidenced by significantly increased plasma damage markers and histopathological examination. We determined the activities and genetic expression levels of Complex I-IV of the mitochondrial Electron Transport Chain (ETC), as well as the protein amounts of Complex-I subunits. Furthermore, we measured the levels of both nuclear and mitochondrial DNA oxidative damage and methylation in both the liver and kidney in all groups. Additionally, we analyzed mitochondrial ATP, oxidized/reduced glutathione levels, malondialdehyde levels, oxidative stress levels, mitochondrial membrane potential, mitochondrial permeability transition pores, and the activity of mitochondrial superoxide dismutase. Our data provide further confirmation that paracetamol-induced nephrotoxicity initiates and progresses through completely different steps.

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P13 | Geno-toxicology & carcinogenesis

P13-01

Exploring antimutagenic effects of 2,6-dimethylpyridine N-oxide using fluctuation Ames test

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Introduction: The mutagenic potential of chemical agents is a profound concern in genetic toxicology. 2,6-Dimethylpyridine N-oxide has been hypothesized to possess antimutagenic properties, which could be incredibly beneficial for pharmaceutical and environmental sciences. This study investigates these properties using the fluctuation Ames test, a sensitive method for detecting mutagenic activity.

Methods: The current study aims to determine the genotoxic and antigenotoxic potential of 2,6-Dimethylpyridine N-oxide using *a fluctuating version of the Ames assay with preincubation in suspension* ofreversion *Salmonella*bacteria. The bacterial mutant tester strains, *Salmonella typhimurium* TA98 with frameshifts a point mutation in hisD3052 and *Salmonella typhimurium* TA100 with base-pair substitution mutation in hisG46, were used to determine genotoxic potentials of the test compound. To determine antigenotoxic potentials of the test compound, the same strains were also used together with positive mutagens 4-Nitroquinoline-N-oxide (4-NQO) for *Salmonella typhimurium* TA100 and 2-Nitrofluorene (2-NF) for *Salmonella typhimurium* TA98 without microsomal fractions of rat liver S9 and 2-Aminoanthracene (2-AA) for both strains with S9.

Results: According to the results, neither of the test compounds showed significant genotoxic activity on both tester strains at the tested concentrations. However, the presence of the 2,6-Dimethylpyridine N-oxide showed slight antigenotoxic activity on 2-AA – or/and 4-NQO or/and 2-NF -induced mutations. The inhibition rates of mutagenesis ranged from 5.56% (before preincubation) to 1.40% (after preincubation) for 4-NQO and from 47.23% (before preincubation) to 31.25% (after preincubation)) for 2-AA genotoxicity with *Salmonella typhimurium* TA100 w/wo S9. The inhibition rates *Salmonella typhimurium* TA98 *strain* of mutagenesis ranged from 12.28% (before preincubation) to 59.52% (after preincubation) for 4-NF and from 17.56% (before preincubation) to 46.59% (after preincubation) for 2-AA genotoxicity with S9 fraction.

According to these results, it is concluded that the test compound 2,6-Dimethylpyridine N-oxide do not have a mutagenic potential on the bacterial strains at the tested concentrations, and have antigenotoxic potentials against 2-AA-, 4-NQO- and 2-NF-induced mutagenesis.

Conclusion: The evidence suggests that 2,6-Dimethylpyridine N-oxide may have a substantial antimutagenic effect, notably diminishing the rate of mutations detected by the fluctuation Ames test. These findings pave the way for further research on the compound's role in mutagenesis prevention and its possible applications in drug development and environmental protection. Our research contributes significant insights to the understanding of antimutagenesis and suggests a promise for 2,6-Dimethylpyridine N-oxide in mitigating genotoxic risks.

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P13-02

Polyhexamethylene guanidine phosphate induces lung fibrosis and cancer via the TAK1-related signaling pathway

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Polyhexamethylene guanidine phosphate (PHMG-p) is a commonly utilized compound in a range of household items, including shampoos, swimming pools, wipes, and humidifier disinfectant (HD). Regrettably, it gained notoriety as the primary causation in the tragic HD disaster in South Korea. Following a comprehensive national-level epidemiological investigation, a significant number of lung cancer cases were identified among users of HDs. However, to date, there has been an absence of research into the carcinogenic effect of PHMG-p in relation to lung cancer. The Organization for Economic Co-operation and Development (OECD) proposes test guidelines (TG) for predicting the carcinogenicity of chemicals, which is recognized as an international standard. Three genotoxicity tests (OECD TG 471, 473, and 474) were conducted for PHMG-p. The results of the TG 471 and TG 474 were negative, whereas the TG 473 was positive. Consequently, no further carcinogenicity tests were conducted. Therefore, we aimed to find out whether PHMG-p has lung carcinogenicity.

In this study, lung lesion changes were observed at 20, 40, and 54 weeks after PHMG-p was instilled through intratracheal intubation using a rat model. We are the first to discover that lung cancer can occur even long after exposure has stopped. Furthermore, we discovered the TAK1 protein by investigating which proteins this chemical binds to, and fundamentally explored the molecular mechanisms involved. In addition to the well-known somatic mutations (Tp53, Sos1, Kmt2d, Mdm2, etc.) associated with lung cancer, we found novel mutations (Rab31, Washc1, Ddx11, etc.) that occurred at a higher frequency, and we found that they were mainly related to the DNA repair system. We also found that exposure to PHMG-p in human lung cells activated TAK1-related key signals, including necroptosis and MAPK signaling pathways, while inhibiting apoptosis. In addition, we identified four genes (*PLAU*, *HMGA2*, *TBX4*, and *GPX3*) that were commonly altered in human pulmonary alveolar epithelial cells and rat lung cancer tissue.These findings will be used as a part of the toxicological evidence base for the South Korean Ministry of Environment (National Institute of Environmental Research) to list PHMG-p as a Group 1 carcinogen, classified by the International Agency for Research on Cancer.

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P13-03

Comparative study of mutagenic/antimutagenic and genotoxic/antigenotoxic activities of ethanolic and aqueous extracts prepared from *Arbutus unedo* fruits

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Arbutus unedo L., a member of the Ericaceae family [1], is known for its fruits, which boast potential medicinal properties and nutritional benefits. These fruits, traditionally used in medicine as antiseptics, diuretics, and laxatives, are also edible [2]. The chemical composition of *A. unedo* fruits comprises phenolic compounds, fatty acids, vitamins, organic acids, sugars, volatile components, and minerals. Notable among these are phenolic acids, flavonoids, and anthocyanins [3]. Despite their historical use in folk medicine and growing popularity in the food industry, comprehensive studies on their mutagenic/antimutagenic and genotoxic/antigenotoxic properties are lacking.

This study aims to compare the biological activities of ethanolic and aqueous extracts from *Arbutus unedo* fruits. It includes Ames [4], alcaline comet [5], and micronucleus [6] tests to assess mutagenicity/antimutagenicity and genotoxicity/antigenotoxicity. High-Performance Thin-Layer Chromatography (HPTLC) was performed for chemical fingerprinting of the extracts and to check the presence of some phenolic compounds. Furthermore, antioxidant activities were explored using DPPH [7], CUPRAC [8], and FRAP [9] assays, alongside total phenolic and flavonoid content analyses, which are crucial for observed antimutagenic and antigenotoxic effects.

The findings of our research revealed that the ethanol extract of the fruit exhibited significantly higher total phenolic and flavonoid contents compared to the water extract. Additionally, the ethanol extract demonstrated superior antioxidant activity, as evidenced by the DPPH, CUPRAC, and FRAP assays. No mutagenicity was observed in either ethanolic or water extracts in tested strains with or without metabolic activation. Both extracts, up to 500 µg/ml, showed no genotoxicity in micronucleus and comet assays. Moderate antimutagenic activity was observed in the TA98 strain at the highest concentration for water and ethanolic extracts, with and without S9 activation, respectively. In the TA100 strain, only the ethanolic extract at the highest concentration with metabolic activation showed moderate antimutagenic activity. Neither the ethanolic nor water extract exhibited protective effects against doxorubicin-induced genotoxicity in micronucleus and comet assays. Taken together, our findings suggest the potential of *Arbutus unedo* fruit extracts, particularly the ethanolic extract, as sources of bioactive compounds with antimutagenic and antioxidant attributes. Further exploration is needed to elucidate their mechanisms and potential applications.

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P13-04
Analysis of cellular key events at the early stages of renal carcinogenesis upon repeated administration of ochratoxin A

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Ochratoxin A (OTA) is a rat renal carcinogen that causes phenotypic changes in carcinogenic target proximal tubular epithelial cells (PTECs) in the outer stripe of the outer medulla (OSOM) of rats after repeated oral administration, such as cell cycle abnormalities, induction of karyomegaly and cellular senescence, enhancement of cell proliferation, and induction of apoptosis. While the induction of deletion mutations might be causally related to the OTA-induced carcinogenesis, the mechanistic relationship of the key cellular and molecular events for renal carcinogenesis remains unclear. At first, to investigate whether OTA induces micronuclei (MN), an indicator of chromosomal instability, in PTECs, we performed an OSOM MN assay in rats treated with OTA, other renal carcinogens, or non-carcinogenic renal toxicants for 4 or 13 weeks. Theassay revealed a dose- and treatment period-dependent increase in PTECs with γ-H2AX$^+$ MN. MN formation was also observed with other renal carcinogens that induce karyomegaly similarly to OTA. These results imply that γ-H2AX$^+$ MN formation by OTA treatment is related to the induction of chromosomal instability accompanying karyomegaly formation. Next, we performed comprehensive gene profiling of alterations in promoter-region methylation and gene expression in PTECs of rats treated with OTA for 13 weeks focusing on the mechanism of OTA-induced carcinogenesis. The OTA-specific gene profile was obtained by excluding genes showing similar expression changes by treatment with 3-chloro-1,2-propanediol, a renal carcinogen not inducing karyomegaly. Then, we validated candidate genes using methylated DNA enrichment PCR and real-time RT-PCR, and identified *Gen1*, *Anxa3*, *Cdkn1a*, and *Osm* as those showing OTA-specific epigenetic gene expression changes. *Cdkn1a* upregulation and increase of p21$^{WAF1/CIP1+}$ karyomegalic PTECs were commonly observed with micronucleus-inducing carcinogens, suggesting that the increase of p21$^{WAF1/CIP1+}$ karyomegalic PTECs links to micronucleus formation that in turn progresses chromosomal instability. The upregulation of *Cdkn1a*-related genes with OTA suggests the acquisition of a senescence-associated secretory phenotype to establish a carcinogenic cellular environment. In contrast, a decrease of GEN1$^+$ PTECs reflecting *Gen1* downregulation and an increase of ANXA3$^+$ PTECs reflecting *Anxa3* upregulation, as well as *Osm* upregulation, were observed specifically with OTA, suggesting OTA-specific cellular mechanisms. OTA may efficiently disrupt pathways for repairing OTA-mediated DNA double-strand breaks involving *Gen1* downregulation and enhance cell proliferation through the upregulation of *Anxa3* and *Osm*. This may in turn contribute to exacerbating the MN formation-induced chromosomal instability from the early stage of OTA-induced renal carcinogenesis before proliferative lesions form.

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P13-05
Neonatal model of Thiacloprid carcinogenesis identification

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Neonatal models of carcinogenesis are focusing on human-relevant modes of action (MoA) vary depending on the type of compound being evaluated and the purpose of the evaluation. Use of special medium-term tests is more justified if the aim is to clear the bio-equivalence in carcinogenic properties of the generic and original substance for which the target organs and MoA have been clarified.

Thiacloprid induces neoplasia in rats (uterine adenocarcinomas and thyroid follicular cell adenomas) and mice (benign ovarian luteomas) and is unlikely to be genotoxic (EFSA, 2013). Induced tumours may have resulted from an endocrine-mediated MoA.

Aim: To identify the carcinogenicity of Thiacloprid Technical, produced by China, compared with the known Thiacloprid carcinogenic properties of other manufacturers using the particular test «Pubertal Development and Thyroid Function in Juvenile/Peripubertal Wistar Han Male Rats».

Material and methods: The study was performed on 45 male Wistar Han rats divided into three groups: Vehicle control (water+OP10), Groups 2 and 3 – Thiacloprid in doses of 1.2 and 48 mg/kg/day by gavage from PND 23 to PND 53. Low and High doses were chosen as NOAEL and effective doses in a carcinogenic study. Age and weight at preputial separation (PPS) were monitored daily beginning on PND 30; ALT, AST, total protein, creatinine, cholesterol, glucose, alkaline phosphatase, urea, and triglycerides were measured, and thyroid gland, testes, epididymis, kidneys were selected for pathomorphological studies.

Results: Thiacloprid caused a statistically significant ($p \leq 0.05$) decrease body weight and body weight gain of animals (16% and 20%) at High dose; affects preputial separation (9%). Significant decrease in absolute weight of testis (6%), epididymis (15%), prostate (28%), seminal vesicles (19%) and LABC (16%); increase the relative weight on the pituitary (30%), thyroid gland (13%) and liver (20%) vs control. Follicular cell hypertrophy/hyperplasia in the thyroid gland and hypospermia in epididymis were revealed in High doses. Significant increases in ALT (10%), creatinine (8%) and a decrease in urea level (23%) were observed. High dose of Thiacloprid shows a non-significant ($p \geq 0.05$) decrease in fT4 (8%) and increase in TSH (29%). Received results suggest that Thiacloprid at High dose has a general toxic effect, suppresses sexual development, induces histopathological lesions in the endocrine and reproductive organs, and changes liver weight, enzyme profile and hormone activity. NOAEL is 1.2 mg/kg.

Conclusions: The obtained results show that the test used in our study is sensitive and acceptable for substances that cause a perturbation of thyroid-pituitary hormone homeostasis, which is a potentially underlying cause for thyroid tumour formation in rats.

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P13-06
Genotoxicity of aqueous metalworking fluids in V79 Chinese hamster cells

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Metalworking fluids (MWF) exposure has been linked to many types of cancers including breast, bladder, larynx, lung, prostate, pancreas, rectum, and skin cancers. However, these links were established mainly with straight, oil-based MWF. Nowadays, oil-based MWF tend to be replaced by aqueous metalworking fluids which are extensively used in various industrial processes. Despite the potential occupational exposure of workers during their use and handling, few is known regarding their toxicological properties and especially their genotoxic potential.

Here, we investigated whether some of these aqueous fluids could affect DNA integrity and could alert thereby on their potential carcinogenic effects, using *in vitro* typical genotoxicity assays in V79 Chinese hamster cells. The comet assay and the micronucleus test were applied on four samples of aqueous fluids from different suppliers before metalworking processes. Our preliminary results show that one of them could induce DNA strand breaks and all samples induced a weak increase of micronucleated cells. Despite the results obtained so far, additional experiments would be require to try to identify which chemicals are responsible for the observed effects. In addition, a larger selection of fresh and used metalworking fluids should be tested to better estimate the potential carcinogenic effects of these mixtures.

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P13-07
Uranium and kidney cancer: insights from genetically engineered mouse models in the UKCAN project

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Exposure to uranium compounds is prevalent in various situations such as nuclear fuel processing, military activities, and environmental context. Uranium has a property to accumulate in the kidneys, yet it's potential carcinogenic effect remains controversial. The UKCAN project (Uranium Kidney CANcerous effects in rodents) is dedicated to investigate the potential carcinogenic effects of low-dose uranium on the kidneys.

To simulate the development of kidney cancer, we employ three genetically-engineered mouse models (GEMs) with mutation in key kidney cancer predisposition genes (*Vhl*±*Pbrm1*, or *Tsc2*). In this experimental study, each of the three GEMs is exposed to repeated uranium intranasal instillation and monitored for 10 to 12 months. Three different doses- 0; 125 or 250 µg/kg/d (n=20/dose/genotype) are being tested.

Our study has the following objectives:

- Evaluate the development of benign and malignant renal tumor lesions over time and dose following uranium exposure: ultrasonography is used throughout the animal's lifespan and postmortem analyses using histological and immunohistological methods provide details characterization of tumor lesions.
- Elucidate the underlying biological mechanisms (initiation, promotion, and proliferation) in renal oncogenesis and carcinogenesis associated with human exposure by clinical biochemistry, molecular biology and protein-array techniques.
- Analyze the concentration of uranium in targets organs and excretory samples using ICP-MS techniques.

Histological analyses showed delineated areas comprising various tumor types within the GEMs. Immunostaining of clinically relevant proteins (CA-IX, CK-7) allows for the identification and quantification of specific tumor subtypes within renal tissue. This approach facilitates the correlation of histological findings with ultrasound imaging data, enabling the tracking of endogenous renal tumors exceeding 200 µm, and characterizing their type, number, and volume. Preliminary results indicate delineated areas comprising various adenomas, carcinomas and cysts with a higher tumor incidence observed in mice exposed to uranium depending on their age. Additionally, screening of genes and proteins by RTqPCR and protein-array enables the identification of various targets involved in renal carcinogenesis and the mechanisms of uranium toxicity, modified by uranium exposure.

These findings will contribute to identifying the most relevant parameters for the experimental evaluation of the potential link between uranium exposure and the development of kidney cancer in our GEMs.

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P13-08
Dibutyl Hydrogen Phosphate (DBP) revisited – new insights from a comprehensive review of the scientific literature, experimental and computational data

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Despite a lack of toxicology data underpinning its regulatory status, Dibutyl Hydrogen Phosphate (DBP, CAS: 107-66-4) is designated as possibly carcinogenic by a variety of stakeholders, including industry and government organizations. This classification of DBP is based on the toxicological profile of tributyl phosphate (TBP, CAS: 126-73-8) a known (non-genotoxic) bladder carcinogen in the rat. DBP is one of the identified metabolites of TBP which supported a read-across hypothesis and justified the carcinogenicity consideration for DBP. TBP-mediated carcinogenicity is considered to be a result of chemical irritation caused by one or more TBP metabolites leading to characteristic pre-cancerous lesions in the rat urinary bladder upon sub-chronic exposure and to various neoplasms (i.e., papillomas, transitional cell carcinomas, squamous cell carcinomas) upon chronic exposure. In this study we revisit the existing scientific evidence for DBP and TBP, including new data from sub-chronic and computational studies supporting an improved assessment opportunity for the postulated carcinogenic potential of DBP. Importantly, the results demonstrate that DBP does not induce the prototypical pre-cancerous bladder lesions associated with TBP exposure. Dosimetric analysis further supports that sub-chronic oral exposure to TBP, but not to DBP, is required to induce the rat urinary bladder lesions. These findings present relevant new insights into the toxicologic characteristics of DBP and raise questions about the current classification of DBP.

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P13-09
Data collection for the compilation of the EFSA pesticides genotoxicity database in IUCLID

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The availability of high-quality databases covering a broad chemical space is a crucial challenge in modern risk assessment, as it can provide the foundation for improving the confidence of (Q)SAR models and

read-across. In particular, genotoxicity information of pesticide active substances and their metabolites needs to be not only accessible but also unambiguous and well-curated. A great amount of genotoxicity data for pesticides is stored in Draft/Renewal Assessment Reports (DAR/RAR), i.e., monographs submitted by different Rapporteur Member States (RMS) for issuing an EFSA conclusion on pesticide active substances upon peer-review. However, fruition of genotoxicity data contained in DAR/RAR documents may be cumbersome, due to the variability of how these data are reported by different RMS. EFSA has hence commenced a 3-year collaborative project (2022–2025) for updating the EFSA genotoxicity database specific for pesticide active substances and their metabolites, and for transferring the full database in an univocal format using the IUCLID (International Uniform Chemical Information Database) platform. Following their entry in IUCLID, genotoxicity data can be further extracted in Excel format or imported in the OECD QSAR Toolbox software. The aim of the current work is to present the compilation of the EFSA genotoxicity database, as performed by the project consortium. The Standard Operating Procedures (SOPs) set up for the collection and entry of data will be outlined; in particular, the minimal information to be gathered for the characterization of a valid genotoxicity study will be illustrated. Specific genotoxicity studies requiring the identification of further experimental details will be also presented. For instance, to ensure the completeness of information for the *in vivo* micronucleus tests, it is crucial to include additional experimental results accounting for the aneugenic potential and the direct or indirect evidence of exposure of the target tissue to the test material. The curation of the database will be pragmatically illustrated by means of three case studies. The first case study provides an example of an active substance negative for genotoxicity, i.e., fluoxastrobin, which gives place to a metabolite, i.e., 2-chlorophenol, raising potential concern for genotoxicity *in vitro* but not *in vivo* in light of a newly-collected positive result in an *in vitro* micronucleus assay. The second and third examples address the broad chemical space covered by the pesticide active substances under study, and these are aluminium ammonium sulphate, i.e., an inorganic compound, and azadiracthin, i.e., a natural extract defined as an UVCB substance. Altogether, the current work provides an excellent example of good data management practices in respect of FAIR data principles, defined as Findability, Accessibility, Interoperability and Reusability of genotoxicity data for pesticides and their metabolites.

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P13-10
Exploring the therapeutic potential of solamargine in glioblastoma: an *in vitro* study

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The complexity of the tumor microenvironment, coupled with the genetic heterogeneity and invasive nature of glioblastoma (GB), distinguishes it among the most challenging neoplasms for anticancer therapy. Currently, the predominant standard treatment for GB involves surgery with extensive tumor resection, combined with radiotherapy and chemotherapy. However, these therapeutic strategies offer only a limited impact on patient prognosis. Confronted with the pressing demand for therapeutic progress, natural compounds arise as a hopeful reservoir for pioneering new strategies in addressing GB, while also mitigating the undesirable effects associated with conventional therapies. This study investigated the effects of the steroidal glycoalkaloid solamargine (SM) on the viability, clonogenicity, morphology, migration, and invasion of cells in 2D cultures of various human glioblastoma cell lines (U-87MG, U-251MG, T98G, and KNS-42), as well as non-tumorigenic human astrocyte cells (NHA). The cytotoxic activity of SM was evaluated under normoxic and hypoxic conditions, as well as its interaction with chemotherapeutic drug temozolomide (TMZ) in GB cell lines. Results under normoxia revealed a reduction in cell viability in all tested cell lines, highlighting the significant influence of SM concerning time and dose. The lowest IC_{50} values were observed after 72 hours of treatment, ranging from 5.04 to 6.95 µM, with the U-87MG line being the most sensitive and a selectivity index of 1.93. Under hypoxia, IC_{50} values ranged from 6.75 to 8.92 µM after 24 hours of SM exposure, being lower than those obtained under normoxia (7.68 to 9.53 µM). TMZ showed no significant cytotoxic effects, while combined treatment revealed an antagonistic interaction between SM and TMZ in all tested cell lines, displaying combination indexes above 1. The clonogenic efficiency assay demonstrated the antiproliferative impact of SM in all tested cell lines, with significant reductions in colonies after 24 hours of treatment starting from 2.5 µM. Morphological changes, such as cellular body contraction and emission of fine filopodia, were observed in different GB cell lines following exposure to SM. Additionally, SM significantly reduced the migration of T98G cells after 24 hours of treatment with 5 µM (26.8%). However, no antimigratory effect of SM was observed in U-87MG, U-251MG, and KNS-42 cell lines. Investigation of the cytotoxic action of SM in U-87MG cells revealed its ability to induce apoptosis, block the cell cycle at the G2/M transition, and induce oxidative stress. These findings underscore the therapeutic potential of SM as a promising option in GB treatment, warranting further investigation in this area.

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P13-11
Hepatic metabolism of copaiba oil and resin oil-based formulation: analysis of the impact on cytotoxicity and cell cycle of human and murine hepatic cells with and without metabolic activation

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Objective: *Copaifera multijuga*, an Amazonian species of copaiba, exhibits extensive use of its oleoresin, particularly in medicinal application. Studies differ on its toxicity, especially in hepatic biotransformation. This study aims to analyze the cytotoxic profile and genetic safety of copaiba oil and its hepatic metabolites. Additionally, it investigates the safety of a resin oil-based formulation (hydrogel).

Methods: V79-4 and HepG2 cells were used for the cytotoxicity assay as a preliminary test, according to the OECD 487 guidelines. Cells were seeded in 6-well plates (9 x 10^6 cells/well), and exposed to 33.0, 16.5, 8.25, 4.13, 2.06, 1.03, 0.52, or 0.26 µg/mL of the oil, hydrogel, and excipient, following by non treated and positive control groups, and incubated for 24h at 37°C. After exposure, Trypan Blue exclusion assay was performed. Obtained data was analyzed for the parameters RICC, RPD and IS%, according to the OECD 487 and ISO 21427-2 2006(E) guidelines. Furthermore, flow cytometry assay using propidium iodide was performed to evaluate DNA fragmentation and the influence of metabolic activation on cell death and cell cycle in V79-4 cells, due to its greater genetic stability. Cells were seeded in 6-well plates (1 x 10^6 cells/well), and exposed to 33.0, 16.5, and 4.13 µg/mL of the oil, hy-

drogel, and excipient, followed by non treated and positive control groups, and incubated for 24h at 37°C. Data was obtained in the FACS-Canto II cytometer and analyzed in FlowJo 10.8 software after voltage adjustments and FL2 intervals.

Results: Determination of IC50 has demonstrated significant variability in cellular sensitivity to copaiba oil. LogIC50 for Copaiba oil was 8.31 (HepG2), 4.33 (V79-4 with S9), and 32.76 (V79-4 without S9). Hydrogel cytotoxicity has demonstrated a LogIC50 of 8.14 (HepG2), 4.38 (V79-4 with S9), and 32.88 (V79-4 without S9). Due to the elevated cytotoxicity, tested concentrations of copaiba could not be exceeded. Variability in the toxicity profile of copaiba was noted among different cells, and due to hepatic metabolism, potentially indicating cytotoxic biotransformation process. Regarding genetic safety, DNA fragmentation was observed in samples with metabolic activation at concentrations of 33 µg/mL and 4.13 µg/mL for both samples. There was also a change in the cell cycle of the V79-4 cells, with a percentage decrease of cells in the S phase at a concentration of 33 µg/mL with metabolic activation. The results may suggest *in vitro* hepatic biotransformation of *C. multijuga*, potentially increasing cytotoxicity and the production of genotoxic metabolites in murine and human hepatoma cells. In conclusion, further studies are required to ensure safety of Copaiba oil.

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P13-12
Detection of DNA damage in *C. elegans* via the automated FADU assay

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C. elegans is a 3R-compliant model organism with growing application in toxicological studies. This project investigates the usage of *C. elegans* in the high-throughput DNA damage assay via automated Fluorometric analysis of Alkaline DNA Unwinding (FADU). The automated FADU assay had been developed as a very sensitive screening tool to monitor various types of DNA damage such as strand breaks (SBs), crosslinks, alkylating adducts, or oxidative damage *in vitro* in human cell culture models and primary lymphocytes. In this project, the amount of DNA SBs, one of the most common genotoxic lesions, has been measured with FADU in *C. elegans* at different developmental stages exposed to genotoxic agents such as hydrogen peroxide (H_2O_2), bleomycin (BLM) or potassium bromate ($KBrO_3$). Additionally, the survival of worms and germline apoptosis were analysed 24 h after genotoxic exposure. According to our data, the automated FADU assay allowed detection of SBs in worms at different developmental stages, including cells isolated from *C. elegans* embryos, larvae as well as young adult (A1) worms. Exposure to known genotoxic agents (H_2O_2 and bleomycin) resulted in a dose-dependent induction of DNA SBs at concentrations exhibiting low general toxicity, indicating high sensitivity of the assay. In turn, exposure to $KBrO_3$ did not increase SBs occurrence, confirming the specificity of the assay. Results from the FADU assay were comparable with the germline apoptosis assay, where the genotoxic effect was observed at similar concentrations. Time-dependent removal of DNA SBs was also observed in worms with the FADU assay, suggesting the possibility of detecting repair of the DNA damage *in vivo*. The deletion of NAD^+-dependent enzymes (sirtuins and poly (ADP-ribose) polymerases) did not affect the worms' H_2O_2 susceptibility, as demonstrated by unchanged SBs occurrence and survival when compared to the WT animals. In conclusion, the FADU assay can be used to monitor SBs in *C. elegans* as an automated alternative to currently used techniques in studies investigating genetic toxicity *in vivo* with a wide range of potential applications.

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P13-13
The use of benchmark dose approach for (non-)genotoxic carcinogens

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The Benchmark dose (BMD) approach was introduced over 40 years ago. It constitutes a refined approach for determination of Reference Points (RPs) for chemical risk assessment. The derived RPs are used for estimating the health-based guidance values (HBGVs) set to protect human health, for example, the acceptable daily intakes or tolerable daily intakes for non-genotoxic substances. The BMD approach was also proposed by the European Food Safety Authority and the Joint FAO/WHO Expert Committee on Food Additives for deriving an RP, which is used to calculate the margins of exposure in the case when chemicals are classified as both genotoxic and carcinogenic. Discussions on the suitability of the BMD approach for (non-)genotoxic carcinogens are still ongoing. Traditionally, the distinction between carcinogens able to interact with the genetic material (genotoxic carcinogens), and that causing tumors by non-genotoxic mechanisms (non-genotoxic carcinogens) acts as a major reasoning to believe that genotoxic carcinogens cause genetic damages linearly related to the dose (and have no thresholds). Meanwhile non-genotoxic chemicals are usually considered to be thresholded. We argue that 1) the issue of a threshold is irrelevant for the question whether or not a BMD analysis is an appropriate tool for risk assessment. In all cases, both the no-ob-

served-adverse-effect level (NOAEL) and the BMD are doses related to a small effect. The difference between the two is that this small effect is quantified for the BMD, but not for the NOAEL. All this is unrelated to the existence of a theoretical threshold. Notably, the (non-)existence of a threshold cannot be proven, and could not be quantified from experimental data. 2) A dose-response may appear to be close to linear when plotted on dose-scale, but on the (more appropriate) log-dose scale the dose-response always has a more threshold-like shape. Biological rationale will be provided showing that, the shape of the dose-response should be considered on the log-dose scale. 3) Analyses of combined dose-response datasets consisting of both genotoxic and non-genotoxic carcinogens show that the shapes of the (log-)dose-response relationships do not differ between both groups of chemicals. This could not have been the case if the (non-)existence of a threshold dose were an existing and discriminating difference between both these groups. To conclude, the authors advocate that the BMD approach is applicable for both genotoxic and non-genotoxic carcinogens. This arrives at a harmonized approach for the hazard characterization between the genotoxic and non-genotoxic chemicals, as well as other chemical classes.

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P13-14

Investigation of genotoxic potential of iron based magnetic nanoparticles in *Drosophila melanogaster* haemocytes by COMET method

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Magnetic nanoparticles (MNPs) have emerged as a new class of nanoparticles (NPs). MNPs are frequently used in many fields such as medical diagnosis and treatment, biosensors, magnetic resonance imaging and drug delivery systems. The widespread use of MNPs today and the fact that they have direct application areas, especially on humans, increases concerns about the potential toxicity of MNPs and encourages new studies in the relevant field. Within the scope of our study, the genotoxic potentials of four different concentrations (1, 3, 5 and 10 mM) of four different iron-based MNPs; Fe_3O_4 NP (14–29 nm), $CoFe_2O_4$ NP (30 nm), $MnFe_2O_4$ NP (55 nm) and $NiFe_2O_4$ NP (25 nm), which have a very common and widespread use, were investigated by means of alkaline single cell gel electrophoresis (COMET) method in *Drosophila melanogaster* haemocytes. The data obtained from the COMET method were evaluated using two different parameters. In terms of the tail length parameter, it was determined that DNA damage was induced statistically significantly at 3, 5 and 10 mM concentrations of $MnFe_2O_4$ NPs, and at all application concentrations of Fe_3O_4 NPs, $CoFe_2O_4$ NPs and $NiFe_2O_4$ NPs. When the data obtained from the tail density (%DNA) parameter was examined, it was determined that genotoxicity was induced in a statistically significant manner at the highest concentration of Fe_3O_4 NPs (10 mM) and the two highest concentrations of $NiFe_2O_4$ NPs (5 and 10 mM). When the data obtained as a result of the study were evaluated, it was revealed that the iron-based MNPs studied within the scope of our research may have genotoxic potential.

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P13-15

Investigation of the α9-nicotinic receptor single nucleotide polymorphisms induced oncogenic properties and molecular mechanisms in breast cancer

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Background: Our previous research has indicated that α9-nAChR, a subtype of nicotinic acetylcholine receptor, is significantly overexpressed in female breast cancer tumor tissues compared to normal tissues. Furthermore, we have found that specific single nucleotide polymorphisms (SNPs) in the CHRNA9 gene are associated with an increased risk of breast cancer in interaction with smoking.

Methods: The study conducted a breast cancer risk assessment of the α9-nAChR SNP rs10009228 (A>G) in the Taiwanese female population, comprising 308 breast cancer patients and 198 healthy controls. Experimental manipulation of α9-nAChR SNP rs10009228 wild (A/A) and variant (G/G) genotypes was performed to overexpress in breast cancer cells for protein functional analysis and to assess variant effects on pathogenic properties. Additionally, four-line triple-negative breast cancer patient-derived xenograft (TNBC-PDX) models with distinct α9-nAChR rs10009228 SNP genotypes (A/A, A/G, G/G) were established and subsequently exposed to chronic nicotine to investigate the synergistic interactions between nicotine and the high-risk allele in promoting tumor progression.

Results: Our investigation confirmed the presence of a missense variation (A>G) in the α9-nAChR SNP rs10009228, resulting in an alteration of the amino acid sequence from asparagine (N442) to serine (S442) to facilitate phosphorylation within the α9-nAchR protein. Our case-control analysis revealed that individuals with the heterozygous A/G or A/A wild genotype have an increased susceptibility to developing breast cancer in the presence of smoking compared to carriers of the G/G variant genotype. Additionally, overexpression of N442 (A/A) in breast cancer cells significantly enhanced cell survival, migration, invasion, and cancer stemness compared to S442 (G/G). Tumors from TNBC-PDX models exposed to chronic nicotine exhibited accelerated growth, particularly in individuals with the A/G or A/A genotype, while no significant response was observed in tumors with the G/G genotype. Nicotine-exposed tumors with the A/A genotype showed sustained activation of the α9-nAChR downstream oncogenic AKT/ERK/STAT3 pathway, contributing to tumor promotion.

Conclusion: Our research has established a link between genetic variations in α9-nAChR and smoking exposure in breast tumor development, emphasizing the potential impact of targeted therapy. This emphasizes the need to consider genetic and environmental factors in devising effective breast cancer treatment strategies.

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P13-16

Studies on the toxicology of electrolyte additives – mechanisms and changes during cycling

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Lithium-ion batteries (LIBs) facilitate our daily lives by their use in a wide field of application: most commonly known are consumer electronics, (plug-in hybrid) electric vehicles and decentralized energy storage. It can be assumed that the market for LIBs will increase, as digitalization is growing and fossil-free traffic is gaining more and more attention. Thus, a release of battery components into the environment is more likely than ever, and the growing need for recycling of the components poses a risk to the workers in recycling plants. Evaluating the toxicological properties of the different parts of LIBs is therefore essential for the risk assessment for these batteries. An important part of the state-of-the-art LIBs is the liquid organic electrolyte with its additives, which increase the lifetime of the batteries. During the use of a battery, the additives degrade to fulfill their

intended function. But even if the additives themselves cannot be detected in the aged electrolyte, potential toxic effects occurred at all life time stages.

To study these effects, cytotoxicity, mutagenicity, genotoxicity and the formation of DNA-adducts were investigated in different *in vitro* assays: *e. g.* the resazurin reduction assay, the Ames test and the Micronucleus test. The studied additives were 1,3-propanesultone (PS), prop-1-ene-1,3-sultone (PES) and 1,3,2-dioxathiolan-2,2-dioxid (DTD). They were used in pristine electrolyte, electrolyte after three charge/discharge cycles and after 200 cycles; the pure additives and degradation products thereof were tested as well.

It was shown that mutagenic and genotoxic activity of most aged electrolytes, in sub-cytotoxic concentrations, strongly differ from the effects of the pristine ones. If so, the pure electrolyte becomes less toxic during the cycling. However, dependent on the used additives, toxic potency does not necessarily change over different life time stages.

To summarize, even if the pristine additives are no longer detectable in the aged electrolyte, some degradation products exhibit strong mutagenic and genotoxic effectiveness. The knowledge about adverse outcome pathways of electrolytes and their additives have to be taken into account in battery development to minimize potential risks by *e. g.* accidents involving LIBs.

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P13-17
Assessment of the efficiency of the literature search of the ten key characteristics of carcinogens in the *IARC Monographs* evaluation process

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Introduction: The International Agency for Research on Cancer (IARC) *Monographs Programme* evaluates agents that are suspected to cause cancer in humans. The evaluation of cancer hazard, as outlined in the *Monographs* Preamble, integrates three streams of evidence: studies of cancer in humans, studies of cancer in experimental animals, and mechanistic evidence. The ten key characteristics of human carcinogens (KCs) are used to identify, organize, and synthesize mechanistic data from across various test systems. Pre-defined KC-based search terms and agent terms were established and are used to perform systematic literature review.

Purpose: As a follow-up activity from a recent IARC scientific workshop on the furtherance of the KCs, with the aim to assess the efficiency of the literature search of available mechanistic information, we performed a preliminary analysis of the published literature searches conducted using the KCs framework.

Methods: Published systematic literature assessments in the open-source Health Assessment Workspace Collaborative (HAWC) content management for IARC Monographs vol 125–131 were analysed for the KC-related information. A first analysis considered 11 agents having at least >100 retrieved references. A comparative analysis was performed between the overall number of retrieved references and actual number of references assigned for the evaluation and those finally considered in the *Monographs*.

Results: We observed that literature searches for KC2 'is genotoxic' included the highest number of pertinent references with an average of 44% of references assigned among the total number of retrieved references. Around 30% was the average of references assigned among the total number of retrieved references for KC6 "Induces chronic inflammation" and KC7 "Is immunosuppressive". 15–19% were the averages for KC1, KC5, KC8 and KC10. Lower percentages (2%–3%) were observed for KC3, "alters DNA repair or cause genomic instability", and KC4, "induces epigenetic alterations". Few references were usually identified for KC9 "causes immortalization". Approximately 18% of the retrieved references were assigned for the evaluation across the ten KCs.

Conclusion: Since its implementation, the KC framework has become a successful tool to identify, screen, and organize available scientific literature. Mechanistic literature searches published in HAWC show that the KC-related literature searches cover a broad spectrum of literature. Based on the number of retrieved references and those assigned for evaluation, consistent differences are identified across the various KCs-related searches, which indeed can limit the final number of evaluated references. In view also of the constant increase of scientific literature in volume, diversity, and relevance, a refinement of search terms and potentially a triage of pertinent assignments are proposed as solutions for a more efficient literature coverage, especially for some of the KCs.

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P13-18
Succinate Dehydrogenase inhibitors (SDHi) fungicides induce mitochondrial dysfunction and metabolic reprogramming in human colon cells

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Succinate dehydrogenase inhibitors (SDHi) are fungicides used to control the proliferation of pathogenic fungi in cereals, fruits and vegetables. Their mode of action is based on blocking the activity of succinate dehydrogenase (SDH), a mitochondrial enzymatic complex involved in both cellular respiration and Krebs cycle. In humans, SDH inactivation leads to a variety of diseases including encephalopathies and cancers. Genetic loss of SDH in neuroendocrine and renal tumors induces an accumulation of succinate leading to oxidative stress, pseudohypoxic phenotype, metabolic, epigenetic and transcriptomic remodeling, and increased migration and invasion capacities of cancer cells. In colorectal cancer (CRC), SDH downregulation is almost always correlated to

a worse prognosis (increased risk in metastasis and significant decrease in survival rate).

The impact of SDHi fungicides on human health remains largely unexplored to date, despite a growing number of studies reporting toxic effects in non-target organisms, supported by the high degree of conservation of the SDH catalytic site (SDHi binding site) during evolution and the ability of SDHi to inhibit SDH in mitochondria of non-target species, including humans.

Most SDHi induce tumors in animals without evidence of genotoxicity. Thus, for these substances, the mechanisms of carcinogenicity are not clearly established. Our work aimed to generate new knowledge on the carcinogenic potential of bixafen in the colon, which is a major target after dietary exposure. We focused on several mechanisms of toxicity not evaluated by the current regulatory assessment processes, such as mitochondrial functions, metabolism, alterations in morphology and migratory phenotype in non-cancer colon epithelial cells (HCEC-1CT), and transformed cells carrying mutations in driver genes of CRC (HCEC-1CTRPA).

In HCEC-1CT cells, bixafen inhibited SDH complex activity leading to an increase in succinate level, associated with a metabolic switch from respiration to glycolysis. Exposure to bixafen led to a metabolic reprogramming, characterized by changes in the levels of metabolites involved in pentose phosphate pathway, nucleotide synthesis and Krebs cycle. Bixafen induced a slowdown in cell proliferation linked to a strong aspartate dependency. Bixafen-treated cells exhibited morphological changes with cell spreading, epithelial-mesenchymal transition, and enhanced migration and invasion capacities supported by a transcriptomic reprogramming in HCEC-1CT cells. Finally, the effects of bixafen were more marked in HCEC-1CTRPA transformed cells than in HCEC-1CT cells, with stronger changes in cell respiration, glycolysis, and migration/invasion capacities.

Altogether our results show that bixafen-induced chemical inactivation of SDH mimics some metabolic and phenotypic features resulting from genetic SDH deficiency and suggest that CRC patients may represent a population particularly vulnerable to exposure to SDHi.

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P13-19
Development of a thymidylate synthase assay to investigate a genotoxicity risk arising from adverse secondary pharmacology and influence drug design

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Background: Thymidylate synthase (TS) plays a central role in the biosynthesis of thymidylate, an essential precursor for DNA biosynthesis. Disruption of the nucleotide pool has been shown to affect genomic stability by altering the rate of DNA synthesis, and multiple TS compounds have positive genotoxicity findings. Off-target inhibition of TS therefore represents a safety concern for therapeutic indications where genotoxicity findings cannot be tolerated. As regulatory genetic toxicology assessments require large amounts of compound they are unsuitable for the screening of compounds early in the drug discovery pipeline. We therefore sought to develop a TS activity assay that can complement a high content *in vitro* micronucleus screening assay, build an understanding of structure activity relationships (SAR) and ultimately aid the selection of compounds without this adverse secondary pharmacology.Methods: The coding sequence for thymidylate synthase was codon optimised for E. coli and synthesised in house. Protein production was caried out with autoinduction at 18°C overnight. The recombinant protein was then purified from lysed cells with IMAC and size exclusion chromatography. Reactions consisted of 10ug/ml enzyme and 100μM of each of the substrates 5,10-methylenetetrahydrofolate and 2-deoxyuridine 5-monophosphate in 50mM Tris buffer with 150mM NaCl. Absorbance at 340 nm was monitored over 20 minutes and percentage activity versus DMSO control was calculated at 5 minutes. For the genetic toxicology screen, A549 cells were incubated with 1mM to 1nM compound. After 24h treatment, compound was removed and cells were incubated for a further 24 hours. Fixed cells were stained with anti-centromere antibodies, anti-γ.H2AX, CellMask and Hoescht. Images were acquired at 20x and analysed in Columbus™.

Results: To confirm the enzyme was active and could be inhibited by known TS inhibitors we ran a dose-response with 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP; the active metabolite of 5-fluorouracil) which resulted in an IC50 of 4.5μM. Ten project compounds which had been run in the genetic toxicology screen were then tested in the TS assay at 10μM. All compounds which were negative in the cell-based screen showed no TS inhibition. 2/3 genetic toxicology -screen positive compounds were identified as TS inhibitors and 1 out of 2 borderline compounds were identified, highlighting that a wider dose range may be required or that mechanisms other than TS inhibition can be responsible for genotoxicity. The TS assay was then extended to a larger shortlist of compounds to explore SAR. Together with computational modelling of the enzyme active site it was possible to identify key regions of the molecule which were responsible for the TS inhibition and move away from this for future design rounds.

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P13-20

Exposure to mixtures of fungicides promotes hallmarks of cancer in human colon cancer cells

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In the current context of global warming, fungicides are massively used in most of crops to prevent or limit the proliferation of pathogenic fungi. SDHi fungicides target the succinate dehydrogenase (SDH), a key mitochondrial enzymatic complex involved in both mitochondrial respiratory chain (MRC) and Krebs cycle. SDH complex is highly conserved throughout evolution, in particular at the catalytic site (which is also the SDHi binding site). It is well-known that genetic-related SDH deficiencies in humans lead to serious clinical consequences, including neurological pathologies and cancers. In tumours, genetic SDH inactivation induces oxidative stress and increased levels of succinate, an oncometabolite involved in the development of a pseudohypoxic phenotype, metabolic and epigenetic reprogramming and enhanced migratory properties of cancer cells.

We recently showed that SDHi fungicides inhibit SDH activity in mitochondria of non-target organisms including humans. We observed that chemical SDH inactivation by SDHi is able to mimic some features of genetic SDH impairment, notably by raising succinate level, increasing mitochondrial reactive oxygen species (ROS) and inducing a metabolic reprogramming in human colon cells (see Duarte-Hospital's abstract).

In marketed formulations of fungicides, SDHi are often combined with strobilurins (STR) that also target mitochondria by inhibiting the complex III of the MRC. We hypothesized that an exposure to such mixtures could increase the adverse effects observed with a single fungicide. Thus, we explored the toxicity of a mixture containing a SDHi (fluxapyroxad or boscalid) and a strobilurin (pyraclostrobin) in HCT 116 human colon cancer cells. We showed that, even at non-cytotoxic doses, an exposure to the mixtures strongly alters mitochondrial function with a metabolic switch from oxidative phosphorylation towards glycolysis and an increase in mitochondrial ROS. Colon cancer cells exposed to the mixtures SDHi/STR also exhibited morphological changes, characterized by cell spreading and actin network reorganization that evokes the implementation of an epithelial-mesenchymal transition, the first step of tumour metastasis.

In conclusion, our preliminary results suggest that an exposure to a mixture SDHi/STR might lead to a reinforcement of the Warburg effect and to morphological changes that promote migration or invasion, both well-known hallmarks of cancer progression. Further studies are in progress to better characterize the cellular and molecular mechanisms involved in the toxic effects of SDHi/STR mixtures on other hallmarks of cancer cells.

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P13-21

Toxicological assessment of porous silica nanoparticles: cytotoxicity, genotoxicity and immunogenicity

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Porous silica nanoparticles (PSNs) hold immense promise as drug delivery carriers owing to their high surface area, accessible silanol groups, customisable pore and particle sizes, and facile surface modification capabilities. However, before their clinical translation, a thorough assessment of PSN toxicity is imperative to ensure their biocompatibility. This study undertook a comprehensive evaluation of various toxicological endpoints, encompassing cytotoxicity, genotoxicity, and immunogenicity, using three cell lines representing potential exposure routes of PSNs. Physicochemical characterisation of the PSNs was conducted employing dynamic light scattering, zeta potential, x-ray diffraction, electron microscopy and fourier transform infrared spectroscopy. Lymphoblastoid TK6, monocytic THP-1, and liver cancer HepG2 cells were exposed to five functionalised PSNs, polyethylenimine (PEI), amine, carboxyl, thiol or silanol at varying concentrations (0 to 200mg/ml) for 24, 48, or 72 hours. Cytotoxicity assessment via the MTT assay revealed varying toxicity among PSN types and cell lines. Notably, PSN-PEI showed significant toxicity towards THP-1 cells compared to TK6 cells. PSN-Carboxyl exhibited toxicity in HepG2 cells, whereas PSN-Thiol demonstrated minimal cytotoxicity across all cell types, suggesting differential cellular responses. Genotoxicity analysis using the cytokinesis block micronucleus assay identified an increased micronuclei frequency induced by PSN-PEI and PSN-Carboxyl in TK6 and THP-1 cells, underscoring the significance of DNA damage evaluation. The correlation between toxicity and cellular uptake was evident in our study, particularly observed in THP-1 cells. Employing encapsulation techniques, we affixed a fluorescence tag to the PSNs, effectively reducing particle size and facilitating their entry into the cells. This enabled the correlation between toxicity and uptake, shedding light on the relationship between cellular internalisation and the potential adverse effects associated with nanoparticle exposure. Immunogenicity studies using western blotting revealed interactions between PSNs and C3b protein. PSN-Silanol showed binding to multiple complement proteins C1q, C3 and MBL, thereby suggesting activation of all three complement pathways – classical, alternative and lectin, respectively. Complement activation could generate anaphylatoxins and PSN opsonisation, affecting the therapeutic effect of PSNs. This study provides crucial insights into PSN toxicity and correlates with cellular uptake studies, ensuring the use of safe and effective nanocarriers for drug delivery. Future research will investigate intracellular signalling events and protein corona associated with toxicity to better understand the impact of PSNs, thus supporting safe nanocarriers.

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P13-22

Genotoxic effects of gliotoxin, ochratoxin A and their combination on neuroblastoma undifferentiated SH-SY5Y cells

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Gliotoxin (GTX) and ochratoxin A (OTA) are naturally produced toxins by fungi and are known for their potential health risks. With the aim of shed some light on the mechanisms by which GTX, OTA, and their combination exert toxicity at neuronal level, the following *in vitro* studies were conducted in SH-SY5Y cells: a) Evaluation of the cytotoxicity using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method, b) study of the genotoxic potential through the *in vitro* micronucleus assay following OECD TG 487 guidelines (OECD, 2016); c) study of the expression of pro-apoptotic genes *Bcl2, Casp-3*, and *Bax* by RT-qPCR, d) intracellular ROS monitorization by the H2-DCFDA rehearsal. GTX and OTA reached IC_{50} values of 1.25 µM and 8.25 µM after 48h and 24 h respectively. Micronucleus formation increased a 30% compared to control when exposed to GTX at 0.4 µM, 43% for OTA at 0.8 µM, with the highest increase observed when cells were exposed to the combination [GTX+OTA] at [0.2+0.4] µM, obtaining a 55% more micronucleus formation. Regarding gene expression, increases in the expression of *Bax* and *Casp-3* genes of 1.3 and 3- folds respectively were observed when cells were exposed to GTX at 0.75

μM, with a more prominent increase after exposure to the combination of both mycotoxins [GTX+OTA] at [0.2+0.1], increasing both 3 and 5-folds more when compared to the control. ROS production was more pronounced when using as treatment OTA rather than GTX in SH-SY5Y cells. Based on the results obtained, we can conclude that for the proposed scenarios of exposure to GTX, OTA, and their combination, genotoxic effects together with oxidative effects at neuronal level in SH-SY5Y cell line, were found to play a key role in their mechanisms of action.

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P13-23
A comparative analysis of the ability of various S9 compositions to metabolically activate N-nitrosamines to mutagens in the bacterial reverse mutation test

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This presentation will concentrate on testing small N-nitrosamines for mutagenicity. The experiments involved different protocols (plate incorporation and pre-incubation methods), solvents (DMSO/water), and S9 conditions to activate the nitrosamines. The various S9 conditions included varying amounts of S9 fraction from hamster and rat, either non induced or induced with Aroclor 1254 or Phenobarbital/beta-Naphthoflavone (PB/NF). Further investigations focused on comparing hamster with rat S9, using non induced hamster S9 as well as PB/NF induced S9 from rat and hamster, respectively.

The results obtained showed that the bacterial reverse mutation assay, applying plate incorporation or pre-incubation protocols, is suitable for predicting the mutagenicity of N-nitrosamines in the presence of PB/NF induced rat liver S9.

The investigation demonstrated that the strains outlined in the OECD 471 guideline are sufficient to record the mutagenic response. TA100 and TA1535 are capable of detecting the activity. Additionally, the results clearly demonstrate that E. coli WP2 uvrA is highly sensitive in detecting the mutagenic activity and should therefore be combined with TA1535 and TA100 for appropriate testing.

Whether the induction of liver enzymes is mandatory for the activation of nitrosamines was challenged by demonstrating that non induced hamster S9 performs equally well as induced hamster S9, casting doubt on the requirement of pretreating animals with enzyme inducers.

Regarding the optimal S9 content in the S9 mix used for activation, the investigation demonstrated that increasing S9 content from 10 to 30% leads to cytotoxicity. Thus, the sensitivity of the Ames test is not improved with increasing S9 content. Therefore, limiting the S9 content to 10% provides reliable results on the potential mutagenicity of the nitrosamines investigated. These results indicate that a smaller number of animals may be necessary for S9 production, aligning with the 3R principles (reduce, refine, replace) for animal testing.

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P13-24
Exploring oxidative stress-mediated micronucleus induction by Acrylamide, Penitrem A and 3-acetyldeoxynivalenol in SH-SY5Y cells

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Oxidative stress induced by food contaminants mixtures poses significant genotoxic risks, particularly in neurological systems. This study investigates the genotoxic effects of acrylamide (AA), Penitrem A (PEN A), and 3-acetyldeoxynivalenol (3-ADON) individually and in combination on SH-SY5Y cells, a neuroblastoma cell line. Acrylamide (AA) is a chemical compound that forms in certain foods during high-temperature cooking processes [1], whereas Penitrem A (PEN A) and 3-acetyldeoxynivalenol (3-ADON) are toxic mycotoxins commonly found in contaminated foods and animal feed [2–5]. Micronucleus assays were conducted after exposure to specified concentrations of t AA at 2500 μM, 1750 μM and 650 μM, PEN A at 10 μM, 5μM and 2,5 μM and 3-ADON at 4 μM, 2 μM and 1 μM individually, as well as in combination at the specified concentrations, evaluating through cytometry the frequency of micronuclei as a sensitive marker of genotoxicity [6]. The levels of ROS were assessed using the DCFH-DA assay and the LPO on SH-SY5Y cells was determined by the TBARS method in the presence of different concentrations of AA (5000 μM, 2500 μM, and 1250 μM), PEN A (20 μM,10 μM and 5 μM), and 3-ADON (4 μM, 2μM and 1 μM), both individually and in combinations [7]. Results show a notable increase in micronuclei frequency in cells exposed to AA+PEN A+3-ADON compared to individual exposures, indicating genotoxicity. The assessment of reactive oxygen species (ROS) production revealed elevated levels, particularly in combination exposures, implicating ROS-mediated damage in micronucleus induction. Furthermore, oxidative stress-induced lipid peroxidation (LPO) was observed in cells exposed to AA, PEN A, and 3-ADON individually and in combinations, suggesting a potential mechanism underlying micronucleus formation. These findings emphasize the importance of considering interactions between chemicals in complex mixtures when assessing genotoxicity and underscore the role of ROS and LPO-mediated oxidative stress in neurotoxicity. Further research is warranted to elucidate underlying mechanisms and expand assessments to other cell types and exposure scenarios, contributing valuable insights for risk assessment and regulatory measures.

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P13-25
Evaluating the effects of food additive E171 on colorectal cancer risk: a human dietary intervention study

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Background: The widespread use of the food additive E171 (titanium dioxide, TiO_2) as a white colorant, particularly in products like sweets and chewing gum, raises public health concerns due to its potential link to colorectal cancer (CRC) and reduced colorectal health. E171 has been shown to induce genotoxic effects *in vitro* and enhance tumor formation in animal models for colitis. Other findings suggest that E171 might be involved in the induction of inflammation in the colon.

Objective: This human dietary intervention study primarily investigates the impact of E171 on whole genome gene expression profiles in rectal biopsies from healthy volunteers, fecal microbiome changes, and effects on systemic and local oxidative stress and inflammatory markers from plasma, blood, and biopsies. Our study aims to bridge the gap between the results obtained by *in vitro* and animal models used for human health risk assessment and the human situation.

Study Design: We employed a randomized crossover design; this four-week study included healthy subjects randomly assigned to intervention groups. The study protocol entailed a daily regimen wherein participants consumed yogurt containing E171 at a relevant human oral intake of 2.0 mg/kg_{bw}/day for 14 days. Subsequently, biopsies, blood, and fecal samples were gathered for analysis. In the study's control phase, participants consumed yogurt without E171. Adherence to the diet was monitored and confirmed via daily dietary assessments.

Study Population: We recruited 31 healthy volunteers who completed the intervention between May 2022 and February 2024 in the Limburg Region, The Netherlands. The participants were, on average, 31.2 (±14.2) years old and had a Body Mass Index (BMI) of 23.1 (±2.0).

Results: We determined the effect of oral intake of E171 on systemic markers for oxidative stress (TEAC, PCCs, TBARS, and superoxide levels) and inflammation (hs-CRP, SAAs, ILs). Whole genome gene expression analysis using Next Generation Sequencing revealed differentially expressed genes, comparing paired measurements before and after the intervention in samples from the same individuals. These DEGs were used for pathway analysis and constructing a gene interaction network, demonstrating the key molecular processes involved in the response. The comparison between these findings in humans and the previous *in vitro* and animal data shows the relevance of these models in evaluating potential risks associated with dietary intake of E171.

Conclusion: This study presents the first *in vivo* results of the effects of E171 on humans through comprehensive genomic, oxidative stress, and inflammatory marker analysis. Our findings illuminate the interactions of dietary E171 exposure on human colon health and its potential role in CRC risk. These insights are pivotal for informing public health policies and guiding regulatory actions concerning E171.

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P13-26
Unveiling the therapeutic potential of cannabinoids in ER$^+$ breast cancer: cytotoxicity and endocrine activity profile

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Introduction: The therapeutic potential of cannabinoids (CBs) has been explored for various diseases and clinical conditions, including cancer [1]. Regarding breast cancer (BC), research has been mainly focused on triple negative and HER2$^+$ tumors [2]. Recently, our group showed that Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) present important anti-tumor effects in estrogen receptor-positive (ER$^+$) BC [3], the most diagnosed BC subtype. Moreover, we verified that CBD could improve exemestane efficacy in BC cells, which may represent a major progress in ER$^+$ BC treatment and in the management of endocrine resistance [4]. However, THC and CBD are just two of more than 120 phytocannabinoids found in the *Cannabis* plant, for which therapeutic properties are yet to be explored. Here, we evaluated the cytotoxic effects and the behavior as ER and androgen receptor (AR) modulators of 10 CBs in non-cancer and cancer models.

Methods: The CBs studied were cannabidiol (CBD), cannabigerol (CBG), cannabidivarin (CBDV), cannabinol (CBN), cannabidiol-C4 (CBDB), cannabidiolic acid (CBDA), cannabidiol monomethyl ether (CBDM), cannabichromenic acid (CBCA), cannabigerovarinic acid (CBGVA) and cannabichromene (CBC). Cytotoxic properties were assessed through MTT/LDH assays in non-tumor cells, human normal mammary epithelial cells (MCF-10A) and human foreskin fibroblasts (HFF-1), and in human ER$^+$ BC cells (MCF-7aro). The binding of CBs to aromatase, ER and AR was initially assessed *in silico*, followed by transactivation assays used to profile their effects on human ER and AR. Moreover, aromatase inhibition was assessed in a radiometric assay using human placental microsomes.

Results: Results demonstrated that all CBs studied, except CBG and CBDM, decreased MCF-aro cell viability, with LDH release detected for CBDB and CBGVA. Moreover, CBD, CBG, CBDV, CBN and CBC are safe for non-cancer cells. Regarding endocrine modulation, most of the *in silico* predictions were confirmed *in vitro*. CBD was the only CB able to inhibit aromatase though all CBs acted as AR antagonists with inverse agonist properties. Additionally, CBDV, CBDB and CBDM displayed agonist activity in ER, while CBDA, CBCA and CBC acted as inverse agonists. CBD and CBN acted as ER antagonists with inverse agonist properties.

Conclusions: Despite the increasing number of studies regarding the benefits of CBs for the treatment of different cancers, information on ER$^+$ BC is still scarce. Here we showed that despite the harmful effects of some CBs in non-tumor cells, they are able to disrupt endocrine signaling, which might be valuable for improving treatment of ER$^+$ BC cases. Thus, this work presents, for the first time, a comprehensive

study regarding CBs and ER+ BC, reinforcing the anti-tumor actions attributed to them and paving the way for novel therapeutic approaches.

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P13-27
Toxicological evaluation of verbascoside from the viewpoint of genotoxicity

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Verbascoside is phenylethanoid glycoside type plant phenolic compound which is predominantly found in some medicinal plants belonging to Verbenaceae, Oleaceae, Buddlejaceae, Lamiaceae, and Scrophulariaceae families [1]. Research has established that verbascoside exhibits a wide array of biological activities, including anti-inflammatory [2–3], anti-ulcerogenic [4], antispasmodic [5], antioxidant [6], antimicrobial [7], and analgesic effects [8]. While many studies have investigated these activities, not enough research has been done on its effects on DNA and whether it can cause or prevent mutations. This study aims to address this gap by investigating the mutagenic/antimutagenic and genotoxic/genoprotective potential of verbascoside.

Verbascoside used in this study was previously purified from *Globularia sintenisii*, and its structure elucidation was performed by NMR[9]. The Ames Assay, using the standard plate incorporation method, was performed to evaluate its mutagenicity/antimutagenicity following OECD test guideline 471. *Salmonella typhimurium* tester strains TA98 and TA100 were used to determine frame shift and base pair mutations, with and without a metabolic activation system. Micronucleus and comet assays were performed in the CHO cell line to evaluate the genotoxic/genoprotective potential of verbascoside.

Results revealed that verbascoside does not induce mutagenicity in both TA98 and TA100 strains with or without metabolic activation. Also, it did not cause genotoxicity in CHO cells in both micronucleus and comet analyses.

On the other hand, no significant decrease was observed against direct and indirect mutagens, indicating the lack of antimutagenic activity in the tested strains. Co-treatment of doxorubicine as a well-known genotoxic compound with verbascoside led to a decrease of the doxorubicine-induced micronuclei, which ranged between 21 and 37% in both micronucleus and comet assays. However these effect were not found dose dependent and statistically significant.

The findings of the present study provide scientific basis to the safety of verbascoside from the viewpoint of genotoxicity risk, and in fact, it was found to be beneficial against genotoxicity.

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P13-28
Lifetime carcinogenicity studies in Han Wistar and Sprague-Dawley rats: historical data for survival, neoplasms and causes of premature death

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Available survival and tumor incidence historical data are useful for interpreting lifetime carcinogenicity bioassays. These data were compiled for control Sprague-Dawley (SD) and Han Wistar (HW) rats in eight 104-week carcinogenicity studies between 2006 and 2018 at CRL Evreux. The breeder was CRL France or Italy.

Mean survival rate of male and female SD rats was similar (46% and 42%, respectively), while male and female HW rats survived longer (75% and 64%, respectively).

The most common causes of unscheduled death were tumors (mostly pituitary gland), non-evident cause, forelimb/hindlimb inflammation, skin ulcer/abscess or urogenital inflammation in SD males, tumors (mostly pituitary/mammary glands), non-evident cause or various non-neoplastic lesions in eyes/liver in SD females, tumors (mostly skin), non-evident cause or urogenital inflammation in HW males and tumors (pituitary gland and reproductive tract), non-evident cause, urogenital inflammation or skin ulcer in HW females.

The most common neoplasms originated from tumors in pituitary gland (adenoma; >46%), thyroid gland (C-cell adenoma; >12%) and adrenal gland (benign pheochromocytoma; 8%) in SD males, pituitary gland (adenoma; >72%), mammary gland (fibroadenoma; 41% and adenocarcinoma; 33%), vagina (benign granular cell tumor; >9%) and uterus (endometrial stromal polyp; 8%) in SD females, pituitary gland (adenoma; >23%), mesenteric lymph node (hemangioma; >9%) and thyroid gland (C-cell adenoma; >9%) in HW males and pituitary gland

(adenoma; 60%), mammary gland (fibroadenoma; 21%), uterus (endometrial stromal polyp; >15%) and thyroid gland (C-cell adenoma; >7%) in HW females.

The incidences of the principal tumors were compared with published data. There were no relevant differences.

These data confirmed the lower survival rate of male and female SD rats when compared to HW rats.

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P13-29

A new mutagenicity NAM: An *in vitro* Transgenic Rodent (TGR) assay for the detection of mutagens and assessment of their mechanism of action

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Mutagenicity is a critical endpoint in the hazard assessment of industrial chemicals, biocides and pesticides. The induction of gene mutation by test substances can be assessed *in vivo* by the transgenic rodent (TGR) assay which has been adopted by the OECD (test guideline no. 488). This assay uses the bacterial lacZ gene as a reporter gene to easily and reliably detect mutations. Multiple copies of this gene are integrated in the mouse chromosome. There is currently no corresponding OECD method *in vitro*. We adapted an *in vitro* mutagenicity assay described by Cox *et al.* (2019) based on the protocol used for the *in vivo* TGR assay (OECD 488). In this *in vitro* version, termed "*in vitro* TGR assay", primary hepatocytes (PHs) isolated from transgenic mice (Muta™Mouse) are treated with the test substances rather than the living animal. Due to the corresponding *in vivo* and *in vitro* target, this approach is aimed to predict the *in vivo* outcome better than other *in vitro* mutagenicity assays. PHs were treated for six hours with N-ethyl-N-nitrosourea, Benzo[*a*]pyrene, Ethyl methanesulfonate, Mitomycin C, Azathioprine, Urea, Erythromycin, Sulfisoxazole, Benzyl alcohol and Diclofenac sodium. Isolated DNA from treated PHs were packaged in λ phages. Mutations of the lacZ gene were quantified by infection of *E. coli* C lacZ-galE- cultures and co-treatment with Phenyl-β-D-galactopyranoside (P-Gal). LacZ mutant frequency (MF) was evaluated by referring the number of phages containing lacZ mutations (selective conditions (with P-Gal)) to the total number of phages (non-selective conditions (w/o P-Gal)). In addition, the induced mutations in the lacZ reporter gene were characterized using microchip electrophoresis and the Illumina next-generation sequencing MiniSeq™ platform for *the in vivo* mutagens.

For the compounds N-ethyl-N-nitrosourea, Benzo[*a*]pyrene, Ethyl methanesulfonate, Mitomycin C, Azathioprine a concentration dependent increase in lacZ MF was detected. The mutations identified for the respective test substances matched the mutagenic mechanisms described in literature. Urea, Erythromycin, Sulfisoxazole, Benzyl alcohol and Diclofenac sodium did not induce mutations. In conclusion, the "*in vitro* TGR" based on primary MutaMouse hepatocytes is a promising New Approach Methodology (NAM) which not only reflects many aspects of the *in vivo* TGR system *in vitro* but also allows for mutation spectra analysis to further evaluate induced mutations.

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P13-30

Applying data-driven analysis to provide mechanistic insights into natural toxins hepatocarcinogenicity: the use case of microcystins

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Microcystins (MCs) are a group of cyclic peptide toxins produced by cyanobacteria well recognized as food and water contaminants and nowadays pose concerns as potential metabolic by-products of certain types of biological pesticides. There are approximately 286 MCs already characterized, yet less than 10% have their toxicological profiles determined. Among these, the MC-leucine arginine (MC-LR) is the most well-known, and it is classified as possibly carcinogenic to humans (Group 2B, IARC), based on liver preneoplastic lesions in animals. To gain mechanistic insight into the relationship of the MCs and the hepatocellular carcinoma (HCC) and gain mechanistic insights into the hepatocarcinogenicity of MCs, we use a data-driven approach, specifically, a Network Medicine approach based on human disease databases. We consider only experimentally validated protein-protein interactions and non-coding RNA-mediated interactions (PPI & NCI) as our underlying network. We mapped from several gene-disease association curated database 89 genes associated with human hepatocytes also expressed in hepatocytes; Disease module's Largestest Connected Component (LCC), its relative size (rLCC) and significance were determined by the NetSci R package. We identified a significant HCC disease module in hepatocytes (LCC 68; rLCC 65%; $p<0.001$). Similarly, we identified four MCs statistically significant disease modules (MC-LR, MC-LF, MC-RR, and unspecific MC). We found a genetic overlap across the different MC types, but no network topological overlap (network separation) was found. We could identify a significant proximity for MCs and HCC related genes, indicating potential toxic effect. Gene Ontology (GO) enrichment analysis revealed shared processes between HCC and MCs, such as response to IL-1 and IL-6, ERK1 and ERK2 cascades, and negative regulation of cell differentiation, indicating potential pathway overlap. Finally, to pinpoint potencial genes mediating the MC-HCC process, we selected genes in the shortest path analysis, identifying 105 genes as possibly associated with the liver disease progression, which can be used as potential molecular targets. Also, the targets GO enrichment showed genes-biological process associations typically reported in literature as dysregulated in HCC. This data-driven approach can help to explore mechanisms related to MCs hepatocarcinogenicity, compare similarities among biological responses from MCs exposure, and guide the development of *in vitro* test strategies to close data gaps on MCs carcinogenicity.

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P13-31

Characterization of human isogenic epithelial cell lines as a relevant tool to study colon carcinogenesis and interaction between genes and environment

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Colorectal cancer (CRC) is the fourth most common cause of death from cancer worldwide. CRC is a multistep and progressive disease where genetic factors are important in the initiation, the development and the progression of the disease. CRC can arise from sequential steps including the acquisition of mutations in the adenomatous polyposis coli (APC), followed by the mutational activation of oncogene KRAS and the inactivation of the tumor suppressor gene, TP53. The occurrence of CRC is largely influenced by the environment, including food contaminants, lifestyle and nutrition. However, the influence of mutations on the response to environmental pollutants is poorly evaluated. Environmental carcinogenesis lacks robust models to explore the interaction between genes and environment and to determine whether genetic mutations associated with colon carcinogenesis generate a particular susceptibility to the harmful effects of pollutants.

Our aim was to characterize an innovative cell model consisting of 6 isogenic human colon epithelial cell lines carrying mutations in key driver genes involved in CRC progression and metastasis. Altogether, these cell lines recapitulate colon carcinogenesis from the healthy, preneoplastic, adenoma and carcinoma stages in a simplified way. We showed that all the cell lines express a battery of detoxification enzymes. They exhibit differences in cell and mitochondrial morphology, in proliferation and migration capacities, and in clonogenicity. They also display a flexible energy metabolism, and differences in sensitivity to genotoxic stress, to mitotoxic stress and to cell death stimuli.In conclusion, this *in vitro* model of colon carcinogenesis may be a powerful and relevant tool to study the effects of environmental pollutants on the colorectal carcinogenesis from the early to the late and metastatic stages, and to evaluate gene-environment interactions in food toxicology.

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P13-32
Bafilomycin induces mitochondrial reorganization, nuclear deformation and γH2AX increase in T24 bladder cancer cells

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Autophagy competence is essential for bladder cells, which are continuously exposed to urinary occurring xenobiotics. Bafilomycin A1 (BAFI), a commonly used autophagy inhibitor, has been previously described to have a significant effect on the mitochondrial morphology and function in T24 bladder cancer cells [1]. Yet, mitochondrial toxicity can have downstream effects on multiple cascades [2]. Untargeted proteomic profiling of T24 cells (24h, 10nM BAFI), returned multiple regulatory events, including a consistent downregulation of several NADH ubiquinone oxidoreductases. According to bioinformatics analysis this signature could be traced back to the regulation of the KEGG pathway "Chemical carcinogenesis – reactive oxygen species" [3,4]. Starting from these data, a potential role for BAFI in the regulation of genotoxic damage in bladder cells was hypothesized. To recreate exposure scenarios in the bladder and to limit possible confounding factors related to long-term autophagy inhibition, experiments were performed privileging short incubation times (4h, 0.1–1–10nM BAFI). Live cell imaging experiments revealed a concentration-dependent rearrangement of the mitochondrial network towards the nucleus (1–10nM BAFI). These results aligned with the proteome signature (24h) showing increased perinuclear mitochondrial protein enrichment. Stemming from the mitochondrial recruitment in proximity of the DNA, a potential involvement of reactive oxygen species (ROS) was verified. DCF-assay revealed no significant increase in ROS production related to the mitochondria rearrangement. In order to assess a potential DNA damage, immunofluorescence analysis of γH2AX signal was performed. In this case, BAFI (10nM, 4h) significantly increased the detection of the biomarker for DNA double-strand breaks. This signature was consistent even in presence of the antioxidant enzyme catalase (100nM), further supporting the view that DNA damage could occur even without direct involvement of oxidative stress. Looking for molecular mechanisms of action potentially sustaining these effects, significant deformation of the nuclear morphology was observed, suggesting a possible involvement of mechanical stress as trigger of the genotoxic damage [5]. In conclusion, the data collected in this study suggest the presence of pathways of relevance for carcinogenesis related to mitochondrial rearrangement and potentially complementary to ROS insults.

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P13-33
Polymer-coated magnetic iron oxide nanoparticles: *in vitro* assessment of cytotoxic and genotoxic effects

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The potential toxic effects of magnetic nanoparticles (NPs) on the environment and human health are of concern despite the advantages of these NPs in medical applications. Magnetic iron oxide (Fe_3O_4) NPs are increasingly being produced and used for medical diagnosis and treatment. In particular, humans are exposed to these NPs via the bloodstream in hyperthermia, MRI imaging and targeted drug delivery, resulting in contact of human blood vessels with these NPs. Therefore,

it is important to elucidate the possible adverse effects of NPs, especially on endothelial cells lining the lumen of human blood vessels. In this context, this study aimed to investigate the possible cytotoxic and genotoxic effects of uncoated magnetic iron oxide (Fe_3O_4) NPs and 3 different synthetic polymers (polyethylene glycol (PEG), polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP)) coated forms of Fe_3O_4 NPs on HUVEC cells *in vitro*.

Results obtained from the WST-1 assay revealed a statistically significant decrease in cell viability with increasing concentration (10, 50, 100 and 500 µg/ml) as the incubation time increased (24, 48 and 72h). In PVA-Fe_3O_4 NPs, statistically significant decreases in cell viability were observed at high concentrations (100 and 500 µg/ml) after 24 and 72 hours of incubation, whereas no statistically significant decrease in cell viability was observed in PEG-Fe_3O_4 NPs and PVP-Fe_3O_4 NPs at all incubation periods and concentrations. According to the comet assay results, uncoated Fe_3O_4 NPs and PEG-Fe_3O_4 NPs induced statistically significant%DNA damage at high concentrations (500 µg/ml), while PVA-Fe_3O_4 NPs did not induce significant%DNA damage, and%DNA damage concentration-dependent increased in PVP-Fe_3O_4 NPs. In the measurement of caspase activities, a statistically significant increase was observed in the activities of all caspase groups (3/7, 8 and 9) at 10 and 100 µg/ml concentrations of PVA-Fe_3O_4 NPs and caspase-8 activity at 10 µg/ml concentration of PEG-Fe_3O_4 NPs. In all groups, caspase-3/7, 8 and 9 activities decreased with increasing concentration.

The results show that the cytotoxic and genotoxic effects of Fe_3O_4 NPs may vary depending on their surface coatings, with PEG-coated NPs showing less adverse effects compared to uncoated or PVA and PVP-coated NPs.

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P13-35
Investigation of the effects of genetic differences on levothyroxine efficacy and toxicity in pediatric patients with congenital hypothyroidism due to dysgenesis

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Thyroid hormones regulate the genes that involved in the differentiation process in many tissues. Dysgenesis is the most prevalent endocrinological problem in the neonatal period and leads to irreversible mental retardation proportionally with delay in diagnosis and treatment. Newborn screening for congenital hypothyroidism is very important, but it is a comprehensive screening program that includes surveillance, diagnosis, treatment, observation and evaluation. Nowadays, the drug that is frequently preferred in the replacement or supportive treatment of hypothyroidism is Levothyroxine (LT4), which has a high affinity for thyroid receptors. When the appropriate dosage cannot be adjusted; adverse effects such as tachycardia, anxiety, fatigue, headache, insomnia, tremor, angina, hair loss may occur. Pharmacological treatments act on and against the background of genetic disposition, with epigenetic annotation resulting from previous experiences. As a result of the studies conducted, inherited variations have been identified in approximately 20 genes that affect about 80 medications and are actionable in the clinic. Current efforts that focus on the processes required to appropriately act on pharmacogenomic variability in the clinic are moving away from discovery and towards implementation of an evidenced-based strategy for improving the use of medications. According to literature knowledge, it has been thought deiodinases (DIO1 and DIO2) and polymorphisms in thyroid hormone receptors (THRA and THSR) are effective in LT4 bioavailability, therefore it leads to dose differences between individuals.

Blood samples were collected from 63 Turkish pediatric patients with congenital hypothyroidism according to the sample number determined by power analysis. DNA was isolated from the blood samples. Genotyping was performed by using real-time PCR for the four genes, including rs225015 (G) for DIO2, rs2235544 (A) for DIO1, rs939348 (A) for THRA, and rs4903957 (A) for TSHR. Of the 63 pediatric patients included in the study, 38 were female and 25 were male. Minor allele frequencies of DIO1 (A), DIO2 (A), THRA (T) and THSR (A) 0.3571, 0.4841, 0.2539 and 0.2619, respectively. Clinical data, including thyroid ultrasound results and patient information, were recorded, and statistical analysis was conducted. These SNP, which have a role in LT4 bioavailability in pediatric patients diagnosed with hypothyroidism related with dysgenesis, were evaluated, to contribute the explanation of genetic background for dose adjustment in pediatric patients and to evaluate the current treatment in terms of pharmacokinetics and pharmacodynamics. Thus, it will be able to enable the development of new diagnosis and treatments methods that will benefit science and all humanity. For more detailed results, new studies should be performed in groups with high participation.

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P13-36
In silico analysis of positive and negative effects of isothiocyanates in breast cancer

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Breast cancer is the most common cancer in women, with a constant trend of increasing incidence, poor prognosis and high death rate. Newer studied show that the application of phytochemicals, such as sulforaphane (SFN) and phenylethyl isothicyanate (PEITC), has a chemoprotective effect, but also that the safety of their application has not been sufficiently tested. Therefore, the aim of this research was to investigate the positive and negative effects of SFN and PEITC on breast cancer using *in silico* methods, i.e., detailed analysis of available toxicogenomic data, with doxorubicin serving as a control molecule. The Gene Expression Omnibus, GEO (https://www.ncbi.nlm.nih.gov/geo/),

a publicly available database, was utilized to procure gene profiles from both malignant and healthy tissues. These profiles were subsequently analyzed using the GEO2R tool (https://www.ncbi.nlm.nih.gov/geo/geo2r/), which identifies genes differentially expressed between tumor and healthy tissue. To assess genes interacting with doxorubicin (as a control), sulforaphane (SFN), and phenethyl isothiocyanate (PEITC), the Comparative Toxicogenomic Database (CTD, http://ctdbase.org/) was used. Interactivenn tool (http://www.interactivenn.net/) helped with obtaining sets of common genes interacting with doxorubicin, SFN, and PEITC, respectively, and which are dysregulated in breast cancer. Toppgene tool (https://toppgene.cchmc.org/) Toppfun function was used to investigate the role of the selected genes in gene ontology processes that could affect cancer progression, while GeneMania (https://genemania.org/) determined genes related to the set of genes and the type of interaction between all of them. A total of 103 genes have altered expression in breast cancer. After analyzing the gene network, it was determined that doxorubicin interacts with 41 genes altered in breast cancer (21 upregulated and 20 downregulated), sulforaphane with 13 genes (6 upregulated and 7 downregulated), and phenylethyl isocyanate with 3 genes (1 upregulated and 2 downregulated). The dominant type of interaction between upregulated and downregulated genes and doxorubicin was co-expression (42.9% and 70.05%), co-expression of upregulated genes and sulforaphane (68.44%), colocalization of downregulated genes and sulforaphane (68.38%), and physical interactions between PEITC and upregulated and downregulated genes (77.64%). Up-regulated genes associated with sulforaphane are involved in the metabolism of amino acids and their derivatives, while down-regulated genes are associated with gluconeogenesis and glycolysis. In a conclusion, doxorubicin and isothiocyanates show a positive chemoprotective effect, however, they also cause negative effects, which affects the safety of application. Doxorubicin exhibits cardiotoxic properties, and sulforaphane can lead to disturbances in glucose metabolism or be associated with metabolic epileptic disorders.

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P13-37
Effects of Zearalenone on the endoplasmic reticulum stress and its relation to the epigenetic mechanisms in HEK-293 cells

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Zearalenone (ZEA) is a non-steroidal estrogenic mycotoxin produced by *Fusarium* species that contaminates cereals and other crops. ZEA has strong estrogenic effects resulting in reproductive disorders. However, little is known about early molecular changes associated with ZEA toxicity. It has been known that the effects of the environmental chemicals on the gene expression may be associated with epigenetic mechanisms such as DNA methylation and histone modifications. It has been investigated the dose-dependent effects of ZEA (0, 1, 10 and 50 μM for 24 h) on epigenetic modifications such as global DNA methylation, global histone modifications (H3K27me3, H3K4me3, H3K9me3 and H3K9ac) and miRNA profile related to cancer pathways using array panels in human embryonic kidney epithelial cells (HEK-293). The effects of ZEA on expression profiles of apoptosis genes (such as *p53*, *Bcl-2*, *Bax*, *Cas3*, *Cas9*) and endoplasmic reticulum (ER) stress related genes (such as *ATF4*, *eLF2*, *GRP78*, *IRE1*, *PERK*, *CHOP*) were also investigated. 10 and 50 μM of ZEA exposure changed the levels of 5-mC% and caused increased H3K27me3, H3K4me3, H3K9me3 levels at ≥ 2-fold and decreased H3K9ac levels at ≤30%, significantly. Also, according to miRNA arrays, it has been observed that ZEA caused alterations on the levels of several miRNA such as let-7b-5p, miR-181a-5p, miR-30c-5p, miR-21-5p (≥ 2-fold change). mRNA levels of *p53*, *Bcl-2*, *Cas3*, *Cas9*, *ATF4*, *IRE1* and *CHOP* were significantly increased, however it has been observed that the expression levels of *Bax*, *eLF2*, *GRP78* and *PERK* changed non-significantly after ZEA exposure for 24 h. Also, ZEA induced apoptosis rate according to Annexin V-FITC/PI test using flow cytometer. Regarding to our results, further investigations are going on the regulations of *Bcl-2*, *p53*, *ATF4*, *CHOP* genes mediated histone modifications by chromatin immunoprecipitation and expression levels of the chromatin modifying enzymes such as *EZH2*, *EHMT2*, *HAT1*, *SIRT1* and *SETD8* in order to give more insight to the role of epigenetic mechanisms in the toxicity of ZEA in HEK-293 cells.

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P13-38
Evaluation of epigenetic alterations as biomarkers in metastatic colorectal cancer patients

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Colorectal cancer (CRC) is one of the most frequent cancers, and the second leading cause of cancer-related mortality worldwide, but early detection is associated with good prognosis. Current screening methods such as colonoscopy are invasive and costly. Therefore, there is a need for non-invasive biomarkers that will facilitate the identification of patients with early recurrence or poor prognosis, and permit earlier diagnosis of the patients with systemic metastases. Recent studies have reported that the presence of microRNAs (miRNAs) in the blood are useful biomarkers for early diagnosis, risk assessment and classification of cancers. Investigation of epigenetic changes in miRNAs is promising in the emergence of highly specific biomarkers due to their ease of obtaining. Regulation of CRC-related miRNAs in mRNAs isolated from peripheral blood samples taken from the non-patient control group and at least 50 volunteer metastatic colorectal cancer (mCRC) patients who applied to the Department of Internal Medicine, Department of Oncology, Istanbul University Cerrahpaşa-Cerrahpaşa Faculty of Medicine, and it was aimed to investigate the effects of these miRNAs on the expression of target genes. According to the data obtained, changes in miR-21, miR-31, miR-143 and miR196a and in the KRAS, HK2, BCL2, PTEN, DNMT3A genes targeted by these miRNAs were observed in colorectal cancer. In light of the results to be obtained from the project; It is thought that miRNAs will contribute to the use of promising non-invasive, prognostic and metastasis predictive biomarkers in mCRC patients.

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P14 | Cardiovascular diseases

P14-01
Age-related effects of AT1 receptor antagonist losartan on cognitive decline in spontaneously hypertensive rats

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Although both hypertension and ageing render the brain vulnerable to cerebrovascular and neurovascular damage, and in particular to cognitive impairment, their relationship is complex and ageing is not a predisposing factor for hypertension. The aim of the present study was to compare the efficacy of the antihypertensive drug losartan in mitigating hypertension-induced cognitive impairment in young adult and middle-aged spontaneously hypertensive rats (SHR).

The expression of amyloid beta (Aβ1-42), CREB and acetylcholinesterase (AchE) activity were examined in the hippocampus, a brain structure closely associated with memory and the frontal cortex (FC). Middle-aged vehicle-treated rats showed poorer performance in hippocampus-dependent memory tasks, the Y-maze test, and the radial arm maze test than their younger counterparts. Supplementation with the AT1 receptor antagonist losartan (10 mg/kg, i.p. for 14 days) corrected age-related memory decline in 14-month-old rats but was ineffective in 3-month-old rats. Changes in memory-related signalling markers were also found in the hippocampus, but not in the frontal cortex between young adult and middle-aged rats. Losartan reversed these memory-related markers to control levels, specifically in middle-aged SHR. Our findings highlight the importance of considering age-associated factors in studying hypertension-induced cognitive impairment and suggest potential therapeutic approaches targeting the renin-angiotensin system for mitigating cognitive decline associated with hypertension.

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P14-02
Contractility-based pharmacological characterization of hiPSC-derived atrial and ventricular cardiomyocytes for preclinical toxicity testing

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Over the past decade, commercial human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) became an important tool for preclinical cardiac risk assessment owing to their human origin and unlimited reproducibility. Mixed cell populations display a commonly used set up with cardiac subtype characteristics of ventricular, atrial and nodal nature. However, diseases such as atrial fibrillation, affecting more than 33 M people worldwide, demonstrate the need for cardiac subtype-specific commercial cell lines.

Here, we compare commercially available ventricular and atrial cell types (Axol Biosciences) regarding their contractile properties using FLEXcyte technology. The cells were seeded on flexible 96-well plates mimicking physiological human heart conditions *in vitro*. General beat shape of both cell types was analysed and compared after six days in culture. Compound-induced effects on contractile properties including beat rate, amplitude and duration were assessed on pre- and post-compound level. Tested compounds include S-Bay, 4-AP, Ivabradine, Vernakalant, Carbachol and Acetylcholine at 5 different concentrations ranging either from 100nM–1μM or 1μM–100μM.

The pre-compound analysis demonstrates cell type specific beat shapes analogue to the respective cardiac action potential, in which ventricular hiPSC-CMs show a calcium influx-related extended plateau phase compared to atrial cells. As an excerpt of the compound analysis, S-Bay K8644 treatment showed an induced concentration-dependent transient increase in beat duration of atrial hiPSC-CMs, whereas ventricular cells showed a physiological increase in beat rate over time. Carbachol treatment produced marked effects on atrial cells, such as increased beat duration alongside a decrease in beat rate over time, but only minimal effects on ventricular cardiomyocytes. 4-AP showed a reduction in beat rate and an increase in beat duration on atrial cells, while ventricular cells showed no change in beat rate and a transient reduction in beat duration.

HiPSC atrial and ventricular cardiomyocytes reproduced the different contractile phenotypes and pharmacological responses of primary cardiomyocyte sub-types suitably. Hence, these cell types provide the starting point to develop more reliable, physiological-relevant research on subtype-specific cardiac diseases.

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P14-03
Addressing cardiotoxicity in environmental chemical regulation: an integrated approach to cardiotoxicity testing

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Cardiovascular diseases are the leading cause of death worldwide, with environmental chemicals contributing significantly to their prevalence. Nevertheless, the consideration of cardiotoxicity in the existing regulatory frameworks for chemicals, biocides and pesticides is currently is inadequate and relies heavily on animal models that may not fully capture the mechanisms relevant to humans.

The EU-H2020 Project ALTERNATIVE (www.alternative-project.eu) aims to rethink regulatory assessment of cardiotoxicity by identifying current regulatory limitations and drafting of an Integrated Approach to Testing and Assessment (IATA) for cardiotoxicity.

Our in-depth analysis of regulations revealed significant shortcomings in the assessment of cardiotoxicity, including the poor predictive power of both animal-based and conventional *in vitro* methods and the

overlooked impact on more susceptible populations, such as the elderly. In order to address this, we developed an IATA that aligns with the Next Generation Risk Assessment (NGRA) principles, employing a tiered strategy that evolves from broad, initial non-testing methods to more detailed evaluations focusing on specific cardiotoxic mechanisms. To create a new basis for cardiotoxicity testing, a comprehensive Adverse Outcome Pathway (AOP) network has been established. The network covers a broad spectrum of cardiotoxic mechanisms triggered by environmental pollutants and utilizes findings from epidemiological, *in vivo*, and *in vitro* studies.

The project developed various non-animal methods and data to support the AOP-informed IATA: Multi omics analysis, biomarkers of effect, a variety of *in silico* models (e.g., QSAR, QIVIVE, PBPK), and a 3D microphysiological model based on human induced pluripotent stem cell (*hiPSC*)-derived cardiomyocytes. This 3D model includes a second variant representing the cardiac tissue of the elderly, thereby addressing the neglected impact on this vulnerable population in current regulations.

Furthermore, we lay out the potential utilization of other existing NAMs in our comprehensive IATA. An extensive review of various databases identified 34 suitable methods targeting 14 distinct Key Events (KEs) within the cardiotoxicity AOP network. Notably, while methods assessing electrophysiological endpoints are well established, sufficient evaluation of contractile and structural cardiotoxicity endpoints requires further development and validation of new methods. Finally, we identified limitations and uncertainties within the established AOP-informed cardiotoxicity IATA.

By shifting away from traditional animal models, this innovative approach promises to enhance cardiovascular safety by providing a more accurate, ethical, and human-relevant framework for assessing the cardiotoxicity of environmental chemicals.

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P14-04
The impact of short telomere length on cardiac disease

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Purpose: Telomeres are short, repetitive hexanucleotide DNA sequences at eukaryotic chromosomes' ends. They progressively shorten with each mitotic cycle, and telomerase counteracts the process by extending telomeres. Indeed, an inverse association between telomeres and cardiovascular disease (CVD) has been highlighted. In addition, classical cardiovascular risk factors, such as aging, hypertension, diabetes, obesity and sedentary lifestyle, have been reported to be correlated with telomere shortening. However, their relationship seems obscure due to conflicting results. This study uses metaphase Quantitative In Situ Fluorescence Hybridization (Q-FISH) technology to elucidate the role of short and critically short telomeres in cardiac diseases.

Methods: Twelve CVD patients (seven men and five women) aged 30 to 70 years old and twelve age-matched healthy individuals were included in the study. Patients were considered to exert cardiac symptoms when there was a diagnosis of at least hypertasis, vascular or CVD disease or atherosclerosis or arrhythmia or cardiomyopathy (after questionnaire completion). Each chromosome's telomere length (TL) from peripheral blood mononuclear cells (PBMCs) was measured using metaphase Q-FISH. An independent samples t-test was used to compare participants' mean or median TL with various medical factors and habits. All analyses were conducted using IBM SPSS Statistics 24.0.

Results: Twelve CVD cases were categorized in an age-dependent manner. Three individuals were involved in the age group between 40–50 years, two other were between 50–60 years and eight others were between 60–70 years. The mean TL of short telomeres in healthy controls was 5682bp in the age group of 40–50 years, 5402bp in the age group of 50–60 years, and 5326 bp in the age group of 60–70 years, respectively. The mean TL of short telomeres showed a remarkable decline with advanced age. In contrast, the mean TL of short telomeres in cardiac diseases patients was 5.544±990 at the age group of 40–50 years, 4253±1325 at the age group of 50–60 years and 4.939±1.150 at the age group of 60–70 years, respectively. The mean TL of whole telomeres in cardiac diseases patients was 9.137±1.426 at the age group of 40–50 years, 7.171±1.636 at the age group of 50–60 years and 7.951±1.652 at the age group of 60–70 years. Accordingly, the mean TL of whole and short telomeres in patients with cardiac diseases showed a similar reduced trend. Although the mean of whole and short telomeres in cardiac diseases patients was lower in comparison to aged-matched healthy controls, there was no statistical significance due to the limited patient sample.

Conclusion: Shorter TL was observed in cardiac diseases patients compared to those of healthy controls by using metaphase Q-FISH. However, more cases need to be studied to elucidate the use of TL as a potential marker for the diagnosis of patients with cardiac diseases and CVD.

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P14-05

Knock-down of FOXO3, GATA2, NFE2L2 and AHR promotes doxorubicin-induced cardiotoxicity in human cardiomyocytes

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The number of cancer survivors has been steadily increasing over the last decades due to improved cancer care. Despite advances in precision medicine, anthracyclines (ACs) remain the cornerstone of standard care for many cancer types. The use of anthracyclines is associated with a dose-dependent and cumulative cardiotoxicity that is evident in up to 10% of the cancer survivors and can ultimately result in heart failure. Good biomarkers to detect antracycline-induced cardiotoxicity (AIC) at an early stage as well as drug interventions to prevent or revert AIC remain scarce.

Mechanisms underlying AIC are partly driven by changes in gene expression which are governed by transcription factors (TFs). To generate new insights into AIC we have analyzed previously generated RNA-seq data from AC-treated human cardiac microtissues. All tested ACs (doxorubicin, epirubicin and daunarubicin) affected the expression of a large number of differentially expressed genes (DEGs) ranging from 1,000–2,500 (false discovery rate<0.01). To be able to assess causality of these DEGs in the cellular response to ACs we first reduced the complexity into TF-gene networks and selected 27 TFs for functional testing. Using focused lentiviral RNA interference screening we identified known (TP53, NFE2L2 and ATF3) and novel (FOXO3, AHR and GATA2) TFs that affect the viability of human cardiomyocytes upon doxorubicin exposure.

Further follow-up experiments showed that knock-down of FOXO3 and GATA2 reduced the IC50 of Dox by ~3-fold, whereas depletion of AHR and NFE2L2 were more subtle. This was accompanied by a significant increase in DNA damage measured by COMET assay upon doxorubicin exposure. Apoptosis assessed by cleaved caspase 3 was not affected by knock-down of FOXO3, GATA2, NFE2L2 or AHR. For NFE2L2 and AHR we observed increased levels of doxorubicin, suggesting that cellular uptake or removal might be affected. FOXO3 depletion showed a stronger transcriptional induction of p53-dependent genes CDKN1A and GADD45A compared to control conditions and knock-down of the other TFs. These data suggest that each tested TF-gene network affects a different molecular mechanism after doxorubicin exposure. We are currently generating RNAseq data from the TF knock-down models to better understand the role of each individual TF in the response of human cardiomyocytes to doxorubicin. We believe that these data will help to discover doxorubicin induced transcriptomic changes that causally affect cardiotoxicity and represent relevant leads for the development of strategies to overcome AIC.

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P14-06

The cytotoxicity of bortezomib in AC16 cardiac cells and possible therapeutic options involving Nrf2

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Cancer survivorship rate has witnessed a notable increase, leveraged by amazing therapeutic advancements. However, oncological treatments present important and clinically limiting adverse effects. Bortezomib (BTZ) is a proteasome inhibitor used in multiple myeloma and mantle cell lymphoma, having its clinical use limited because of its cardiotoxicity. Nuclear factor erythroid 2–related factor 2 (Nrf2) is a transcription factor that plays a crucial role in maintaining redox homeostasis e.g. influencing glutathione (GSH) pathway, among others.

The aims of this study to investigate the influence of Nrf2 modulators [cheirolin (CH), SK-119, SH-29, and dimethyl fumarate (DMF)] on the cytotoxicity induced by BTZ in human differentiated AC16 cardiac cells.

AC16 cells were differentiated with horse serum and then exposed to clinically relevant doses of BTZ (0.01–20 μM) for 24 or 48h, after which two cytotoxicity assays were performed: the MTT reduction and the neutral red uptake assays. Then, two concentrations were chosen (1 and 0.01 μM) to be co-incubated with the modulators. Furthermore, in the former conditions, the levels of GSH, Nrf2 and p62 were analyzed.

A time-dependent cytotoxicity was observed for the concentrations tested; however, a concentration-dependent cytotoxicity was only observed for the lowest concentrations tested (0.01 to 0.5 μM). Regarding the cytotoxicity elicited by BTZ, CH (10 μM) proved to be partially protective against the toxicity elicited by BTZ (1 μM). Incubation with BTZ alone led to a reduction in Nrf2 levels, but its co-incubation with CH increased Nrf2 levels, also affecting GSH levels. SK-119 and SH-29 did not induce significant changes on any parameters assessed.

In conclusion, the protection conferred by CH against BTZ-induced cytotoxicity appears to be related to its ability to increase overall levels of Nrf2. Therefore, Nrf2 modulation emerges as a potential therapeutic approach for mitigating BTZ-induced cardiotoxicity.

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P15 | Immune toxicology

P15-01

Incidence of neutralizing adeno-associated viral antibody subtypes in cynomolgus monkeys of Cambodian, Mauritius, and Philippines origins

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Preclinical safety assessment studies utilizing cynomolgus macaques (*Macacafascicularis*) are an instrumental part of the drug development process. Safety assessment of gene therapy (GT) products, especially those utilizing adeno-associated viral (AAV) vector-based therapeutics, requires prescreening of many animals to obtain adequate numbers for study assignment due to the presence of naturally occurring neutralizing antibodies (nAb) against AAVs. Preexisting antibodies against AAV vectors can impact the effectiveness of gene therapies with the main challenges being loss of efficacy and loss of durability of the GT. Due to the increased demand for cynomolgus macaques, attributed in part to unforeseen global factors (e.g., the COVID pandemic and spe-

cific border restrictions), continued use of cynos for AAV-based gene therapy has necessitated exploring utilization of animals from other origins such as Mauritius and the Philippines. Genetic and environmental variability between origins can complicate data interpretation and generation of reference data is essential for informed study design of new toxicology programs. Given the unique challenges of working with AAV vector-based test articles, a review of the prescreening nAb data, collected from a large number of toxicology studies performed in the past few years, was conducted, with the aim of identifying origin-specific differences in the percentages of nAb negative animals for utilization on AAV studies. AAV neutralizing antibody cell-based assay (ID50 at ≤1:10 serum dilution) was used for confirming negative or low viral titers. Review of the data set indicates no substantial differences in seronegativity rate between origins tested – Cambodian, Mauritian, and Philippines, with the exception of AAV9 where some variability was noted ranging from 40% (Mauritian) to 79% (Philippines). Variations between serotypes were noted, with AAV8 having the lowest seronegativity rate and AAV5 and AAV6 having the highest. In conclusion, prior to the initiation of a program utilizing AAVs, it is important to understand the necessity and constraints of screening animals for pre-existing antibodies against the specific AAV serotypes. This data compilation serves as an important reference for estimating animal use numbers and selection during the initiation of preclinical safety studies for gene therapies utilizing AAV gene delivery modalities.

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P15-02
Assessing immune-related effects of nanoplastics on primary human monocytes *in vitro*

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Introduction: The global concern regarding plastic pollution has brought focus on micro- and nanoplastics, which have gathered significant scientific attention for their potential impact on human health [1]. These plastic particles have been shown to trigger inflammatory immune responses by activating cells like monocytes and macrophages [2,3]. Our understanding of the immunotoxic effects of micro and-nano plastics in humans is still in its infancy with much yet to be unraveled [4,5]. We aimed to investigate the immunomodulatory effects of nanoparticles derived from polyethylene (PE) and polypropylene (PP), on monocytes *in vitro*.

Methods: Peripheral blood mononuclear cells (PBMC) were prepared from fresh blood samples collected from six healthy individuals using SepMate™ tubes. Monocytes were harvested using EasySep™ Human Monocyte Isolation Kit from STEMCELL Technologies. Monocytes were exposed to different concentrations of PE (350nm, 82µg/ml) and PP nanoplastic particles (180nm, 41µg/ml). Lipopolysaccharides (LPS, 100ng/ml) served as positive control. Cell cytotoxicity was evaluated by measuring cell viability over time using the PrestoBlue HS assay, while high-plex Luminex assay was used to quantify cytokine secretion after 48hrs in cell supernatants. Surface expression pattern of specific markers on monocytes was assessed by multi-color flow cytometry.

Results: Three concentrations (1µg/ml, 10µg/ml, 20µg /ml) of PE and PP were tested to assess cell viability and a significant decline was observed after 48 hours of exposure across all the concentrations ($p<0.0001$) for PE and PP. Monocyte viability declined substantially at 10µg /ml of PP ($p<0.0001$) compared to PE at the same concentration over different time points. PE and PP exposure induced secretion of cytokines such as IL-1β, IL-6, IL-4, TNF-α, GMCSF, IL-1α, IL-2, VEGF, IL-8 with no production of IFN-γ, IL-5, IL-10, and IL-12. Multi-color flow cytometry revealed a proportionate decline in CD14 expression level at 20µg/ml of PE ($p=0.0175$) and at 10µg/ml of PP ($p<0.0001$). Expression of HLA-DR, and CD86 did not differ upon PE and PP exposure compared to control. However, the expression level of the co-stimulatory marker CD80 was upregulated with increasing concentrations of PE (20µg/ml; $p<0.0001$) and PP (10µg/ml; $p<0.0001$). Interestingly, CD11b expression exhibited a gradual decline for PE and PP at the same concentrations.

Conclusion: Our findings highlight the cytotoxicity of PE and PP particles on monocyte viability *in vitro* in a concentration-dependent manner. Both PE and PP induce the secretion of cytokines and modify surface marker expression, potentially triggering an inflammatory immune response. However, further research is needed to better understand the mechanisms underlying these immune effects and long-term consequences of chronic exposure to nanoplastics.

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P15-03
Immunotoxicity as an endpoint: on the lookout for contaminants of emerging concern for drinking water quality

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Water contamination is a pressing global issue that poses a potential health risk to populations worldwide. A number of contamination sources, from industrial discharges and agricultural runoff to improper waste disposal and accidental spills, contribute to this global problem. Ensuring safe and high-quality drinking water is essential to protect public health. However, both legacy and emerging chemicals are known to contribute to the health risks associated with water contamination, which requires research. Legislative frameworks, such as the EU Drinking Water Directive and national drinking water regulations, are constantly revising the legal parameters required for public health

considerations. Recently, several water quality parameters have been added to these legislative frameworks due to the immunotoxic potential of certain chemicals, including per- and polyfluoroalkyl substances (PFAS) and bisphenol A (BPA). This emphasizes the need for proactive identification of immunotoxic substances in order to be prepared for challenges in drinking water production. The current work focuses on the immunotoxicological properties and risk-based monitoring of drinking water-relevant substances with an outlook on effect-based monitoring methods to improve our understanding and prediction of chemical immunotoxicity in relation to drinking water quality. To achieve this, a literature search was conducted on peer-reviewed publications from (inter)national (meta) databases and websites, as well as reports from reputable institutes and health authorities, complemented with other relevant databases. The results provide a comprehensive overview of the available literature on the immunotoxic potential of selected Dutch water micropollutants. Additionally, effect-based trigger values (EBTs) for bioassay endpoints related to immunotoxicity and analogues that currently have limited data will be derived. The method used for EBT derivation can be applied to similar data-poor bioassays with immunotoxicological endpoints to facilitate water quality monitoring by drinking water companies. The approach outlined in this study holds promise for broader international applications in water quality monitoring by leveraging immunotoxicology testing methods and effect-based monitoring strategies. The knowledge gained from this research has the potential to further advance water quality management practices at local and global level, securing water quality and the well-being of the populations.

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P15-05

Comparison between bisphenol A and its major substituent, bisphenol S, in terms of affecting the inflammatory process through the production of inflammatory mediators

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In recent decades, the production of bisphenol A (BPA) in industrial applications has been restricted by many countries and organizations around the world. The most prevalent substituent is bisphenol S (BPS). Bisphenols are used as plasticizers in a wide range of consumer products. The biggest problem in the usage of these compounds arises from leaking monomers into food under certain conditions. In this study, we wanted to prove that highly restricted BPA can be more immunotoxic to inflammatory processes than its major substituent (BPS), which appears to be a safer option. As a laboratory model, we chose human umbilical vein endothelial cells (HUVEC), as they appear to be the most suitable model for researching the inflammatory process. We cultivated this cell line according to previously established protocols. After cultivating, the treatment of HUVEC cells with bisphenol A and bisphenol S lasted for 24 hours. In treatment, we used the same concentrations for both bisphenols: 50, 25; 10; 1; 0.1; and 0.05 μM. The measured parameters for this study were the production of prostaglandin I2 (PGI2, prostacyclin) and the production of prostaglandin D2 (PGD2). For the assay, we used commercially available ELISA kits. Production of prostaglandin I2 after 24 hours of treatment with BPS was increased in all concentrations. Only statistically significant values were obtained from a concentration of 0.1 μM with a level of significance (**P<0.01). On the other hand, concentrations of PGI2 in samples treated with BPA were significantly higher. At concentrations of 50 and 10 μM, we recorded significantly higher production of PGI2 (*P<0.05). Even higher differences appeared at concentrations of 25μM (**P<0.01), and the most significant changes were displayed at concentrations of 0.1μM with a level of significance of ***P<0.001. In the second monitored parameter (PGD2), treatment of BPS revealed no significant results, although the level of prostaglandin fluctuated. In the 24-hour treatment with bisphenol A, the production of the inflammatory mediator PGD2 was notably higher. At a concentration of 50 μM, the production was significantly higher at a level of (*P<0.05). 0.1 and 0.05 μM of BPA resulted in an even higher volume of PGD2 with a higher level of significance (**P<0.01). Concentrations of 1 and 10 μM treatment caused a rise in PGD2 production at the level of significance (***P<0.001). Our findings proved that BPA is way more noticeably harmful in terms of increased production of inflammatory markers than its major substituent, BPS. The use of bisphenol S as a substitute for bisphenol A can be characterized as a safer variant, but the results point to its similar endocrine-disrupting effect, just to a lesser extent than that of bisphenol A.

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P15-06

Amoxicillin-induced liver injury: characterisation of amoxicillin-specific T-cells in healthy donors reveals unexpected HLA class I restrictions

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T-cell mediated adverse drug reactions are a major concern to patients, clinicians, and researchers in the field of drug development. They are associated with significant patient morbidity and mortality as well as challenges in identifying appropriate future therapies. However, the growing identification of strong human leukocyte antigen (HLA) class I restrictions linked with aberrant T-cell responses demonstrate translational clinical benefit and utility in pre-clinical drug screening. Indeed, mandatory screening of Southeast Asians for HLA-B*15:02 prior to administration of carbamazepine has already proved successful in reducing severe drug reactions in the population. The beta-lactam antibiotic, amoxicillin, is the most common culprit drug responsible for a hypersensitive phenotype. The HLA-A*02:01 allele is widely associated with amoxicillin (clavulanate) induced hepatotoxicity with negative predictive values as high as 99%. This study aimed to investigate the stringency of this association as well as the mechanisms of interaction of the drug antigen with HLA class I molecules in CD8 MHC I restricted T-cell clones (TCCs) from a healthy donor expressing HLA-A*02:01. HLA genotyped immortalised B-cells and transfected C1R cells were used to present antigens to TCCs and responses were detected by proliferative and cytokine-based assessment (IFN-γ secretion in ELISpot), in addition to flow cytometry. Responses were absent in the presence of the HLA-A*02:01 positive mismatch donor, however, T-cell responses were observed within HLA-A*02:01 negative mismatch donors. Proteomics analyses examining intracellular protein binding, and the elution of MHC I bound drug-modified peptides from the surface of immortalised B-cells were also conducted to examine mechanistic differences in drug antigen presentation between allogenic donors. Here, we report that amoxicillin-associated HLA restrictions may not be as stringent as previously suggested with T-cell activation occurring in the presence of additional HLA alleles. Future work aims to develop a screening system with the potential to infer the likelihood of common HLA risk alleles presenting novel drug antigens to cytotoxic T-cells.

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P15-07
Bridging the gap: uncovering the mechanisms of action underlying antibody reduction following PFAS exposure

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Per- and polyfluoroalkyl substances (PFAS) are a group of man-made chemicals prevalent in the environment, associated with a wide range of adverse health effects, including immunotoxicity. We have previously demonstrated that PFAS can directly affect the antibody production inducing a reduction of IgM and IgG in human peripheral blood mononuclear cells (PBMCs)-derived B cells from male and female donors. However, the molecular mechanisms underlying PFAS-induced immunotoxicity, remain poorly understood. This study aims to bridge this knowledge gap starting from RNA sequencing (RNAseq) data to explore the impact of PFAS on gene expression. To identify a subset of differentially expressed genes (DEGs) potentially implicated in the reduction of the antibody production, PBMCs were exposed for 24 hours to PFOA and PFOS and then stimulated with the CpG oligodeoxynucleotide ODN2006 (ODN2006) and rhIL-2 for 6 days. Initial events were identified after 24 hours of exposure, while the long exposure was assessed after a total of 7 days of exposure. In these short-term studies, the effects of PFOA and PFOS on gene expression were most similar to those of the Peroxisome Proliferator-Activated Receptor alpha (PPARα) agonist GW7647. While, in the long-term exposure the gene expression induced by PFOA and PFOS appeared to be related to glucocorticoid receptor (GR) signaling, similar with the agonist Dexamethasone. The genes selected from RNAseq analysis were confirmed and studied by examining their modulation through PCR analysis and the two selected pathways of activation were confirmed using pharmacological inhibitors and analyzed the effect on IgG and IgM release. Our findings suggest that PFAS exposure disrupts the expression of key genes within these pathways, potentially leading to altered lipid metabolism, immune cell proliferation, antibody, and cytokine production. These disruptions may contribute to the observed immunotoxic effects of PFAS. This study not only sheds light on the molecular mechanisms of PFAS-induced immunotoxicity but also identifies potential biomarkers for assessing PFAS exposure and its health impacts.

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P15-08
Establishing the efficacy of the Rho-kinase inhibitor fasudil as a protective measure against wildfire smoke-induced neuroinflammation

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Purpose: Wildfires across the United States have steadily increased over the past 40 years. Smoke from wildfires can cause cardiovascular and respiratory diseases, with new studies highlighting potential neurological outcomes[1]. The purpose of the following studies are to delineate the dynamics of peripheral immune populations in response to woodsmoke, the mechanism of infiltration into the brain, and the treatment options available. This study is the first to deliver a comprehensive timeline of neuroinflammation following wildfire smoke inhalation, collecting tissue from animals 1, 14, and 28 days after exposure. Air pollution has previously shown to impact the blood-brain barrier which serves an essential role in mitigating neuroinflammation. Therefore, we introduced the Rho-kinase inhibitor fasudil to strengthen this barrier and demonstrate an endothelial-dependent mechanism of peripheral immune infiltration.

Methods: Fasudil will be administered via osmotic mini pumps which provide a consistent dose over 21 days. Mice are exposed to either filtered air or a woodsmoke exposure (0.5mg/m^3 every other day for 14 days, 4hr/day). Timeline studies will follow these mice over the course of exposure, and extend past 1, 14, and 28 days after exposure. Left hemispheres will be collected for high dimensional flow cytometry analysis using the Aurora Cytek. Markers for peripheral immune infiltration included T cells (CD3,CD4, CD8), adhesion molecules (VCAM, ICAM) resident immune cells (CD11b, TMEM119, ACSA-2) and other inflammation-dependent markers.

Results: Results demonstrate the presence of CD4 T cells in response to woodsmoke inhalation and the decrease of this population over time. VCAM and ICAM expression also showed similar trends, peaking 1 day after our 14 day exposure paradigm. Treatment with fasudil demonstrates the depletion of VCAM expression which indicates the endothelial-dependent nature of woodsmoke-induced neuroinflammation. Overall, we demonstrate the ability of woodsmoke to cause indirect neuroinflammation through the recruitment of peripheral immune populations. With this knowledge, we will continue our investigation of a blood-brain barrier dependent mechanism of neuroinflammation through imaging of the right hemisphere for albumin leakage, glial activation, and presence of CD4 T cells. Further research detailing the mitigation of toxicity could lead to public health-based recommendations following air pollution events.

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P15-09
Immunometabolism and phenotype modulation of human dendritic cells exposed to Nickel and Cobalt

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Allergies represent a major public health problem. Nickel (Ni) and cobalt (Co) are two transition metals known for inducing allergic contact dermatitis (ACD) and constitute the leading cause of occupational disease linked to the skin [1,2]. Exposure to Ni and Co are known to modify dendritic cells (DC) phenotype leading to their maturation [3,4]. Adaptive immune response triggered by the DC is linked to changes in their phenotype and immunometabolism [5,6,7]. Our objective is to identify new pathways and early biomarkers in response to chemical sensitizers. Consequently, this study aims to investigate the effects of increasing concentrations of Ni and Co (150, 250, 500µM) on the modulation of DC phenotype, glycolytic metabolism, and oxidative phosphorylation. DC were generated from monocytes purified from human PBMC of blood donors and treated with Ni or Co. Our results showed a dose-dependent modulation of the DC phenotype with an increase of CD83 and CD86 expression. In addition, a dose-dependent decrease in total ATP was observed to be associated with an increase in extracellular lactate production without any severe cytotoxicity. These observations may highlight the metabolic switch of DC stimulated by Ni and Co, which are known to be human TLR4 ligands and activators [8,9,10]. Additional studies are planned to measure extracellular ATP, to study disturbances in the citric acid cycle and the mitochondrial chain and the link to DC phenotype modifications.

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P15-10
How does exposure to cigarette smoke or e-cigarettes impact mechanisms relating to viral susceptibility and severity?

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Currently, associations between smoking and COVID-19 susceptibility are conflicted and there is limited research especially on the impact of vaping. With e-cigarettes increasing in popularity, understanding toxicological impacts on viral susceptibility or severity is essential to improve public health guidance.

Human bronchial epithelial cells, HBEC3-KT, were grown at the air-liquid interface for 14d to allow for differentiation into a pseudostratified epithelium. Cells were exposed to e-liquid, e-cigarette condensate or tobacco cigarette condensate for 2h. Transepithelial electrical resistance was measured, LDH assay was performed and cells were harvested 24h post-exposure. qPCR was used to analyse any changes in the expression of key genes and pathways of interest. Further work then included an infection with SARS-CoV-2 (Victoria strain) 24h post-exposure with the same endpoints 24h post-infection.

Data suggested non-significant changes to SARS-CoV-2 entry mechanisms following e-cigarette condensate exposure with varied responses to tobacco cigarette condensate. Of interest, ACE2 fold change trends were different between e-cigarettes and tobacco cigarettes, suggesting that SARS-CoV-2 entry is altered following exposure with differing impacts. Physiological impacts of these changes to viral entry may be revealed following planned infection studies. Genes relating to toxicity such as DNA damage and oxygen species were upregulated following exposure to higher doses of tobacco condensate with varied changes following e-cigarette condensate exposure, suggesting exposure to high doses of tobacco condensate may increase the severity of COVID-19. It is worth noting the non-significant changes following e-liquid and e-cigarette exposure are likely due to the variability in data due to batch variation and a lack of regulation on e-liquid composition. This will become clearer during planned GC-MS investigations. Additionally, results demonstrated differing trends in key genes of interest following exposure to e-liquid vs e-cigarette condensate. This shows the importance of the exposure methods and how it can alter the conclusions gained.In summary, many key genes and pathways of interest involved in SARS-CoV-2 viral entry and disease severity are altered following exposure to cigarette smoke or e-cigarettes, sometimes differently. This demonstrates the need for further research into e-cigarettes and their individual toxicity. Despite many genes of interest having non-significant changes following exposure, physiological impacts of these genetic changes to viral entry may be revealed following planned infection studies and the variability between e-liquid bottles needs to be investigated further.

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P15-11

Comparison between senescence-accelerated mice and usual aging mice in development of *Dermatophagoides farinae*-induced asthma and hapten-induced allergic contact dermatitis

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In the current aging society, the development and safety evaluation of therapeutics for the elderly are highly demanded for both humans and companion animals. Nowadays, Senescence-Accelerated Mice (SAM) are commonly used in aging research[1], however, it has not been well confirmed that age-dependent changes, particularly in immune function and associated allergic pathogenesis, exhibited by SAM correspond to those in usual aging mice. Therefore, the objective of this study is to compare between SAM mice and usual aging mice in development of *Dermatophagoides farinae (Derf)*-induced asthma and hapten-induced allergic contact dermatitis (ACD).

SAM is broadly classified into the SAMP (Senescence-Accelerated Mouse Prone) strain, which exhibits accelerated aging and a short life span, and the SAMR (Senescence-Accelerated Mouse Resistant) strain, which exhibits normal aging. Each is further subdivided, with SAMP1 reported to be highly sensitive to immune function. In this study, 8- and 20-week-old SAMP1 and SAMR mice were used. As a usual aging mouse, 8- and 80-week-old C57BL/6 mice were simultaneously used and evaluated. Allergic asthma symptoms were developed by repetitive intranasal sensitization (25 μg/dose) and challenge (5 μg/dose) of *Derf*. After percutaneous arterial oxygen saturation (SpO_2) was monitored, hilar lymph node (LN), bronchoalveolar lavage fluid (BALF), lung tissue, and serum samples were collected for histological and immunological analysis. ACD was also developed by repetitive topical sensitization (5%) and challenge (0.5%) of toluene-2,4-diisocyanate (TDI). After the ear swelling response was monitored, ear skin, auricular LN, and serum samples were collected for histological and immunological analysis.

To understand the basic age-dependent changes in both strains, immunological evaluations of untreated SAMP1 and C57BL/6 mice were initially conducted. Naive CD4- and CD8-positive T cells of the spleen and blood in the aged groups for both strains were significantly decreased compared to the younger groups, whereas effector CD4- and CD8-positive T cells and regulatory T cells were significantly increased in both strains of the aged group compared to the younger groups. In the pathogenesis of asthma including SpO_2 and the number of group 2 innate lymphoid cells in the lung, there was no reaction to *Derf* exposure in the aged group of C57BL/6 mice, whereas significant reactions were observed in the aged group of SAMP1 mice. The pathogenesis of ACD including the ear swelling response and the number of IgE-positive B cells in the auricular LN act in a similar fashion to asthma development that significant response to TDI exposure was only observed in the aged group of SAMP1 mice.

Our findings indicate that SAMP1 and usual aging mice differ in the development of allergic responses, while a similar trend of age-dependent changes was observed in both strains of untreated mice.

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P15-12

Omega-3 fatty acid metabolite resolvin D1 modulates organic dust-induced pulmonary and neurological inflammation

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Particulate matter exposure in agriculture workers, particularly organic dust exposure (ODE), is a significant cause of chronic inflammatory respiratory disease, of which there are limited effective therapies [4,5,7]. ODE in agriculture settings has also been linked to an increased risk of developing dementia and other neurodegenerative diseases, and patients with chronic lower respiratory diseases often experience memory loss and confusion [2,8]. There is emerging but limited research on the lung-brain axis inflammatory response of inhalant toxicants and even less research on proposed therapies to mitigate chronic inflammation and tissue damage caused by ODE. Omega-3 fatty acid-derived specialized pro-resolving mediators (SPMs) have demonstrated efficacy in multiple inflammatory disease models but have not been extensively studied as therapeutics for pulmonary and neurological inflammation in ODE [3,6]. The SPM Resolvin D1 (RvD1) has shown efficacy as an exogenous therapy in a chronic ODE-mediated pulmonary carcinogenesis model but has not been investigated in ODE-mediated neuroinflammation [1]. C57BL/6J mice were intranasally instilled with agriculture dust extract (DE) in saline and injected with 50 ng, 100 ng, or 250 ng RvD1 intraperitoneally (IP) 5 days/week for 3 weeks. Mice were allowed to recover for 3 days before sacrifice to assess the prolonged immune response and the tissue repair response. Bronchoalveolar lavage fluid (BALF), lung tissue, and brain tissue were collected. BALF cytokines were evaluated via ELISA, which revealed that the anti-inflammatory cytokine interleukin-22 (IL-22), which is involved in tissue repair and homeostasis, was upregulated in DE-exposed animals. BALF cell counts were also evaluated and the data revealed that neutrophil counts in DE-animals remain elevated after a 3-day recovery, but are significantly reduced in RvD1-treated animals, independent of dose. Lung tissue was stained with hematoxylin and eosin and evaluated for histopathology. DE-animals displayed increased alveolar space, parenchymal nuclear counts, and peribronchiolar and perivascular inflammation. Animals that received 250 ng RvD1 demonstrated decreased peribronchiolar inflammation. Brain tissue was stained with immunohistochemistry for S100 calcium-binding protein β (S100β) to assess astrocyte inflammation. Animals exposed to DE exhibited a significantly increased number of astrocytes in the hippocampus and frontal cortex, with animals given 250 ng RvD1 displaying decreased number of astrocytes. These data demonstrate DE contributes to neurological inflammation in individuals chronically exposed to organic dust and that 250 ng RvD1 appears to be the most effective dose in reducing inflammation. RvD1 attenuates the pulmonary and neurological inflammation and suggests it may be a viable novel therapeutic for ODE-induced inflammation.

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P15-13
Perfluoroalkyl substances and immunotoxicity: an *in vitro* structure-activity relationship study in THP-1-derived monocytes and macrophages

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Per- and polyfluoroalkyl substances (PFAS) comprise compounds with fluorinated carbon chains and have a wide range of industrial applications due to their high thermal stability, waterproof, and oil-repellant properties. Historically, concerns about PFAS toxicity were centered around their hepatotoxicity. More recently, epidemiological data have demonstrated a correlation between PFAS blood concentration and reduced vaccine response. In turn, PFAS immunotoxicity is becoming the major source of concern over hepatotoxicity. However, there is currently a lack of data regarding the effect of PFAS on the various cell types involved in the immune response.

The present study aims to investigate the effect of PFAS on the innate immune system, specifically the monocytes and macrophages, using THP-1 cells as models. Furthermore, using a structure-activity approach, it aims to understand better the effect of common linear PFAS structures, such as the headgroup and chain-length, on their immunotoxicity. Well-established toxicological assays are employed to assess cell viability (MTT), oxidative stress (DCFH), cytokine release (ELISA), and NF-κB activation (Western blot/QUANTI-Blue™). Thirteen linear PFAS are tested, with chain-length ranging from 4 to 10 carbons and with either carboxylic (PFCA), sulfonic (PFSA), or alcoholic (FTOH) headgroups.

The study outlines a clear relationship between PFAS structure and their toxicity to monocytes and macrophages derived from THP-1. Cytotoxicity and reactive oxygen generation (ROS) positively correlate with carbon chain-length up to 9 carbons. Moreover, all tested headgroups led to reduced cell viability, with FTOH having higher toxicity than PFCA and PFSA. However, where PFCA and PFSA lead to ROS generation, FTOH does not. The present study demonstrates that while PFAS with an alcoholic headgroup previously demonstrated no toxicity to HepG2, they might contribute to effects on the native immune system.

PFAS also demonstrate potential immunomodulatory potential as they reduce LPS-induced cytokine release in macrophages. In macrophages, IL-6, IL-8, IL-1β, and TNF-α release is reduced after exposure to 6:2 FTOH, PFOS, and PFOA. Comparable to the cytotoxicity data, FTOH displayed a higher inhibiting potential than PFCA and PFSA. Our data show that the reduced cytokine release appears to be due to the immunomodulatory effect of PFAS on the NF-κB pathway. Additionally, exposure to FTOH, PFCA, and PFSA result in lower response after LPS and TNF-α exposure.

The present study demonstrates the potential toxicity of PFAS toward the innate immune system and a structure-activity relationship for all tested endpoints. Moreover, to the author's knowledge, this is the first time FTOH toxicity has been demonstrated in the innate immune system, and a potential mechanism of action has been outlined with an important role for the NF-κB pathway inhibition.

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P15-14
Characterisation of liver-derived T-cells during and upon resolution of CFTR modulator induced liver injury

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Cystic fibrosis (CF) is a life-limiting condition caused by defects in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Ivacaftor, Lumacaftor, Elexacaftor and Tezacaftor, known collectively as CFTR modulators, have transformed the treatment of CF by targeting the underlying defect in the CFTR protein. T-cell mediated drug hypersensitivity reactions, such as drug induced liver injury (DILI) and skin rash, have been reported following treatment with CFTR modulators and severe reactions can result in cessation of treatment. This work aimed to characterise liver derived T-cells isolated from a punch biopsy obtained from a CF patient following a DILI reaction after exposure to the CFTR modulator triple therapy, Kaftrio (Elexacaftor, Tezacaftor and Ivacaftor).

Upon resolution of the DILI reaction, a biopsy was taken with the aim of expanding and assessing the phenotype and function of the T-cells residing in the liver. T-cells were isolated from the liver biopsy and flow cytometry was performed to determine their phenotype. Activation, memory and liver specific migratory T-cell markers were identified suggesting that the T-cells involved in the DILI reaction remained within the liver following resolution. Liver derived T-cells were stimulated with the selective T-cell mitogen Phytohemagglutinin and incorporated into an ELISpot assay to measure the cytokine profile. A mixture of CD4 (T-helper cell) and CD8 (Cytotoxic T-cell) cytokines were secreted including granzyme B and IL-13. This provided evidence that the liver derived T-cells were functional and able secrete cytokines when stimulated.

Our findings suggest T-cells involved in DILI reactions remain in the liver following resolution. The T-cells implicated in the reactions secrete both CD4 and CD8 cytokines which may give rise to the severe phenotype seen. The same patient was later given the dual CFTR modulator therapy, Symkevi (Ivacaftor and Tezacaftor). They developed DILI in response to Symkevi and a further biopsy was taken. T-cells have been isolated from the liver biopsy. Ongoing work is being conducted to allow the comparison of liver derived T-cells after resolution of DILI and during active DILI. Future work aims to incorporate liver derived T-cells within functional T-cell assays to determine the mechanisms behind the T-cell mediated hypersensitivity reactions.

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P15-15
Unveiling the therapeutic potential of MSC-derived conditioned media and exosomes towards COVID-19-induced neurological sequelae

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SARS-CoV-2 spike protein has been proposed to have neurotoxin-like motifs [1] which may alter neuronal cell function and contribute to neurological sequelae associated with acute and long-COVID. Microglia, that sustain neuronal networks, when dysregulated affect the proliferation and differentiation of neural stem cells (NSCs), ultimately impacting in adulthood neurogenesis [2] and brain homeostatic balance. Remarkably, mesenchymal stem cells (MSCs)-derived conditioned media (MSC-CM), mainly their exosomes (MSC-EXO), demonstrated immunomodulatory and regenerative properties. Furthermore, MSC exposure to preconditioning strategies, including pro-inflammatory cytokines, has been shown to improve their therapeutic properties [3,4].

With this work, we aimed to develop a stem cell-based therapeutic product and to understand the neuroregenerative and immunomodulatory effects of MSC-CM and MSC-EXO in a microenvironment that recapitulates the cytokine release syndrome of COVID-19. As such, MSCs were preconditioned with the inflammatory cytokine IFN-γ (10 ng/mL) to produce MSC-CM or MSC-EXO (CM-I and EXO-I, respectively). We evaluated if CM-I and EXO-I were able to revert an overreactive state of microglia, as compared with control CMs (CM-C) and EXOs (EXO-C), produced without any preconditioning strategy, and if these MSC-derived products induced the proliferation of undifferentiated mouse NSCs (mNSCs). The results showed that CM-C/I and EXO-C/I showed a trend to regulate the excessive phagocytic activity of microglia. Moreover, besides not revealing cytotoxicity, CM-C/I and EXO-C/I significantly induced mNSC proliferation. The MSC products derived from the IFN-γ-preconditioning strategy produced highest effects in mNSC proliferation, particularly at 15 mg/mL for EXO-I and 5 mg/mL for CM-I. Proteomic analysis revealed that CM-I induced the expression of proteins involved in innate immunity (*e.g.,* STAT1, HLA-A, B2M) and with antiviral activities (*e.g.,* GBP1 and GBP2), envisioning a role against SARS-CoV-2 infection. Overall, these data show that MSC preconditioning is a promising strategy to produce novel targeted therapeutics. Specifically, it shows that the secretome of MSCs can regulate the microglial phenotype, which may influence neurogenesis, and directly regulate NSC proliferation. Consequently, exploring MSC-CM/EXO as a therapeutic strategy holds promise against neurological COVID sequelae.

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P15-16
Deoxynivalenol detoxification by *Chlorella sorokiniana* leads to inhibitory effects on the Deoxynivalenol induced allergic contact dermatitis symptoms in a mouse model

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Deoxynivalenol (DON) is a type B trichothecene mycotoxin and produced by *Fusarium spp*., specifically *Gibberella zeae*. Contamination of grains such as wheat, barley, and corn with DON has long been recognized as a risk to human and animal health. DON is mainly categorized as vomitoxins because of their tendency to induce vomiting when ingested; however, DON is also recognized as immunotoxic, with several studies reporting that its oral administration abnormally stimulates the immune system. Our previous studies also indicated that acute and subacute oral DON administration significantly exacerbated allergic contact dermatitis and allergic asthma responses by activating Th2-immune responses. However, the current countermeasure for DON contamination is random inspection of samples prior to shipment. Several previous reports indicated the detoxification potential of *Chlorella* to environmental contaminants such as Aflatoxin B1 and cadmium. In this study we focus on *Chlorella sorokiniana* (*CS*) from Ishigaki Island in Japan and examine its detoxification to DON and inhibitory effects on the DON induced allergic contact dermatitis symptoms in a mouse model.

Detoxification of *CS* was demonstrated by toxicokinetics study in male ICR mice (8 wks old), 0.5, 2 and 24h post oral administration of 5 mg/kg DON and/or 500 mg/kg *CS*. DON or DON metabolites were analyzed in plasma and urine samples. Allergic contact dermatitis model was generated in female BALB/c mice (8 wks old) by repetitive dermal sensitization (5%) and challenge (0.5%) of Th2 type hapten toluene diisocyanate (TDI). Once daily oral administration of DON (0.068 mg/kg/day) and/or *CS* (100 mg/kg/day) was performed during the experiment. After ear swelling response and itch behavior (30 min) were monitored, serum, ear skin and auricular lymph nodes (LN) were collected for histological and immunological evaluations.

DON and DON metabolites in plasma (30 min after DON administration) was significantly reduced by *CS* co-administration in toxicokinetics study. In a mouse model of allergic contact dermatitis, ear swelling response and itch behavior were significantly reduced by *CS* co-administration compared to the DON only treated group. Amelioration of dermatitis symptoms was corroborated by histological evaluation of the skin, including significant inhibition of the edema, ulcers and inflammatory cell infiltration in the epidermis and dermis. Local immune responses related to allergic development including type 2 conventional dendritic cells, effector helper T cells, IgE positive B cells and IL-4 production by T cells in LN were also significantly inhibited by *CS* co-administration compared to the DON only treated group.

Our findings suggest that co-administration of *Chlorella sorokiniana* significantly inhibits the systemic absorption of DON and correspondingly leads to a significant inhibition of development of DON-induced allergic contact dermatitis symptoms.

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P15-17
In vitro immune off-target toxicity assessment using morph_ONE for parenterally administered antibody-drug conjugate test item

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Antibody-drug conjugates (ADC) are monoclonal antibodies (mAbs) that have been developed to combine cytotoxic agents with the unique targeting capabilities of mAbs. Current uses for ADC's include cancer treatments, due to their ability to target specific cancer antigens without harming healthy cells. While being effective in targeting tumour cells, some ADCs appear to have significant off-target effects detected in animal *in vivo* studies. Trastuzumab deruxtecan (T-DXd) is one such example, with pulmonary toxicities frequently observed in the preclinical monkeys/rat studies. Pathologies are often characterized by diffuse lymphocytic infiltrates/fibrosis, with T-DXd shown to be distributed in alveolar macrophages. In a global phase II study in patients with breast cancer, T-DXd- related interstitial lung diseases were reported in 13.6% of patients.

The study aimed to (i) validate a human *in vitro* system for predicting the pulmonary toxicity observed in *in vivo* toxicity studies of T-DXd, and (ii) understand the impact of the ADC vs individual components on respiratory inflammation and other potential off-target effects characterized by changes in macrophage morphology.

ImmuPHAGE™ (alveolar macrophage-like cells) was exposed to the T-DXd (ADC), deruxtecan (drug) and trastuzumab (mAb) in a range of concentrations 0.000001 – 0.1 mg/mL for 48 h. Cells were stained with a cocktail of fluorescent dyes to identify cellular features (nucleus, cytoplasm, mitochondrial activity, membrane permeability). Images were captured using the InCell Analyser 6000 and analysed using InCell Toolbox v1.9.2.

Cell health was affected in a dose dependent manner for deruxtecan but not for the ADC or mAb. A significant increase in membrane permeability, decrease in cell count and mitochondrial activity were observed for 0.1 mg deruxtecan, indicating that the drug alone displayed a cytotoxic effect. In contrast, when conjugated with the mAb evidence of toxicity on macrophages was not observed at the same concentration of deruxtecan. While the ADC did not affect cell health, changes in macrophage morphology were present, namely increased cell area accompanied by increased number and area of vacuoles was observed. The population phenotypes reported were dissimilar the cytotoxic (apoptotic) control, implying ADC even at its highest concentration of 0.1 mg is not cytotoxic, but generates adaptive morphological responses in macrophages. Further work is required to understand the significance of these responses to the long-term impact on respiratory health.

This study showed that the morph_ONE assay is a suitable tool for identifying changes in macrophage health and morphology resulting from ADCs treatment. Implementing this tool in the early stages of ADC development may help to detect potential off-target toxicity in the airways before progressing to animal studies, aiding candidate selection and increasing the chances of success in the drug development process.

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P15-18
Evaluation of transferability and within laboratory reproducibility of an alternative test method with THP-1 cell line for predicting immunotoxicity

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The IMMUNOTOX-T assay method was recently developed for *in vitro* screening immunotoxicity through profiling 24 cytokines from THP-1 dendritic cell line activated with lipopolysaccharides in the presence or absence of test chemicals. Considering pre-validation of the method, the assay method was transferred to a GLP laboratory, and transferability and within laboratory reproducibility (WLR) were evaluated. THP-1 cell line with acceptable doubling time (35–50h) was used for the evaluation. Concerning 75% cell viability (CV75), the CV75 values for dexamethasone, chlorambucil, and glycerol were within 1 digit variation ranges with the reference values decided at the time of method development. Proficiency was tested through calculating the relative cytokine production level versus vehicle control (RCPL%) using two different batches of THP-1 cell line. Three cytokines including Interleukin (IL)-6, IL-8, and tumor necrosis factor-alpha were evaluated. The three chemicals above were resulted in acceptable RCPL% ranges within 3 standard deviations of RCPL% established at the development stage. The WLR was further investigated using 9 coded chemicals (cyclophosphamide, dexamethasone, tacrolimus as immunosuppressants, butyl alcohol, ethyl vanillin, lactic acid as non-immunotoxicants, and 5,5-diphenylhydantoin, nonylphenol, phorbol 12-myristae 13-acetate as immunomodulants). The CV75 values for 9 test substances were within 1 digit variation ranges with the reference values decided at the development stage. Correlational analysis between the duplicated experiments with two different batches of THP-1 cell line was conducted, and mean RCPL%s of duplicated experiments were found well correlated for the 8 chemicals except cyclophosphamide, indicating no significant differences between the duplicated results. In addition, consistency on RCPL%s of 24 cytokines for 9 test substances with 3 different concentrations (0.01x, 0.1x, and 0.5x of CV75) were examined through t-test, resulting in no significant differences in 98% of the duplicated results. Overall, the IMMUNOTOX-T could be appropriately transferred with reliable proficiency and WLR.

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P15-19
Comparison of cytokine production in young and adult mice following intranasal exposure to polyhexamethylene guanidine

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Polyhexamethylene-guanidine is a polymer with guanidine groups. It is widely used as a biocidal disinfectant due to its low toxicity, however, exposure to the lungs causes a fatal lung disease with persistent lung inflammation and fibrosis. The toxicity of PHMG has been primarily studied using adult laboratory animals. The purpose of this study is to compare the immune responses of neonatal and adult mice following intranasal exposure to PHMG. Newborns are not born with fully developed organs at birth: lung development is complete by 730 days, and immune system development continues until 12 years of age. Therefore, the toxicity of PHMG in neonates is expected to be different from that in adults. Neonatal (7–10-day-old) and 8-week-old mice were intranasally instilled with PHMG (3 mg/kg, 4.5 mg/kg, and 6 mg/kg) and necropsied at days 4 and 15. Histopathological examination was conducted and lungs and spleens were harvested to analyze cytokine production. Splenocytes were isolated from the spleens of both newborn and adult groups to determine the ability to produce cytokines. They were treated with lipopolysaccharide (LPS), R848, and concanavalin A (ConA) and incubated for 44 hours. And then cytokine production was measured. Minimal to moderate lymphoid atrophy in the spleen and minimal to moderate granulomatous inflammation and fibrosis in the lungs were detected in the adult groups. In contrast, minimal inflammation was observed in the newborn groups. Proinflammatory cytokine production increased at day 4 but returned to normal at day 15 in the lungs of newborn mice. However, increased cytokine secretion persisted from day 4 to day 15 in the lungs of adult mice. Next, we checked the ability of splenocytes to secrete cytokines. Interestingly, cytokine production in response to LPS was greater in newborn mice than in adult mice. In addition, IL-10, an anti-inflammatory cytokine, was also increased more in male newborn mice than in male adult mice. When splenocytes were treated with R848, IL-10 was elevated more in male newborn mice than in male adult mice. These data suggest that neonatal immune cells can respond to pathogens and are biased toward Th2 response. Due to the Th2 bias in newborns, it is expected that when neonatal mice were exposed to PHMG, inflammation resolved rapidly and caused lower pulmonary toxicity than in adults.

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P15-20
The cytotoxic and immunotoxic effects of primary and secondary micro- and nanoplastics on human THP-1 macrophages

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The presence of micro- and nanoplastic particles (MNP) in our environment has raised increasing public concern and a growing body of evidence suggests that humans are exposed to these particles daily. The MNP can be intentionally added to products (primary MNP) or they can be formed from larger plastics through fragmentation or degradation (secondary MNP). Macrophages can potentially activate the innate immune system and trigger inflammation upon encountering MNP. However, very little is known about to which degree and due to which MNP properties such an activation occurs, especially regarding the secondary MNP. Therefore, this study investigates whether various primary and secondary MNP have cytotoxic and immunomodulatory effects on human macrophages.

Human THP-1 macrophages were used to conduct uptake and toxicity studies after up to 24 hours of exposure to 1, 10 and 100 µg/ml of mechanically degraded secondary polyvinylchloride, polypropylene and polyamide particles (PVC, PP; <1 µm and 1–5 µm, PA; 1–5 µm), in addition to primary polystyrene particles (PS; 0.05, 0.2 and 1 µm) and titanium dioxide (TiO_2; <0.01 µm) particles. Uptake was determined through flow cytometry and confocal microscopy, and immune activation and toxicity were measured via lysosomal activity, mitochondrial activity, lactate dehydrogenase (LDH) leakage, NF-□B activity and cytokine secretion.

MNP were taken up in a concentration-, time-, and size-dependent manner by macrophages. In addition, MNP increased lysosomal activity, suggesting potential accumulation of the particles in the lysosomes. Depending on the concentration, secondary MNP decreased mitochondrial activity and increased LDH leakage, specifying their cytotoxic potential. However, at these levels, they did not significantly induce NF-κB activity and cytokine production (IL-6, IL-1β, TNF-α).

Our research suggests the lack of a direct pro-inflammatory response by macrophages after exposure to primary and secondary MNP. However, at higher concentrations of secondary PVC, PP and PA, cytotoxicity is observed which might indirectly influence the functioning of the immune system. For further investigations, it is imperative to examine environmentally weathered MNP to shed more light on their altered properties and potential health impact.

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P15-21
Chemical respiratory sensitization: current status, challenges, and future opportunities

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Respiratory conditions linked to chemical exposures pose a growing health concern. Chemical respiratory sensitization manifests clinically mostly as occupational asthma. The chemical cause of diseases frequently remains unrecognized due to the latency of symptoms. No comprehensive and validated approaches are currently available for the prospective identification of chemicals that induce respiratory sensitization, while the expectations of New Approach Methodologies (NAMs) are high. An Adverse Outcome Pathway (AOP) connected to respiratory sensitization (AOP 39) is available in the AOP-Wiki, but it is still in the process of being finalized.

The Partnership for the Assessment of Risks from Chemicals (PARC) respiratory sensitization group works on 3 objectives: (i) NAMs, (ii) AOP and (iii) effect marker development.

1. No universally accepted or validated NAMs are currently available, including both *in vitro* and *in silico* approaches. A number of cell models consisting of lung epithelial and immune cells will be exposed to chloramine-T and piperazine and different readouts will be assessed with the aim to compare the results and develop more robust methods.
2. AOP 39 shares similarities with the skin sensitization AOP 40, as both describe the pathway from the binding of chemicals to proteins, through dendritic cell and T cell activation leading to the respective adverse outcome. We provide suggestions to include additional relevant key events to extend AOP 39, such as e.g. loss of barrier function and recruitment of inflammatory cells.
3. For the effect marker identification, we created an inventory of measurement methods potentially applicable as an effect marker connected to AOP 39 based on information found in AOP-Wiki and a comprehensive literature search in PubMed. Abstract screening and data extraction from selected literatures is still in progress.

The incomplete understanding of the molecular, cellular, and systemic processes leading to the adverse outcome pose a challenge for regulation and the development of testing methods, as well as for clinical diagnostics.

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P15-22
Particle-induced systemic autoimmune features in C57BL/6J and NOD/ShiLtJ mice

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Introduction: Occupational exposure to crystalline silica is associated with an elevated risk of autoimmune diseases in susceptible individuals, yet underlying mechanisms, likely influenced by gene-environment interactions, remain unclear. We aimed to investigate the systemic immunotoxicological effects of exposure to crystalline silica, and a co-exposure with diesel exhaust particles (DEP), considering the effect of genetic background in C57BL/6J and NOD/ShiLtJ mice.

Methods: C57BL/6J and NOD/ShiLtJ mice were oropharyngeally exposed to a total dose of 4 mg quartz (median size about 2 µm) and/or 40 µg DEP (size ranging from 5.3 to 110 µm) through repeated instillations with animal ethics committee approval of KU Leuven (P111/2021) [1]. Ten weeks after the last exposure, bronchoalveolar lavage fluid (BALF), blood, urine, spleen, lungs and kidneys were collected to evaluate BALF and serum anti-nuclear antibodies, proteinuria, spleen weight and lung and kidney histology.

Results: Silica-exposed C57BL/6J mice showed significantly increased spleen weight, suggesting systemic immunological activity. In NOD/ShiLtJ mice no noticeable differences in spleen weight were found between exposure groups. In BALF, the silica-exposed NOD/ShiLtJ mice showed significantly higher mean ANA scores, while silica-exposed C57BL/6J mice had similar mean scores as vehicle-exposed NOD/ShiLtJ mice. Silica-exposed NOD/ShiLtJ mice also developed systemic ANAs present in serum, with scores reaching up to 4, whereas silica-exposed C57BL/6J mice only reached scores up to 2–3. The silica-exposed groups seemed to have a higher urine protein concentration suggesting potential kidney damage, being more obvious in the NOD/ShiLtJ mice. Locally, silica exposure led to typical lung inflammation that was more obvious in the NOD/ShiLtJ and systemically, only NOD/ShiLtJ mice of all groups showed inflammatory infiltrates in the kidney tissue. Besides that, no significant acute tubular or glomerular injury was noted in either strains.

Discussion: The autoimmune-prone background of NOD/ShiLtJ mice, characterized by an H2(g7) MHC haplotype and genetic variants impacting tolerance and T cell functioning, seems to render them more susceptible to developing an inflammatory phenotype possibly facilitating the development of autoantibodies and distant autoimmune organ damage. A co-exposure with a relatively low dose of DEP, not able to provoke an inflammatory or autoimmune-like effect by itself, also did not intensify these effects in silica-exposed mice. Possibly the applied dose was too low to induce any measurable effect.

Conclusion: The genetic predisposition in NOD/ShiLtJ mice significantly contributes to their vulnerability to silica-induced systemic autoimmune features. A low dose of 40 µg DEP did not significantly worsen the observed effects in the mice.

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P15-23
In vitro evaluation of immunotoxicity of bisphenol A substitutes using THP-1 cell line

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Due to the growing knowledge of the harmful effects of bisphenol A (BPA), legislation on its presence in plastics and thermal paper is increasingly restricted. Based on observations from animal studies that BPA can increase the number of Th17 cells, EFSA proposed to reduce its tolerable daily intake from 4 µg/kg to 0.2 ng/kg in 2023 [1]. Unsurprisingly, more and more substitutes are emerging to replace BPA, albeit without prior research into their safety. The endocrine modulating effects of BPA and its substitutes on reproductive health have been widely studied, but its effects on immune system function are still poorly understood [2].

Besides BPA, a set of 25 substitutes was chosen based on EPA's list of BPA alternatives in thermal paper and ECHA's list of compounds for which hazards cannot be clarified due to the lack of data and analysed with Endocrine Disruptome [3] to predict *in silico* the binding affinity to 12 nuclear receptors. Based on predicted interactions with most receptors, we selected 11 BPA substitutes (BPAP, BPE, BPAP, BPG, BPP, BPPH, BPS-MAE, BPS-MPE, TCBPA, BTUM, PF201) for *in vitro* assays and examined their effects on cytokine release from THP-1 derived macrophages.

Comparing to BPA, BPP, BPPH, BPG, BPAP, BPZ, BPS-MPE and TCBPA were more cytotoxic. BPG, BPPH and BPP had IC50 values about five to ten times lower than BPA. PF201 and BPS-MAE showed comparable cytotoxicity to BPA, while BPE and BTUM affected the viability the least.

Next, THP-1 derived macrophages were pre-treated with increasing concentrations (0.01 μM; 0.1 μM; 10 μM) of BPA substitutes for 2 h and then stimulated with 10 ng/mL lipopolysaccharides for 24 h. Supernatants were collected by centrifugation and IL-1β, IL-6, IL-8, and TNFα were measured using cytokine multiplex assay accordance to manufacturer's instruction.

BPA significantly reduced IL-1β at 10 μM and similar trend was observed also for BPAP, BPG, BPP, BPPH, BPS-MAE and BPS-MPE, although not all changes at 10 μM were significant. Interestingly, BPG, BPP, BPPH and BPS-MPE elevated IL-1β in nanomolar concentrations. TCBPA, BTUM and PF201 showed different mechanism of action, as they were prone to the elevation of IL-1β in all concentrations tested. Regarding the IL-6, BPPH affected it with hormetic response by significant increase at 10 nM and decrease at 10 μM. BPS-MAE, TCBPA, BTUM and PF201 increased IL-6 at 10 μM while BPS-MPE was even more potent as it already reached significant increase at 100 nM. On the other hand, BPZ also increased IL-6 at 10 μM but also showed the decrease at 10 nM. TNF-α and IL-8 were relatively unaffected and only BPPH showed dose response inhibition of TNF-α and 10 μM TCBPA elevated IL-8.

In conclusion, several BPA substitutes were identified to influence macrophage function. In order to get a more complete picture of the immunotoxicity of BPA substitutes, the presented results will be evaluated with the results obtained on Jurkat and lymphoblastoid cell lines.

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P15-24
Contact sensitizers modulate non-coding RNA expression and secretion of proinflammatory mediators in human keratinocytes

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Keratinocytes (KC) play a crucial role in epidermal barrier function and act as sentinels on the alert for environmental insults. Alteration of the cutaneous barrier contributes to the development of skin pathologies, such as allergic contact dermatitis (ACD), occurring upon exposure to contact sensitizers (CS). We previously demonstrated that CS induces a pro-inflammatory phenotype in KC. Since non-coding RNAs (lncRNA and miRNA) are crucial in initiating early stress response during inflammation, we then study the expression of non-coding RNA in the response of KC exposed to CS in relation to the inflammatory cytokines' profiles.

Primary Keratinocytes pool (HEKp) were exposed to well-known CS [methylisothiazolinone (MIT), cinnamaldehyde (CinA),orhydroxycitronellal (HC)] for 3,6 or 24 hours. We then measured the expression of non-coding RNA and pro-inflammatory cytokines mRNAs by RT-qPCR. Extracellular expression of inflammatory cytokines has been measured using the cytokines human proteome profiler XL (R&D systems). Our results show that CS were able to induce an up-regulation of mRNA *IL-1α, IL-6, IL-8, IL-18, IL-33, IL-24* cytokines in KC. Some non-coding RNAs such as WAKMAR2 and MALAT and the miR-21-3p were differentially regulated upon CinA and HC depending on the concentration. Our preliminary data using proteome profiler analysis performed on KC supernatants showed that CinA induced the secretion of some pro-inflammatory mediators in KC (IL-1α, IL-8, MIF, osteopontin…).

In conclusion, CS are responsible for modifications in the expression of non-coding RNA in KC and the profile of pro-inflammatory mediators. We are currently investigating the mechanistic link between non-coding RNA modifications and the production of pro-inflammatory cytokines by the KC. These results will contribute to a better understanding of the early molecular mechanisms in KCs in response to chemical stress that could contribute to developing allergic skin conditions.

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P15-25
Short-term effects of polystyrene nanoplastics on lymph node endothelial cells: insights on oxidative and inflammatory potential

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Micro- and nanoplastics (MPL and NPL) have become persistent pollutants, predominantly originating from discarded packaging materials, and are undoubtedly one of the major threats to the impressive biological diversity of our planet. With sizes less than 5 mm and 100 nm, respectively, MPL and NPL can cross cell membranes and interact with cellular biomolecules and molecular processes. In this context,

the current research aims to assess the effects of polystyrene nanoplastics (PS-NPLs) on an immune system-related cell model, for understanding the involved toxicity mechanisms and implications for human health.

In this study, cells from an endothelial cell line (SVEC4-10) derived from axillary lymph nodes were exposed to different concentrations of PS-NPLs (0, 1, 10, 50, 100, and 500 μg/mL) for 24, 48, and 72 hours. Cell viability (by MTT assay), nitric oxide (NO) production (by Griess assay), as well as cell morphology (by optical microscopy), were analyzed in both treated and untreated cells (control). The cellular redox status was assessed by measuring the levels of reduced glutathione (GSH) and malondialdehyde (MDA). The protein expression of markers involved in the inflammatory response (NF-κB, IL-1β, caspase-1, and cathepsin B) was also revealed by Western blot.

The results indicated that PS-NPLs cause no cytotoxic effects at concentrations up to 100 μg/mL. However, upon exposure to a concentration of 500 μg/mL, cell viability significantly decreased by approximately 30% compared to untreated cells after 24 hours. On the other hand, at the concentration of 50 μg/mL, an elevation by 11% and 54% of NO production after 48 and 72 hours respectively, was registered in the culture media of SVEC4-10 cells, suggesting the activation of a possible inflammatory response. Microscopic analysis demonstrated cell morphological changes consisting of cell shrinkage, cytoplasmic vacuolation, nonspecific elongation, and reduced cell volume. Although the concentration of GSH has not been changed, the MDA level has increased by 38% in cells incubated with 500 μg/mL of PS-NPLs, suggesting the peroxidation of lipids and induction of oxidative stress in the presence of PS-NPLs. These findings were correlated with an elevation in cathepsin B and caspase-1 protein expression in cells exposed to concentrations of 50 μg/mL and 500 μg/mL. Up-regulation of these proteins indicates a possible activation of the inflammasome in endothelial cells. Notably, the expression levels of NF-κB and IL-1β remained unchanged under these experimental conditions.

The outcomes of the study indicated that PS-NPLs can cause an amplification of cellular oxidations and trigger inflammatory responses dependent on their concentration in endothelial cells as potential toxic mechanisms.

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P15-26
Interleukin-2 – induced vascular leak syndrome recapitulated on a patient-derived lung-on-chip

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For the development of drugs that target the immune system, such as immunotherapy, preclinical *in vitro* and animal models used for safety assessment often fail to predict complications in humans. Adverse events can occur in the main organs, among which the lungs, where drugs such as the first approved immunotherapy Interleukin-2 (IL2, Proleukin®) have led to unwanted side effects. In fact, approx. 1/3 of the patients undergoing IL2 therapy develop vascular leak syndrome (VLS), a dose-limiting side effect that increases vascular permeability and can lead to pulmonary edema and organ failure. This led to a limited clinical use of IL2 therapy but also called for the development of IL2 variants with decreased toxicity and improved anti-tumor efficacy.

To recapitulate this condition, Roche partnered with AlveoliX and developed an *in vitro* assay with the aim to benchmark such variants to IL2 regarding the VLS risk.

The AlveoliX breathing chip model of the alveolar-capillary barrier, using patient-derived primary alveolar epithelial cells (AX-hAEpC) from several donors in triple-culture with endothelial cells and peripheral blood mononuclear cells (PBMC) was treated with IL2 at physiological dose.

IL2 treatment caused the main hallmarks of VLS, interestingly, only in a proportion of tested donors: barrier impairment measured by TER and permeability assay, and cytokines upregulation detected via Luminex Immunoassay. PBMC harvested from the chips revealed significant T-cell and NK-cell activation upon IL2 treatment (flow cytometry). Moreover, baseline levels of cytokines were analysed to search for safety biomarkers candidates and/or to better understand the mechanism behind this pathological state.

As comparison, the proinflammatory control TNFα was administered to the chip barriers, and robustly caused barrier impairment and inflammation regardless of the donor, proving the donor-specificity effect of IL2.

These results highlight the predictive potential of the AX-Lung-on-chip technology, which reliably reconstructed the healthy alveolus, its impairment upon IL2 treatment, and most importantly, patient-to-patient variability as observed in the clinics. This model could therefore help advancing cancer immunotherapy drug development, contributing to selecting drugs with a better benefit/risk ratio than the initial approved therapy.

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P15-27
Does activation of N9 microglia by thimerosal increase GL261 glioblastoma cell death?

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Introduction: Microglia is a crucial glial component that has important functions in the Central Nervous System (CNS), being responsible for the activation of immune responses and playing essential role in the maintenance of brain homeostasis. Furthermore, activation of microglia is important for the removal of unnecessary cells and pathogens and thus, it also has a neuroprotective role against CNS diseases including malignant brain tumours.

Thimerosal (TM) and its metabolite ethylmercury (EtHg) are controversial compounds in terms of neurotoxicity in relation to the administration of thimerosal-containing vaccines (TCV), although epidemiological studies did not provide solid evidence of that effect in humans.

Objectives: This study aims to determine the activation and/or autophagic effects of TM and EtHg, on N9 microglia cell line and to evaluate in co-cultures of N9 and GL261 the antitumoral effects of these compounds.

Materials and Methods: The viability results were obtained through the MTT method and flux citometry (co-culture studies). The expression of IL-1β , iNOS and TNF-α citokynes was evaluated by PCR. The quantification of IL-6 and IL-10 was performed by ELISA in the co-cultures' media.

Western Blot analysis of Beclin-1 was used to quantify its expression.

Results: The viability results obtained through the MTT method, indicate that TM is the most toxic compound for N9 cells (GI_{50} 1.4 μM) in comparison to EtHg (GI_{50} 2.3 μM) at 24h.

By using PCR, it was verified a higher expression of interleukin IL-1β specially at 3h, which indicated that microglia are activated by TM

and its metabolite. In the case of iNOS, another inflammatory marker, there is also an increase of expression at 3h. TNF-α appears as a consequence of a more critical inflammation state and its concentration was very low at all timepoints studied.

To evaluate microglia autophagy the expression of Beclin-1 was determined through Western Blot analysis as this protein is responsible for the begging of autophagosome process. The results obtained at 3h and 24h, showed that TM at 2μM leads to an increase of Beclin-1 expression.

Comparing the results of GL261 monocultures with microglial co-cultures, we verified that the presence of microglia decreased even further the number of GL261 viable cells and increased the number of GL261 dead cells after 24 h of exposure. In short, it is possible to conclude that TmHg can activate microglia and this activation favors the elimination of glioblastoma cells.

Conclusions: Our study demonstrates that, TM and its metabolite EtHg, activate microglia by eliciting inflammatory responses and this new data should be considered on the TM mode of action. On the other hand, the results obtained are also be of outmost importance for the development of new therapies for glioma tumours as hard-repurposing of thimerosal as microglia activation may constitute a second line of treatment or a combination therapy with temozolomide may also be considered.

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P15-28
Classification of megakaryocytes in the bone marrow of rats with deep learning

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Due to variability in diagnostic accuracy stemming from pathologist training and experience, there exists a demand for enhanced diagnostic tools. Leveraging advancements in computer vision and artificial intelligence, digital pathology facilitates automated classification and segmentation of microscope images. In particular, the integration of artificial intelligence into digital pathology, utilizing whole slide imaging (WSI), holds significant promise in medical image analysis. In this study, we assessed the potential of employing the Inception-v3 deep learning model to classify megakaryocytes in the sternum of SD rats using WSI and evaluated its accuracy. The sternums were excised, fixed in neutral buffered formalin, subjected to decalcification, underwent processing and embedding in paraffin, followed by staining with hematoxylin and eosin (H&E). Subsequently, the H&E-stained slides were digitized into WSI, and the resulting images were resized to 79x79 pixels. These images were then partitioned into training and test datasets, comprising 13,630 and 20,790 images, respectively. The visualization of megakaryocyte-containing images within WSIs was conducted through the addition of colored markers to designated patches. Subsequently, the classification of images containing megakaryocytes was performed utilizing the Inception-v3 model, and the accuracy was determined. Upon evaluation of images depicting megakaryocytes in sternums, the model exhibited a high accuracy rate of 92.01±3.43%. In the present investigation, the Inception-v3 deep learning model demonstrated proficiency in discerning megakaryocytes from non-megakaryocytes within rat sternum tissue samples. Collectively, the deep learning model successfully differentiated megakaryocytes from non-megakaryocytes in rat sternum specimens.

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P16 | Systemic toxicology

P16-01
Potential androgenic effects induced by pure cyanotoxins (microcystin-LR, cylindrospermopsin) *in silico*, *in vitro* and *in vivo*

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Potential androgenic-disrupting properties of cyanotoxins, such as microcystin-LR (MC-LR) and cylindrospermopsin (CYN) are of concern due to their increasing occurrence, the scarcity of reports on the topic (particularly for CYN) and the impact on human's health. Thus, this work performed for the first time the *in silico* molecular docking procedure of CYN and MC-LR in complex with the androgen receptor (AR), the AR transactivation assay in AR-EcoScreen cells following the OECD test guideline 458 and the Hershberger bioassay in rats, following the OECD test guideline 441, to explore the androgenic properties of MC-LR and CYN. *In silico* results revealed potential binding complexes of both toxins with the AR. CYN demonstrated a higher propensity to form stable complexes with AR, establishing a complex with a free binding energy of -8.2 kcal/mol while when MC-LR was docked, the free binding energies were -7.2 kcal/mol. *In vitro* results showed a reduction in the AR-EcoScreen viability of more than 50% after 24 hours of exposure to 75 and 6 μM of MC-LR and CYN. Non-cytotoxic concentrations of the toxins were selected to perform the AR transactivation assays. Respect to the agonist activity, for both toxins the maximum levels of response induced were less than the 10% of the response induced by the positive control (DHT). For the antagonist assay the toxins did not cause an inhibition higher than 30% of the response induced by the positive control (HF). Therefore, CYN and MC-LR did not cause any effect to the AR *in vitro*. Regarding to the *in vivo* experiments, 2 groups of castrated rats for the agonist assay were established and treated by gavage with 75 and 300 μg/kg of MC-LR or CYN. For the antagonist assay, 3 groups treated with 75, 150 and 300 μg/kg of MC-LR or CYN and 0.4 mg/kg of testosterone propionate. The parameters measured were weight, water and food consumption, and the weight of 5 androgen-dependent tissues. In addition, the levels of thyroid hormones (T3, T4 and free-T4) were measured. Results revealed a few significant changes in some of the weights of the different tissues after the treatments with the toxins but the criteria of acceptation (significant changes in the weight of two of five tissues) were not reached. Respect to the levels of thyroid hormones, only one significant change in comparison to the negative control was found in the 75 μg/kg CYN group (agonist assay). Taken together, these results point out that MC-LR and CYN are not androgenic compounds at the conditions tested. However, more studies are necessary to determine the potential androgenic properties of the toxins.

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P16-02
Subchronic intramuscular toxicity and immunogenicity study of a plant-based SARS-CoV-2 vaccine in Sprague-Dawley rats with a 4-week recovery period

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The COVID-19 pandemic, which originated from a pneumonia outbreak in Wuhan, China, has highlighted the urgent need for effective vaccination strategies to achieve herd immunity in the absence of established treatments. In the midst of this global health crisis, the development of plant-based vaccines represents a significant innovation that offers a cost-effective and scalable solution compared to traditional vaccine production methods. Using Agrobacterium tumefaciens-engineered transgenic plants, this novel approach enables the rapid generation of vaccine antigens, including the S1 antigen of SARS-CoV-2, at reduced costs. Despite the promising advantages, research on the toxicity of plant-based vaccines is severely limited. The purpose of this study was to conduct a comprehensive evaluation of the repeated toxicity of a plant-based vaccine in Sprague-Dawley rats following three intramuscular doses administered at two-week intervals over a four-week period. Ten animals per group were sacrificed at the end of the four-week treatment period, and the five rats were sacrificed after a four-week recovery period. Mortality, clinical signs, body weight changes, food consumption, ophthalmologic examinations, hematology, serum biochemistry, urinalysis, organ weight measurements, gross finding, histopathologic examination and immunogenicity were evaluated. The results showed significant increases in anti-S1 IgG levels after vaccination, along with transient reductions in body weight and food consumption in the vaccinated groups – changes that were not considered toxicologically significant because they resolved during the recovery period. In addition, gross finding and histopathologic examination indicated moderate to mild immune responses at the injection site, consistent with expected local immune responses to vaccination and suggesting the absence of toxicological changes. In conclusion, the plant-derived vaccine studied showed no major systemic adverse effects. This research contributes to the advancement of plant-based vaccine safety and highlights its potential in addressing global health emergencies such as the COVID-19 pandemic.

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P16-03
Predicting systemic toxicity for impurities from medical devices with (Q)SAR and read-across

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The need for speed in risk assessment of chemical substances and materials urges the development, validation, and regulatory acceptance of New Approach Methodologies (NAM) as alternative to traditional costly and time-consuming animal studies [1].

In silico technologies play an important and still increasing role in NAM approaches under several regulations, including REACH, ICH M7 and the latest ISO 10997-17:2023 standard for toxicological risk assessment of medical device constituents. This can either be as a combination of *in vitro* and in silico methods, or as high quality in silico modelling and data integration.

The OECD Integrated Approaches to Testing and Assessment (IATA) (OECD, 2020) recommendations for read-across analysis and the newly published (Q)SAR Assessment Framework (OECD, 2023) guidance, provide excellent workflows for assessing the reliability and ensuring transparency and structured documentation of the in silico predicted data. This is an important step towards more widespread regulatory acceptance of data obtained by gap filling.

Read-across analysis has shown to be the preferred method for obtaining estimates of point of departures (PODs) for risk assessment purposes, in cases where no data are available for the compound of interest. In recent years, much research effort has been devoted to developing better frameworks for grouping compounds and for identifying adequate source (analogue) compounds for read-across analysis [2].

Drawing on our experience in consultancy, this poster discusses estimation of POD values for systemic toxicity endpoints. The focus is on data gap filling for repeated-dose, developmental and reproductive toxicity for impurities, such as leachables and extractables from medical devices.

Through examples, it is illustrated how adequately performed read-across analysis, assisted by (Q)SAR, provides a framework for predicting reliable estimates for POD values. More specifically, NOAEL (no-observed adverse effect level) values for systemic toxicity endpoints are predicted for “6-ethyl-m-cresol”. Considering the analysis performed and general experience with predicting POD values by read-across, the potential, pitfalls and uncertainties associated with such analysis are discussed.

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P16-04
Co-exposure of polystyrene nanoplastics and carbon nanoparticles induces abnormal neurobehavior via alteration of neurotransmitter and inflammatory markers in rats

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Background and Aim: Recently, polystyrene nanoplastic (PSNP) and carbon nanoparticles (CN), a model particle of air pollution, have emerged as environmental pollution issues. Their combined presence may concern with potential risks to organisms. However, the combined toxicity and mechanisms of PSNP and CN remain unclear. The present study was designed to investigate the effect of co-exposure to PSNP and CN on neurobehavior and possible mechanism of neurotoxicity in rats.

Materials and Methods: Four-week-old Sprague Dawley male rats were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan) and used in this study. The rats were allotted into four groups: 1) clean air and sterile water group (control), 2) clean air and PSNP group (45 nm, 50 mg/Kg, 3 d/wk for 4 wks, oral), 3) CN group (40 nm, 20 microg/m^3, 5 h/d, 5 d/wk for 4 wks, inhalation) and sterile water group, and 4) PSNP (oral) + CN (inhalation) group. After completion of exposure, at the age of 9-week-old, open field test and novel object recognition test were performed. Then, the hippocampus from each rat was collected to detect the expression level of neurotoxic marker glutamate, synaptic proteins, neurotrophic factors, inflammatory cytokines, antioxidant enzymes, microglial markers using ELISA, real-time RT-PCR and immunohistochemical analyses.

Results and Discussion: In open field test, PSNP, CN or PSNP+CN-exposed rats showed significantly decreased number of entry and time spent in the center compared to the control. In novel object recognition test, PSNP alone-exposed rats showed poor discrimination ability between familial and novel objects. Increased glutamate concentration, decreased-glutamate receptor NMDA subunits (*NR1*, *NR2B*) and transcription factors *CREB1* and *CAMKIV* mRNAs in the hippocampus were observed in CN alone or PSNP+CN-exposed rats. Expression level of anti-oxidative enzymes (SOD, CAT) were significantly lower whereas inflammatory cytokines (*IL-1β*, *TNF-α*) and microglial marker (*Iba1*) mRNAs were significantly higher in CN alone or PSNP+CG-exposed rats. Moreover, neurotrophic factors (brain derived neurotrophic factor, nerve growth factor) were significantly lower in CN alone or PSNP+CN-exposed rats. Our study revealed the synergistic toxic effects of co-exposure to PSNP and CN *compared to PSNP alone*.

Conclusion: Our results indicate combined exposure to PSNP and CN induced learning and memory impairment by dysregulation of neurotransmitter system, memory function-related gene expression, inflammatory markers, oxidative stress markers and microglia activation in the rat hippocampus.This study would be helpful to understand the association between co-exposure of environmental nano-sized particles and increasing neurological disorders like dementia and Alzheimer's disease in human.

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P16-05
Exposure to carbon nanoparticle impairs cognitive functions by modulation of neurotrophic factors in rat hippocampus

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Background and Aims: Carbon-based nanomaterials have paid a great attention in biomedical applications such as advanced imaging, tissue regeneration, and drug or gene delivery. The toxicity of the carbon nanoparticle (CN) remains unclear. In the present study, we aimed to examine the neurotoxic effects of inhalation exposure to CN using high spatial resolution phase-contrast X-ray imaging, behavioral test and gene expression assay in rat models.

Materials and Methods: Four-week-old Sprague Dawley male rats were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan) and used in this study. The rats were allotted into clean air (control) and CN inhalation exposure group (40 nm, 20 micro-g/m^3, 5 h/d, 5 d/wk for 4 wks). After completion of exposure, at the age of 9-week-old, novel object recognition test was performed. Rat's brains were extracted under deep anesthesia and fixed with 10% formalin for imaging. Crystal X-ray interferometer-based phase-contrast X-ray computed tomography (C-PCCT) was performed at High Energy Accelerator Research Organization (KEK, Tsukuba)to detect the morphological changes in the brain. Then, the hippocampus from each rat was collected to detect neurotrophic factors using real-time RT-PCR method.

Results and Discussion: In novel object recognition test, CN-exposed rats showed poor discrimination ability between familial and novel objects. In the C-PCCT image, enlarged perivascular spaces (Virchow-Robin spaces) were observed around the hippocampus fissure of CN-exposed rats. Enlarged perivascular spaces are associated with neurodegeneration and cerebrovascular pathology. Expression level of neurotrophic factors (brain derived neurotrophic factor, nerve growth factor) were significantly lower in CN-exposed rats compared to the control.

Conclusion: Our results indicate that inhalation exposure to CN impaired learning and memory functions by inducing enlarged perivascular spaces and suppressing the neurotrophic factors in the rat hippocampus.

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P16-06
Food Contact Chemicals (FCCs): risk assessment of combined exposure to endocrine disruptors chemicals

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In recent years, considering the FCCmigex database data, there has been a growing concern over Food Contact Chemicals (FCCs) in Europe, as some chemical substances have been discovered in Food Contact Articles (FCAs) made principally by paper, board and plastic. Prominent studies in Europe have identified and measured a variety of chemical compounds in FCAs that pose potential health hazards, including carcinogens, mutagens and endocrine disruptors. Thus, we performed a risk assessment protocol to evaluate the combined exposure to polyfluoroalkyl substances (PFASs) as FCCs, considering the endocrine activity as endpoint.

The first step was to use the FCCmigex infromation to identify the PFASs molecules able to migrate from paper, board and plastic materials and classified under the Regulation CE 2008/1272 CLP (Classification Labelling and Packaging) as Endocrine Disruptors Thyroid Chemicals (EDTCs).Further, Material Exposure Scenarios (MESs) were created for each material (paper, board and plastic) under investigation, using the worst-case approach which involves the highest concentrations observed/detected for the EDTCs investigated.

The level of concentration was referred to EDTCs migrated into food and/or food simulants and we ruled out the data came from extraction procedure, focusing on the repeatedly used FCAs.The Hazard Index (HI), Target-Organ Hazard Index (Target-Organ HI) and the Combined Margin of Exposure (MOE(T)) methodologies were employed for the EDTCs selected and included into the MESs. To this end, the selection of the dose descriptor/s was/were a crucial aspect of the overall procedure.

The NOAEL, LOAEL, BMD and, in case of availability DNEL/DMEL as defined in the Regulation CE 2006/1907 REACH (Registration Evaluation Authorization of Chemicals), were used together with the level of exposure esteemed, assuming the 100% as GI adsorption.

The investigation revealed a high potential of endocrine disruption activity at the Thyroid level, also considering low concentrations of the PFAS (EDTCs) investigated.

The data highlighted a concrete endocrine risk and, considering the relevance of the data, at the end of the theoretical procedure we deemed necessary to refine the level of GI adsorption by physico-chemical parameters. Further, we planned MTT/CDK8, Live and Dead cell and Lactate dehydrogenase assays to improve the cell proliferation and cytotoxicity data in order to thoroughly investigate potential thyroid neoplastic lesion as well.

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P16-07
Toxicological evaluation of anisotropic cross-linked polyvinyl alcohol hydrogels intended for humic substances immobilization and topical skin disease treatment

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Most skin diseases and wounds progress through four main phases of healing: coagulation, inflammation, proliferation and remodeling. The prolonged inflammation stage leads to chronic skin diseases and wounds which are usually characterized by disorders of skin microbiome, high oxidative stress and are additionally infected with biofilm bacteria, creating additional barriers to wound healing [1]. The most prospective way to treat such chronic skin diseases is topical dressings with antioxidants and antimicrobial substances combinations and simultaneous photothermal therapy features [2]. In the current study, we propose biocompatible polyvinyl alcohol (PVA) cross-linked hydrogels as a base for new topical skin disease remedies, in combination with humic substance complexes as prospective candidates with antioxidant and anti-inflammatory activities, possibly combined with light-based photothermal therapy. Humic substances (HS) are natural organic high-molecular compounds with a wide range of biological effects [3]. To use HS as components for novel skin disease treatment hydrogels, the key task is to study their safety and biological effects. Several studies have shown that HS does not have a toxic effect on the organs and systems of experimental animals, which makes them promising targets for our further investigations [4]. By now, we have prepared hydrogels from PVA acylated derivative with a content of unsaturated groups of 4.5%. The concentration of the polymer solution was 10%. The “hydrogen peroxide – Mohr’s salt” system was used as the initiator of radical polymerization during cross-linking.To assess the toxicity of the hydrogel samples obtained as a result of cross-linking, a set of methods was used, such as: studying hemolytic activity (hemolytic test), and cytotoxic action while determining the toxicity index when exposed to a biological cellular test object. The hemolytic effect of aqueous extracts was assessed in *in vitro* experiments with isolated rabbit erythrocytes. The percentage of hemolysis of extracts of all samples did not exceed values from 0 to 0.15%, with an acceptable value of 2%. Cytotoxicity was assessed on a short-term suspension culture of motile germ cells prepared from frozen cattle semen and no negative impact on the viability of the cell test was observed (toxicity index was 75.0–88.0%). The acute toxicity, irritant and sensitizing activity of the polymer hydrogel samples were evaluated *in vivo* on mice. The animal experiment was carried out according to all needed ethical rules and sanitary standards. No death or external manifestations of intoxication were registered. There was no skin reaction at the application sites and at the site of preliminary sensitization and provocative intradermal test. The prospective investigations will be directed to the preparation of HS-loaded PVA cross-linked hydrogels and highlighting their therapeutic potential as a topical remedy for skin disease treatment.

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P16-08
Pseudomonas aeruginosa mannose-sensitive-hemagglutinin (PA-MSHA) causes anemia in rats after subacute exposure

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Pseudomonas aeruginosa mannose-sensitive-hemagglutinin (PA-MSHA) is adjuvant cancer therapy with immune-modulating properties. Clinical studies indicate its ability to enhance chemotherapy efficacy and stimulate the immune system against cancer, with mild-to-moderate immune-related side effects such as fever and skin irritation. However, the safety profile of PA-MSHA is not well understood and requires further investigation. Hence, the current study aimed to explore the effects of PA-MSHA on parameters of anaemia in a rat model after 28 days of exposure. Moreover, the study intended to explain gender-specific differences when animals were exposed to three increasing concentrations of PA-MSHA (PA-MSHA 1 (0.09 x 10^{11} CFU/ml), PA-MSHA 2 (1.8 x 10^{11} CFU/ml), and PA-MSHA 3 (3.6 x 10^{11} CFU/ml)), administered intraperitoneally once a week on the 1st, 7th, 14th, and 21st day. At the end of the study period, blood samples were obtained through cardiac puncture and further used for subsequent haematological analysis. The results revealed PA-MSHA’s potential to cause anaemia in a dose- and gender-dependent manner. In females, both PA-MSHA 1 and

PA-MSHA 2 significantly decreased the level of erythrocytes and haematocrit, while in males the drop of these parameters was seen only in PA-MSHA 3 group. The PA-MSHA treatment did not impact haemoglobin levels in both genders. However, while male rats experienced a significant dose-dependent decrease in iron blood levels in all investigated groups, the iron levels in female rats did not differ from the control. Mean Corpuscular Volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) parameters were also measured. Male rats showed a dose-dependent increase only in MCHC, while female rats experienced an increase of MCH in the PA-MSHA 2 group and MCHC in all investigated groups. Finally, conducted Benchmark analysis (PROAST Web, version 70.1; https://proastweb.rivm.nl/) revealed that lower concentrations of PA-MSHA can cause a 5% change in effects level related to anaemia in female rats compared to male animals. Calculated BMDL concentration for erythrocytes and haematocrit levels were, respectively: 2.5e-05 x 10^{11} CFU/ml, 7.06e-6 x 10^{11} CFU/ml for female rats and 0,000858 x 10^{11} CFU/ml and 9.39e-05 x 10^{11} CFU/ml for male, while BMDL for MCHC was 0.00947 x 10^{11} CFU/ml in female animals and 0.0234 x 10^{11} CFU/ml in males. These findings suggest gender-specific responses to varying doses of PA-MSHA and further underscore the significance of taking gender-specific factors into account when designing and analysing therapeutic approaches that involve PA-MSHA.

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P16-09
Comparison of mRNA vaccine toxicology evaluation in the rabbit and minipig

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Vaccines developed using mRNA/LNP have tremendously increased in recent years. Numerous studies have demonstrated the considerable potential of mRNA vaccines to elicit protective immune responses against pathogens or cancers in non-clinical studies and clinical trials. Non-clinical studies with vaccine candidate are usually performed in rabbit or rodent species. In this work, we investigated whether an alternative species, the minipig, would enable better transposition thus prediction of the vaccine effects in humans. Therefore, local and systemic tolerance of an mRNA vaccine formulation was evaluated in minipigs and results were compared with those from rabbits, and the most relevant biomarkers and endpoints for each species were evaluated.

The vaccine formulation tested in the minipig and rabbit was a modified mRNA, encoding for an antigen, encapsulated in a proprietary lipid nanoparticle. Vaccine formulation at 45 µg mRNA/dose (minipig) or 50 µg mRNA/dose (rabbit) was given twice or once, respectively, by intramuscular injection, in a dose volume of 0.5 mL (human dose volume). The following features/parameters were monitored in both species: clinical observations, injection site observations, body weight, food consumption, body temperature, clinical pathology (hematology, coagulation, clinical chemistry, C-Reactive Protein and Haptoglobin, and PIG-MAP in minipigs only), immunogenicity, macroscopic and microscopic observations.

The results showed few differences between the 2 animals models after intramuscular injection of mRNA vaccine formulation. Local findings at the injection site (in-life and at terminal necropsy 2 days after injection), an increase in body temperature and changes in some clinical pathology parameters were noted in the minipig but not in the rabbit, and they were all considered to be related to the inflammatory response, as anticipated following a vaccine injection. Similar microscopic changes at the injected site were noted in both species, but with a slightly higher severity at terminal sacrifice (2 days after injection) in the minipig compared with the rabbit. Increased cellularity of the germinal centers, considered to reflect the vaccine-related stimulation of the immune system, was observed 2 days after injection in the minipig only (lymph nodes draining the injection site), and 2 or 4 weeks after injection in the lymph nodes (both species) and spleen (rabbit only). Biomarkers of inflammation such as CRP provided a sensitive indication of the acute phase of inflammation for both species. In addition, PIG-MAP also appeared to be a sensitive marker of inflammation in the minipig. These results confirm that the minipig can alternatively be used for mRNA vaccine toxicology evaluation, including a panel of inflammatory parameters.

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P16-11
Effects of subacute oral exposure to sulforaphane on hematological parameters in rats

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The rising incidence of non-communicable diseases and the decreasing effectiveness of traditional therapies have led to increased focus on utilizing numerous plant-derived chemicals (phytochemicals) believed to have significant positive effects on disease prevention and therapy efficacy for various ailments. One of these chemicals is sulf raphane (SFN), an isothiocyanate commonly found in broccoli, which represents a promising phytochemical with chemoprotective, antioxidant, and anti-inflammatory effects. However, the potential negative effects, especially for synthetic SFN, as well as the appropriate doses for safe use in therapy, are still not sufficiently investigated. Hence, this research aimed to investigate the impact of increasing doses of SFN on hematological parameters in subacutely treated rats (28 days) and to assess the safety profile for therapeutic application of synthetically obtained SFN. Male and female Wistar rats were divided into four groups (n=5) – control group treated with deionized water, SFN1 group receiving 0.5 mg/kg body weight/day, SFN2 group receiving 2 mg/kg body weight/day, and SFN3 group receiving 5 mg/kg body weight/day of SFN. Animals were euthanized after 28 days of oral administration. Blood samples were collected for analysis of hematological parameters. In male rats, a statistically significant decrease in the percentage of basophils in the SFN1 group and the absolute number of basophils in the SFN2 group compared to the control group was observed, suggesting a modulation of allergic or hypersensitivity reactions after the exposure to SFN. Additionally, percentage of lymphocytes was increased in SFN3 group compared to the control, suggesting a heightened immune response. Unsaturated iron-binding capacity, UIBC values were significantly higher in the SFN2 group compared to the control, signifying alterations in iron metabolism, possibly indicating changes in iron absorption or utilization. The alterations in hematological parameters among females showed significant divergence from those observed in males and changes in less parameters were observed. In females, a statistically significant increase in the absolute number of neutrophils was observed in the SFN3 group compared to the con-

trol, which could imply an enhanced immune response to the treatment, similar to the leukocyte increase observed in males. The conducted *in vivo* study revealed significant alterations in hematological parameters in response to varying doses of sulforaphane (SFN) in male and female Wistar rats, suggesting potential gender-specific effects and highlighting the need for further investigation to elucidate the safety profile and therapeutic potential of chemically synthesized SFN.

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P17 | Epidemiological toxicology studies

P17-02
Study of forensic research via omics markers in environmental health vulnerable area

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Around the world, the needs for research on environmentally vulnerable areas is emerging. In this regards, this research group (FROM) aimed to develop biomarkers of exposure to environmental hazards, to assess environmental diseases, and verify them in the environmentally vulnerable areas.

Environmentally vulnerable areas, such as abandoned metal mine, refinery were chosen. Between June 2021 and October 2023, a total of 1,157 adults who had lived in the areas over ten years were recruited (Exposed area: refineries, abandoned metal mines, coal-fired power plant, Waste incinerator, cement factories etc. Control area: areas without facilities with environmental vulnerability) and epidemiological investigations were conducted. Personal characteristics such as demographics, dietary habits, and disease history were collected via survey questionnaires. Blood and urine samples were also obtained. Using the biological samples, environmental chemicals (i.e. heavy metals, Organic compounds, adducts) were analyzed and epigenomes, transcriptomes, proteomes, and metabolomics were performed for investigating the exposure markers.

The sex distribution in the exposed area (men: 34.8%, women: 65.2%) was similar to that in the control area (men: 38.2%, women: 61.8%). The mean age in the exposed area (70.4 years) was higher than in the exposed area (68.7 years). In the diagnoses of chronic diseases, pulmonary diseases, asthma, allergic conditions, and arthritis showed higher rates of diagnosis in the exposed area compared to the control area. Blood lead (exposure: 1.91 μg/dL, control: 1.26 μg/dL), blood cadmium (exposure: 1.41 μg/L, control: 1.05 μg/L), urinary cadmium (exposure: 1.82 μg/g cr, control: 1.50 μg/g cr), and urinary total arsenic (exposure: 162.58 μg/g cr, control: 156.62 μg/g cr), were higher in the exposure area than in the control area.Monitoring for the environmentally vulnerable areas are required. The epidemiological information as well the discovered biomarkers will be used to evaluate the risk of the occurrence of environmental diseases, and to assess the impact on residents' health in environmentally vulnerable areas.

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P17-03
Toxic and essential metals in breast milk: Preliminary results from Bosnia and Herzegovina

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Results of numerous studies have shown many benefits of breastfeeding for infants, and mothers as well. Breast milk is optimal, natural food for infants, but at the same time different organic and inorganic pollutants from the environment, including toxic metals, can be excreted in breast milk. Presence of toxic metals in breast milk does not necessarily mean that toxic effects will occur, but since there is a biological probability, their content should be continuously monitored. Breast milk is a significant indicator of the mother's exposure to metals during the prenatal period, and at the same time it can be a source of exposure for the infant. Toxic compounds exposure assessment and related health effects, especially in sensitive population groups, are public health priorities.

In this study cadmium (Cd), copper (Cu), iron (Fe), lead (Pb), mercury (Hg), manganese (Mn), and zinc (Zn) were determined in samples of human milk in order to assess infants' exposure. The nationally representative sample (N=48) was collected in accordance with official methodologies, respecting ethical standards for this type of research, taking into account regional and socio-economic aspects. The content of selected metals in the samples was determined using the atomic absorption spectroscopy method, the graphite furnace technique (GFAAS) for Pb, Cd and Mn; flame AAS for the Cu, Fe and Zn, and the FIAS (Flow Injection for Atomic Spectroscopy System) for Hg, after wet digestion with the microwave system.

The average content (range) of analyzed metals was 0,917 μg/kg (0–2,0 μg/kg); 225,9 μg/kg (0–746,0 μg/kg); 143,3 μg/kg (0–1162 μg/kg); 0,391 μg/kg (n.d.–3,0 μg/kg); 0,231 μg/kg (n.d.–1,0 μg/kg); 6,313 μg/kg (0–106,0 μg/kg) and 1927,7 μg/kg (56,0–3 619 μg/kg) for Cd; Cu; Fe; Pb; Hg; Mn and Zn, respectively. The estimated intake levels for both, non-essential (Cd, Pb, Hg) and the essential (Cu, Fe, Mn, Zn) elements were within the safety limits.

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P17-04
Associations between PFAS serum levels and breastfeeding during the first three months in Swedish first-time mothers

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PFAS exposure is associated with reduced breastfeeding duration in humans and with insufficient mammary gland development in mice. The aim of the present study was to examine associations between maternal serum PFAS levels in Swedish first-time mothers and both mastitis and extent of breastfeeding during the first three months. In addition, we investigated if serum PFAS levels were associated with volume of milk donated by the mothers.

In total, 869 first-time mothers from Uppsala County, Sweden were recruited during 1996–2022 within the Persistent Organic Pollutant in Uppsala Primiparas (POPUP) study. PFOA, PFNA, PFDA, PFUnDA, PFHxS and PFOS were analysed in serum sampled three weeks after delivery. During this week the mothers sampled breast milk daily, storing it in glass bottles in the freezer in their homes. Three months after delivery the mothers answered questionnaires about infections during the first three months and the extent of breastfeeding for each week during this period (full nursing, partial nursing or no nursing). Occurrence of mastitis (yes or no) and breastfeeding duration (full nursing or partial/no nursing) were evaluated using logistic regression analysis with ln-transformed serum PFAS levels, adjusted for the potential confounders age, pre-pregnancy BMI, weight gain during pregnancy, weight reduction after delivery until sampling, education level, smoking and fish consumption. The total volume of donated breast milk (a proxy for milk production) were divided into quartiles in the statistical analysis and the association between quartiles and PFAS levels were evaluated using ANCOVA and a linear model, adjusted for the same variables as above. POPUP is a temporal trend study and mothers have been recruited for 27 years, therefore a second statistical analysis including "sampling year" in the models was made as a sensitivity test.

The odds ratio for occurrence of mastitis was 0.59 (95% CI 0.36/0.97) for PFOA but the association was not significant after adjustment for sampling year. No significant associations were observed for other PFAS. The odds ratio for partial nursing was 0.72 (0.56/0.93) for PFOS but after adjustment for sampling year the association were no longer significant. No significant associations were found between breastfeeding duration and maternal serum levels for the other studied PFAS. Mothers who donated the highest breast milk volumes (the highest quartile) had significantly higher levels of PFOA, PFOS and PFAS4 (sum of PFOA, PFNA, PFHxS and PFOS) compared to those donating the lowest volumes (the lowest quartile), which became non-significant when sampling year was included in the statistical models.

The results showed no association between maternal PFAS exposure and reduced breastfeeding ability. However, only mothers with functional breastfeeding were recruited in the study, therefore the results may not be representative for the entire population of first-time mothers in Uppsala County.

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P17-05
The T cell response to SARS-CoV-2 mRNA vaccine in adults with high exposure to perfluoroalkyl substances from Ronneby, Sweden

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Background: Perfluoroalkyl substances (PFAS) have been associated with impaired antibody levels after childhood vaccinations [1–3] and immunosuppressive effects [4] in animals. However, the *in vivo* effects of PFAS on antigen specific human T cell responses have not been investigated in adults. In Ronneby, Sweden, the drinking water of one of the water works was previously highly contaminated with primarily perfluorohexane sulfonic acid (PFHxS) and perfluorooctane sulfonic acid (PFOS) [5]. The COVID-19 vaccination scheme presented the possibility to assess antigen specific T cell function after vaccination in adults with high PFAS serum levels.

Objectives: To investigate the relationship between PFAS exposure and T cell responses, in a population with a wide range of PFAS exposure levels, after administration of two doses of COVID-19 mRNA vaccine.

Methods: This is a nested cohort study within the PFAS Immune Response After COVID-19 Vaccination cohort (PIRVACoV) [6], that includes 116 COVID-19 naïve individuals from Ronneby and a reference group with background PFAS exposure. All participants received two doses of Spikevax® (Moderna) vaccine. Blood T cells were stimulated with overlapping peptides based on the SARS-CoV-2 spike protein and their production of the cytokines IFN-γ, IL-2, and TNF were measured. Adjusted mixed linear regressions were fitted against measured, address-based and prenatal PFAS exposure indices.

Results: PFAS median serum levels differed greatly between participants ever having had contaminated drinking water at home (PFOS 47 ng/mL, 5th to 95th percentile 6–221 ng/mL) and the background group (PFOS 4 ng/mL, 2–9 ng/mL). PFAS exposure was not associated with T cell cytokine responses (e.g., measured PFOS to IFN-γ: +3% per interquartile range PFOS, 95% confidence interval: -10, 17).

Discussion: This study indicates, in concordance with the PIRVACoV antibody study and other antibody PFAS/COVID-19 studies [7–9], that PFAS exposed, healthy adults mount adequate immune responses to mRNA COVID-19 vaccination.

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P17-06
Development and health of children 3-5 years of age in relation to exposure to environmental contaminants

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Background: Exposure to Persistent Organic Pollutants (POPs) during fetal and early life can affect child development/health. POPs include lipophilic compounds (e.g. organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), and brominated flame retardants (BFRs)) and amphiphilic compounds, e.g. per- and polyfluoroalkyl substances (PFASs).

POPs are resistant to degradation and bioaccumulate ubiquitously in the environment, animals, and humans. Humans are exposed to POPs through food intake, but exposure by use of personal care products, other consumer products, and electronic devices can also occur.

The Inuit population is at substantial risk of POP exposure due to their traditional food intake, including marine mammals (whales and seals) and seabirds high on the food chain. Even though the intake of traditional food has declined in Greenland, high levels of POPs are still observed in the population. POPs are transferred through the placenta and breast milk to developing offspring.

Aim: The present study examines the associations between POP exposure and development and health in children (3–5 years) of the ACCEPT cohort.

Methods: The ACCEPT birth cohort was established 2010–2015 as a prospective mother-child cohort in Greenland. Of the 614 ACCEPT pregnant women, 102 families (mothers, fathers, and children) were followed up 3–5 years after birth. The POPs (serum) and heavy metal (whole blood) levels were measured in mothers at pregnancy, and at follow-up for both the mothers and fathers. The mercury levels in the children's hair were also measured. Parents answered questionnaires on the children's development (motor and behavioral) and health (infections, allergies etc.), and anthropometric data and blood pressure were measured. The associations between POP exposures and child health outcomes were assessed with linear and logistic regressions with adjustment for relevant confounders.

Results: Prenatal exposure of OCPs associated significantly with increased risk of behavioral problems and hyperactivity in the children. We found no consistent evidence of associations between PCBs, PFASs, or heavy metals and behavioral problems and hyperactivity. The PCB and OCP levels were also associated with higher risk of child referral to a psychologist.

Mothers and fathers' blood arsenic levels at follow-up were positively associated with the time that the child start standing. The mother's blood lead level at follow-up and the child mercury hair level were also positively associated the time that the child started standing.

Mothers blood mercury levels at follow-up were negatively associated with the children's diastolic blood pressure, and a similar tendency was seen for the association between child hair mercury level at follow-up and diastolic blood pressure.

Conclusions: Exposure to POPs and heavy metals in fetal and early life can affect the child development and health at 3–5 years of age and may affect the child later in life as well.

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P17-07
Prenatal exposure to acrylamide and metabolic status at 20 years of age: a prospective biomarker-based cohort study from Denmark

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Background: Obesity and metabolic diseases in adulthood may be influenced by prenatal exposure to dietary factors. Acrylamide (AA) forms in a wide variety of commonly consumed carbohydrate-containing foods during frying or baking at high temperatures. Prenatal exposure to AA is of concern as AA is neurotoxic and classified as a probably carcinogenic in humans. AA crosses the human placenta and maternal intake of AA from diet during pregnancy has been associated with low birth weight and childhood overweight/obesity. AA disrupt metabolic homeostasis and induces adiposity in offspring of mice following gestational exposure, but the evidence from studies with hemoglobin (Hb) adducts (internal dose)in adult humans are mixed and it is unknown if prenatal exposure to AA increases the risk of development of obesity and related metabolic outcomes in adult humans.

Objectives: We aim to examine associations between prenatal exposure to AA and metabolic outcomes measured in offspring at 20 years of age in a biomarker-based cohort study.

Methods: Hb adducts were measured in blood from pregnant women with singletondeliveries in 1988–1989 in Aarhus, Denmark. Information on maternal diet, lifestyle and socio-economic status was collected from questionnaires in gestational week 30. Offspring weight, waist circumference and height were recorded (n=586) and biomarkers of adiposity and cardiometabolic risk factors including blood pressure, insulin, leptin, adiponectin and lipid concentrations were measured in fasting blood samples collected from a subset of the offspring at 20 years of age (n=374) in 2008. We fitted AA adduct levels and the met-

abolic outcomes as continuous scale variables in multivariable linear regression with adjustment for smoking, education, and income to examine the relation between prenatal exposure to AA and metabolic outcomes.

Results: AA was detected in all women with a medium level of 84 (range: 27–650) pmol/g Hb. Higher prenatal exposure to AA was associated with larger waist circumference and higher levels of low-density lipoprotein cholesterol at age 20. For every 10-pmol/g Hb increase in adduct levels the mean low-density lipoprotein cholesterol concentration was increased by 0.01 (95% confidence interval: 0.0003, 0.03) mmol/l. These preliminary analyses, however, do not suggest that higher AA levels were associated with higher offspring BMI, weight, blood pressure, insulin, leptin, adiponectin, high-density lipoprotein cholesterol and total cholesterol. Analyses with splines, categorical exposure and outcomes variables e.g. obesity, sex-stratified analyses and analyses restricted to offspring of non-smokers are ongoing.

Conclusions: Our study provides some evidence that prenatal exposure to AA increases the risk of large waist circumference and of levels of low-density protein cholesterol.Further studies are needed to fully understand the potential role of prenatal AA exposure, if any, in the development of poor metabolic health.

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P18 | Clinical toxicology

P18-01
A 2-year review of severe poisonings referred to a hospital based poison service

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Objective: Historically poisoning accounts for 1 percent of all emergency department (ED) visits at this cluster of hospitals [1]. This study aims to better appreciate the demographics of severely poisoned patients who were co-managed by toxicologist from a newly established hospital cluster-based poison consultation service in collaboration with referring hospital specialists.

Method: A retrospective chart review of all patients referred to the hospital cluster-based poison service from May 2019 to April 2021. Severe poisoning cases were identified if patient required admission or transfer to intensive care units (ICU) or other high acuity areas such as high dependency (HD) at any point during their hospital stay. Patient demographics, exposure patterns, clinical presentation, interventions received, and outcome are presented.

Results: A total of 151 severe poison cases consulted over the 2-year period accounting for 16.6% of all calls to the toxicology service. Patients' average age was 42 (range 1–95 years), 58.3% male. Most calls were initiated from the ED (86.1%) and majority patients conveyed by ambulance (67.5%). Predominantly intentional poisoning (68.9%) with oral route of exposure accounting for most (84.8%) with 4% presenting within the hour post ingestion. Most (45%) had consumed one toxin with sedatives hypnotics, cardiac medications, antidepressants, and antipsychotics being predominantly involved.

Patients who were admitted to HD/ICU spent a median of 2 days there (IQR=1–3.25days, Range=1 to 17days). 13 cases (12 from general ward and one from emergency short stay unit deteriorated) soon after admission requiring transfer to ICU (4 cases)/HD (9 cases). All these patients survived.

Therapeutic interventions included activated charcoal for decontamination (17.9%), enhanced elimination (8.6% with 10 cases requiring haemodialysis, 2 cases requiring urine alkalinization and 1 case given multidose activated charcoal) and antidotes (54.3%). 31.8% were intubated, 17.2% had inotropic support and 3 cases (2%) underwent ECMO. There were 9 deaths in this cohort of patients.

Conclusion: Severe poisonings involved young males with sedative hypnotic, cardiac medications, and antidepressant / antipsychotics toxins commonly involved. Majority had a short length of stay in ICU/HD with quick turnaround times demonstrating the severe but transient nature of toxic effects. The treating physician should be mindful of the potential for unanticipated deterioration of poisoning cases and hence have a high level of vigilance which is critical for patient safety.

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P18-02
Evaluation of clinical and laboratory data in the acute thallium poisoning diagnosis (group criminal case)

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Among acute criminal metal poisonings, thallium (TI^+) compounds have prevailed over the past 20 years. In most cases, acute TI^+ poisoning is diagnosed late, because usually the first 5–7 days the victims have symptoms of food infection. Thus, late treatment leads to a high mortality rate of victims. The problem of poisonings has also become relevant in connection with russia's terrorist activities in Ukraine.

Aim: Based on the analysis of clinical, laboratory and instrumental data, identify early diagnostic criteria of acute TI^+ poisoning to optimize diagnosis and treatment.

Material and methods: Six victims of criminal acute TI^+ poisoning after consuming alcohol containing it were examined. Laboratory tests were performed to determine the TI^+ concentration of the alcohol, its level in the blood and urine of the patients (atomic emission spectrometry method, "Shimadzu ICPE-9820), biochemical analysis of blood and urine. Clinical examination, electroneuromyography and pathological examination of organ tissues were performed.

Results: Of the six poisoned patients (5 women, 1 man who later died), 4 were admitted to the hospital with pronounced signs of poisoning: nausea, vomiting, headache, abdominal pain, diarrhea, fever. Chemical analysis of the poisoned alcohol showed a TI^+ concentrations 10,011–10,035 mg/l. In women the level of TI^+ in the blood was 30,0–460,0 μg/l (depending on the volume of alcohol consumed), in the urine 2710,0–7640,0 μg/l. In men who drank 2/3 of a bottle of alcohol, TI^+ concentrations was 1019,0 μg/l and 9090,0 μg/l respectively. The most pronounced clinical symptoms in all victims were: gastrointestinal disorders, painful paresthesias in the limbs and oral cavity, intence burning pain in scrotum and mammary glands in the first 2 days. On the 3–4th day, head paresthesias and skin pigmentation appeared. Diffuse alopecia, mainly in the parietal-occipital region, began on the 6–8th day after poisoning. Ataxia, dysarthria, paresis of upper and especially lower limbs, decreased visual acuity joined after 8–10 days. In patient who later died, laboratory tests showed toxic hepatopathy, nephropathy with the formation of acute renal failure, and signs of

pulmonary and cardiac failure. Due to suspected TI^+ poisoning all patients were started on treatment with the antidote Ferrocyne in a dose 3,0–6,0 g per day, that saved the lives of four of the five victims.

Conclusions: Early diagnosis of TI^+ poisoning is essential for effective treatment. Evaluation of clinical symptoms in the first days of the disease is extremely important for diagnosing intoxication before the onset of alopecia, as well as before obtaining laboratory tests of TI^+ concentration in the blood and urine.

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P18-03
Ferroptosis mediated by mtROS is involved in the Ag2Se QDs induced NLRP3 inflammasome activation in BV2 cells

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Silver selenide quantum dots (Ag2Se QDs), show great advantages in the imaging of deep tissues and tiny vascular structures and therefore its biosafety has attracted wide attention. Previously, we showed that Ag2Se QDs increased IL-1β secretion in the hippocampus of mouse in acute toxicity tests. Therefore, finding targets to reduce IL-1-mediated inflammatory responses has become the next research focus. In this study, using microglial BV2 cells, decreased cell viability, the cytosolic iron overload, glutathione (GSH) depletion, lipid peroxidation (LPO) were observed after Ag2Se QDs treatment. The pre-treatments of a specific ferroptosis inhibitor Ferrostatin-1 (Fer-1) and an iron chelater Deferoxamine mesylate (DFO) not only inhibited cell death, but also alleviated iron overload, LPO and alternations in ferroptosis biomarkers in BV2 cells. Meanwhile, Fer-1 and DFO inhibited the activation of NLRP3 inflammasome and IL-1β release. This indicates Ag2Se QDs-induced ferroptosis promotes NLRP3 inflammasome activation and IL-1β release. We then explored the mechanism of ferroptosis. Mitochondria-targeted ROS scavenger (Mito-tempo) improved mitochondrial antioxidant capacity, reversed biological changes of ferroptosis in BV2 cells, suggesting that Ag2Se QDs-induced microglial ferroptosis could be attributed to mitochondrial oxidative stress. Mito-tempo also reversed NLRP3 inflammasome activation and IL-1β release. In summary, Ag2Se QDs exposure causes ferroptosis in BV2 cells by inducing mitochondrial oxidative stress and ultimately leads to NLRP3 inflammasome activation and IL-1β release. Our study provides a basis for alleviating the hippocampal inflammatory response induced by Ag2Se QDs in biological imaging, and also extends the role of ferroptosis in inflammatory responses triggered by quantum dots.

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P18-04
Ketoprofen lysine salt has no gastroprotective effect in comparison with ketoprofen in femalerats after ethyl alcohol intoxication

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Ketoprofen lysine salt is a new non steroidal antiinflammatory drug (NSAID) competing with ketoprofen on the market. The former is believed to have gastroprotective properies, the latter to kill acute pain. In East Europe binge drinking and taking NSAIDs after is common. The aim of the study was to verify the hypothesis about the gastroprotective effect of ketoprofen lysine salt after exposure to 50% alcohol. The experiment was carried out on 36 female Wistar rats divided into 6 groups of 6:

1. ethanol 50%
2. NaCl 0.9%
3. NaCl 0.9% and ketoprofen
4. ethanol 50% and ketoprofen
5. NaCl 0.9% and ketoprofen lysine salt
6. ehtanol 50% and ketoprofen lysine salt

On day 7 animals were sacrificed. Their blood was obtained to measure blood morphology and biochemical parameters. Stomachs were dissected for histopathological examination. Microscopic examination of stomachs from groups 1,3,4,5,6 revealed non-specific, high- grade lymphocytic-plasmocytic inflammation of the gastric mucosa. Ketoprofen and alcohol limited animals body mass gain (p<0.05 vs ethanol) and lowered albumin concentration (p<0.05 vs ketoprofen). Conclusions Ketoprofen lysine salt and ketoprofen damage gastric mucosa in female rats after and without alcohol intoxication. Ketoprofen lysine salt has no gastroprotective effect.

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P18-05
Modern aspects of the use of poisonous substances in the war zone in Ukraine

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Over the past decades, fundamental changes have occurred in the weapons systems of many countries worldwide. New special tools based on irritants, algogens, and malodorants have been created, and the tactics of their use have changed. Starting from February 24, 2022, Ukraine constantly records cases of the Russian army using these chemicals in the war zone in Ukraine.

The aim is to analyze the nature of chemical incidents associated with the Russian Federation's use of irritants, malodorants, and phosphorus in the war zone.

Materials and methods: analysis of sources of open information and operational data of units of the Defense Security Service of the Armed Forces of Ukraine, the Security Service of Ukraine, and the Ministry of Foreign Affairs of Ukraine regarding the use of chemical substances by the Russian Federation's army in the war zone.

Results: As of January 1, 2024, hundreds of cases of the use of chemical substances by the Russian army were recorded. According to the results of research on the remains of explosive devices, chemical substances were found that mainly belonged to substances with an irritating effect. In a separate case, malodorants were found. These substances are considered non-lethal weapons. However, they are capable of causing a temporary loss of combat capability of personnel, which poses a direct threat to the health and life of soldiers. During the study of cases of chemical damage to the personnel, there were noted cases of irritants getting into the eyes, resulting in severe burns of the cornea and burns of open areas of the skin. When providing first aid, it is necessary to consider the conditions of using poisonous substances with an irritating effect and the speed of manifestation of symptoms. All mechanisms of action of irritants, algogens, malodorants, etc., lead to excitation of the nociceptive system, which causes local sensations of irritation and pain, as well as reflex motor, secretory, vegetative, and somatic reactions. Therefore, although the duration of action of irritating poisonous substances is not long, their sudden use during an enemy attack can negatively affect the performance of military tasks. Cases of the use of phosphorous ammunition and other incendiary mixtures by the army of the Russian Federation, which caused III-IV degree burns to the soldiers, are constantly being recorded in the war zone. Mixtures of highly toxic chemicals and their combustion products pose a separate threat.

Conclusion: The enemy's use of combat poisons of non-lethal action on the battlefield (particularly of irritant action) requires readiness for immediate use by the personnel to protect the respiratory and visual organs, especially the ability to provide first aid. Another important aspect is the recording of the facts of chemical attacks and the thorough collection of evidence to hold the Russian Federation accountable for war crimes committed on the territory of Ukraine.

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P18-08
Novel metabolic signatures in renal cell carcinoma for precision diagnostics and therapeutics

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Introduction: Renal cell carcinoma (RCC) is characterized by alterations in angiogenesis, energy metabolism, and redox regulatory pathways [1]. This heterogeneity poses a challenge in achieving durable responses to treatment, despite advances in targeted and immunotherapeutic agents [2]. Understanding the unique metabolic features of RCC is critical for refining diagnostic tools and identifying novel treatment options. This study used a metabolomics approach to map the metabolic reprogramming that occurs in RCC tumors and to explore how these changes correlate with urinary phenotypes.

Methodology: Matched tumor and non-tumor kidney tissues were collected from 18 patients submitted to nephrectomy at the Portuguese Oncology Institute of Porto (IPO-Porto). Urine samples were also collected from the same patients. Ethical approval was obtained from the Ethics Committee of IPO-Porto (238/2018), and written informed consent was obtained from all participants. Metabolites of both tissue and urine samples were extracted using a methanol-water method, followed by gas chromatography-mass spectrometry (GC-MS) analysis. Statistical analyses, including multivariate and univariate methods, and pathway analysis were performed to evaluate the metabolic dysregulations associated with RCC. Correlation analysis was performed between the significantly altered metabolites in tissue and metabolites identified in urine.

Results: Major changes in RCC tissue included a significant decrease in amino acid levels (alanine, asparagine, aspartate, serine, tyrosine, among others), except for β-alanine and glutamate, which showed increased levels. Dysregulations of organic acids were also observed in tumor tissue, including significant decrease in fumarate and gluconate and increase in 3-aminobutyrate, citrate, and lactate. In addition, increased levels of glucose and maltose were found in RCC tissue, while other sugar derivatives such as *myo*-inositol and *scyllo*-inositol showed decreased levels. These results revealed a metabolic reprogramming in RCC characterized by multiple alterations in pathways related to amino acid metabolism, energy metabolism, and disturbances in sugar and inositol phosphate metabolism. Our investigation identified, for the first time, significant dysregulations in asparagine, urea, and 3-aminoisobutyrate levels in RCC tissue. Interestingly, the significantly altered metabolites found in tissue correlated with several metabolites in urine (threonic acid, *scyllo*-inositol, serine, citrate, creatinine, and glucose), suggesting a possible biochemical relationship with potential implications for non-invasive RCC detection in urine.

Conclusions: These findings provide new insights into the metabolic reprogramming associated with the pathogenesis of RCC. Understanding these changes provides valuable information for identifying novel therapeutic targets and potential diagnostic biomarkers that may have implications for RCC management.

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P18-09
Quantitative analysis of micro and nanoplastics in biological tissues: implications for environmental and human health

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The escalating global presence of environmental micro- and nanoplastics (MNPs) raises significant concerns for human health. While MNPs have been detected in every major organ, a comprehensive quantitative comparison of their distribution across organs is lacking. This study addresses this gap by employing Pyrolysis Gas Chromatography/Mass Spectrometry (Py-GC/MS) to isolate and quantify MNPs. This research delves into two distinct yet critical aspects of MNP accumulation in biological systems: the placenta during pregnancy and decedent tissue samples, comparing accumulation in kidneys, livers, and brains.

In addressing the challenge of robust quantification methods, we employed innovative Pyrolysis Gas Chromatography/Mass Spectrometry (Py-GC/MS) techniques, enhancing detection sensitivity for trace amounts of MNP with identification of up to 12 different polymers in each sample analyzed. Our investigation of 62 placental samples revealed the ubiquitous presence of MNPs, predominantly polyethylene (PE), with concentrations ranging from 6.5 to 685 μg per gram of tissue. Furthermore, Attenuated Total Reflectance-Fourier-transform infrared spectroscopy (ATR-FTIR) visually confirmed translocation of various plastic polymers (>1μm) to the placenta, showing that about 75% of particulates were polymer-based.

Expanding our inquiry utilizing decedent (autopsy) samples from the Office of the Medical Investigator in Albuquerque, NM, collected in 2016, we assessed MNPs in the brain, liver, and kidney tissues stored in formalin. During our investigation, we found that the brain exhibited significantly higher concentrations, at 5,742 μg/g, ranging as high as 23,979 μg/g isolated from the frontal cortex of MNPs compared to the liver and kidneys. Kidneys and liver samples exhibited similar concentrations to what were seen in palcentas (145 and 182 μg/g respectively). The mean concentration of MNPs in adult brains was 0.5% by weight, with some samples exceeding 1%. Polyethylene was the dominant composition for MNPs in all tissues, but more abundant in brain tissues compared to liver and kidney tissues.

No apparent influence of demographic factors (age, sex, ethnicity/race) or causes of death was observed for MNP concentrations. Our findings raise concerns about possible implications for neurological health. While the exact implications remain unclear, there is emerging evidence that MNPs may potentially disrupt neurometabolic processes and promote pathological protein aggregation.

In conclusion, this study emphasizes the urgent need to address the escalating challenge of MNP pollution. Our innovative quantification methods shed light on the extent of contamination in prenatal and lifetime bioaccumulation contexts, emphasizing the potential health risks MNPs pose. Addressing these risks is of the utmost importance to safeguarding human health and environmental integrity in the face of this emerging environmental threat.

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P18-11
Determination and analysis of γ-hydroxybutyric acid in blood by MPFC-QuEChERS with GC-Q-TOF/MS

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γ-hydroxybutyric acid (GHB) is an endogenous short-chain fatty acid found in various mammalian tissues, with unique neuroregulatory effects. Xyrem is used for the treatment of alcohol withdrawal syndrome and maintaining abstention in alcoholics. However, in recent years, there have been reports of sexual assaults on female victims in entertainment venues where GHB is used as an abused drug. Similarly, various cases of drug driving and death often involve GHB. A new method was established for the determination of GHB in human blood by gas chromatography-high resolution mass spectrometry (GC-Q-TOF/MS) with a kind of purification column (MPFC-QuEChERS). First of all, GHB-D_6 was spiked into 0.1 mL blood sample as the internal standard, and then the analyte was extracted using ethyl acetate efficiently. After that, the supernatant was allowed to flow through the MPFC-QuEChERS purification column and evaporated to dryness. Finally, the dried residue was derivated with trifluoroacetamide (BSTFA) and analyzed. The GHB content in 205 postmortem blood samples was measured by the established method, and Statistical analysis and correlation analysis of the content values and possible influencing factors were conducted. Experimental results showed that there were linear relationships for the spiked human blood samples in the range of 0–60 mg/L (R^2>0.9962). The limits of detection and quantification were 0.03 mg/L and 0.15 mg/L, respectively. The inter-day relative standard deviations (RSD) and intra-day RSD were both less than 15%. The matrix effect ranged from 101.1% to 109.7%. The range of GHB content in 205 postmortem blood samples is 1.3–99.7 mg/L with a median of 9.0 (5.3–19.0) mg/L. The sex, age, and mode of death of the samples were not associated with GHB content, but the intake of toxic drugs before death may affect the GHB content. It was found that there was a statistically significant correlation between ethanol and GHB content through univariate and multivariate linear regression models. The results indicated that the developed method was improved and optimized by traditional liquid-liquid extraction method, it could be applied to the rapid and effective determination of GHB in human blood samples of actual cases with its good stability and high sensitivity. The results and analysis of GHB content in postmortem blood provide a certain research basis for the determination of endogenous and exogenous GHB.

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P18-13
The use of *Aloe vera* gel as anti-inflammatory adjunctive herbal remedy for lobular capillary hemangioma: a case report

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Purpose: Lobular capillary hemangioma which is known as pyogenic granuloma (PG) is a kind of oral tumor- like lesions with friable and ulcerated surface and colored in ranges from pink to purple. PG can be appeared in oral captivity with inflammatory responses due to reactive oxygen specious (ROS) formed by thermal trauma. In this study the anti-inflammatory effects of Aloe vera gel as adjunctive treatment on a patient affected by PG was shown.

Case Report: The patient was a 54-year-old man weighing 80 kg with a primary diagnosis of hemangioma. The patient `s history showed

that after thermal trauma with drinking hot liquid, the lesion was formed on dorsal surface of his tongue and had grown to attain the size of 0.7×0.5×0.2cm.The lesion was removed by surgical procedure. The presence of widen inflammations and wounds was managed by following treatment protocols based on informed consent of patient.

Methods: The treatment was based on putting a layer of *A. vera* leaf gel with the size 5×2×1 cm for 4 times a day on tongue and allowed the jel to cover up all affected area of tongue for few minutes. The patient was requested to take all other conventional medicines for infection prevention. The procedure was continued for 14 days.

Results: The symptoms of induced inflammations including redness, swelling as well as the developed wounds after surgery well disappeared and healed. Therefore, *A.vera* can be considered as adjunct therapy for some exophytic oral lesions such as PG.

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P18-14
Monitoring of cortisone and cortisol levels in hair during pregnancy

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Purpose: Pregnancy is a period of intense hormonal changes, which cause intense stress and can affect the emotional health of the pregnant women. In every stressful situation, the adrenal glands secrete adrenaline and cortisol. Cortisol is the main natural glucocorticoid synthesized in the adrenal cortex and it is secreted during physical and psychological stressful situations by compensating and metabolizing them in cortisone by 11beta-hydroxysteroid dehydrogenases (11betaHSDs). This study aimed to determine the levels of cortisol and cortisone in the three trimesters of pregnancy using hair analysis.

Materials & methods: The hair sampling was performed the period 2022–2023. Total length hair samples were collected from 49 mothers during the second week after delivery. Since hair growth rate is about 1 cm per month, the first 9 cm from the root are representative of the pregnancy period. Hair samples were divided into three segments (3 cm per segment), washed and extracted by methanolic liquid-solid extraction for 4 hours in an ultrasonic bath. The analysis was performed by liquid chromatography–mass spectrometry (LC–MS).

Results & discussion: Our results showed that the% detection frequencies of cortisone and cortisol were 49.0% and 36.7%, respectively for the first trimester, 63.3% and 49.0% for the second trimester, 77.1% and 62.5% for the third trimester. The cortisol concentration levels for the first trimester were 7.2 pg/mg, for the second trimester 11.7 pg/mg and for the third trimester 11.0 pg/mg. In correspondence, the cortisone concentration levels for the first trimester were 11.2 pg/mg, for the second trimester 16.1 pg/mg and for the third trimester 23.0 pg/mg.

Conclusion: The findings of the study showed an increase in both cortisone and cortisol levels and detection frequencies, indicating the increase of maternal stress as labor approaches.

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P18-15
Determination of tramadol and its metabolite O-desmethyltramadol in blood and vitreous humor samples

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In recent years, there has been increasing interest on the use of alternative biological materials in forensic toxicology. Vitreous humor is one of them, which, due to the closed cavity where contained, has a low degree of contamination and high purity that makes it ideal during toxicological investigation of forensic cases with post-mortem samples. The aim of this study was the development and validation of a gas chromatography/mass spectrometric (GC/MS) method for the determination of both tramadol and O-desmethyltramadol in vitreous humor and blood samples and its application to real biological samples from forensic cases in order to investigate the distribution of the two substances in this alternative biological fluid.

A GC/MS method was developed, validated and applied to post-mortem blood and vitreous humor samples obtained from 12 forensic cases. The presence of tramadol in these samples had been confirmed during general screening of blood or urine samples or the intake of tramadol had been reported in the case history. The sample preparation procedure included solid-phase extraction and derivatization using N,O-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylsilyl chloride prior to GC/MS analysis.

The method was fully validated according to international guidelines. For both analytes, the LOD and LOQ were 1.50 and 5.00 ng/mL, respectively. The calibration curves were linear ($R^2 \geq 0.992$) from 5.00 to 1000.0 ng/mL, and absolute recoveries were higher than 85%. Accuracy and precision were within the accepted range. Both substances were found to be readily distributed in vitreous humor, since even in cases of very low concentrations of the analytes in blood, their detection was also possible in vitreous humor. The blood concentrations of tramadol and O-desmethyltramadol were ranged from 136.8 to 1888 ng/mL and from 5.6 to 239.3 ng/mL, respectively. The respective vitreous humor concentrations were found to be for tramadol from to 58.4 to 1254ng/mL, and for O-desmethyltramadol from 5.2 to 287.1 ng/mL. In addition, the ratios of vitreous humor concentrations to the respective blood concentrations were calculated in order to study the distribution of tramadol (0.43–2.29) and O-desmethyltramadol (0.53–2.16) in the vitreous humor. The mean values were found to be 0.91 and 0.94 for tramadol and O-desmethyltramadol, respectively, while the median values were 0.75 and 0.83, respectively.

The study of showed the importance of using vitreous humor as an alternative biological material either in cases where blood and urine samples cannot be collected or for drawing safer conclusions since vitreous humor is much less affected than blood by the phenomenon of sepsis and post-mortem redistribution. However, in order to establish therapeutic and toxic concentrations of tramadol and its metabolite in vitreous humor, further analysis of biological samples from a larger number of forensic cases, and in particular cases of overdose, seems necessary.

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P19a | Risk prediction and Assessment/ Risk assessment using New Approach Methodologies

P19-01
Potentially toxic elements in muscle of fish species from the Miliç Wetland in Samsun, Türkiye: a probalistic human health risk assessment

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Fish are crucial for a nutritious diet since they include abundant omega-3 fatty acids, proteins, vitamins, and minerals, all of which contribute to their extensive range of health advantages. Consequently, there has been a substantial rise in global fish consumption. However, fish can acquire trace metals and other environmental contaminants. Excessive trace element contamination of fish might lead to detrimental health consequences. The study conducted on potentially toxic elements (PTEs) in the muscle tissue of various fish species (*Carassius gibeli*, *Esox lucius*, and *Squalius cephalus*) from the Miliç Wetland in Samsun, Türkiye, aimed to assess the probabilistic human health risks associated with the consumption of these fish. Local communities frequently visit the Miliç Wetland for fishing and other recreational activities because it is an essential habitat for a variety of aquatic species. The research focused on analyzing the concentrations of PTEs, such as mercury, lead, cadmium, and arsenic, in the muscle tissues of selected fish species. The levels of PHEs (mg/kg) were as follows: Zn (4.98) >Fe (4.24) >Al (1.65) >Mn (0.60) >Cu (0.20) >As (0.13) >Hg (0.06) >Ni (0.03) >Pb (0.02) >Cd (0.01). The metal hazard index (MPI) and target hazard quotients (THQ) for all PTEs resulting from the ingestion of fish species were found to be less than 1. This indicates that there is no risk associated with consuming these fish in terms of metal intake. The carcinogenic risks (CR) from exposure to inorganic arsenic ($_i$As) were within tolerable limits (10–4 to 10–6) for all the fish species tested. Furthermore, given that the hazard index (HI) is below 1, it has been concluded that the consumption of specific fish species does not pose a threat to the health of the general population. The estimated daily consumption of PTEs in each fish species was far below their respective permissible daily intake levels. This suggests that consuming fish does not pose a health concern to consumers in terms of PTE intake on a daily basis. Furthermore, the multivariate data analysis provided evidence that PTEs originated from both anthropogenic and lithogenic sources.

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P19-02
Health risks caused by nicotine in pouches

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Nicotine pouches for oral consumption do not contain tobacco and are therefore marketed as “healthy” alternatives to cigarettes and other tobacco products on the ground that these products cause less harm to consumers. However, it is still under discussion, if nicotine pouches can be considered as ‘safe’. Objectives of the presented study were to determine the nicotine content in several pouches, to estimate the corresponding nicotine uptake and finally to assess the potential risks for human health.

31 samples of nicotine-containing pouches were collected from distributors in Bavaria from 2019–2023. The median nicotine concentration per pouch was determined to be 9.04 mg with a range of 2.20–56.00 mg nicotine/pouch. When a single daily use of only one pouch and a nicotine release during usage of 50% was assumed, the resulting nicotine uptake from pouches with a median nicotine concentration was calculated to be 65 µg/kg bw (range 16–400 µg/kg bw) for an adult of 70 kg bw. However, similarly to tobacco products, consumption of more than one pouch per day has to be expected, resulting in a significantly increased nicotine uptake during one day for high consumers.

Possible health risks after oral nicotine uptake include cardio-vascular (i.e. elevated heart rate) and embryotoxic effects. EFSA and BfR proposed an oral acute reference dose (ARfD) of 0.8 µg nicotine per kg bw that was based on an observed elevated heart rate in a study with human volunteers. A similar effect was observed in humans after acute consumption of nicotine pouches. Long-term studies with nicotine pouches, however, are still lacking. A continuous elevated heart rate is associated with an increased mortality rate in humans and similar effects were already observed after long-term consumption of the similarly used product category snus.Even when a daily use of only one nicotine pouch with low nicotine levels was assumed, the ARfD is still exceeded by a factor of at least 20. With increasing nicotine concentrations in the consumed pouches and a more frequent consumption vast ARfD exceedances are the consequence. Taken together with evidence from observed effects after consumption of nicotine pouches and snus, an increased nicotine related risk for health injuries after consumption of the investigated pouches has to be expected.

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P19-03
Integration of transcriptomics data for ED assessments using an Adverse Outcome Pathway Network approach

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Omics technologies can be used to generate large amounts of data on biological effects caused by exposure to chemicals. However, the integration of data from omics studies in regulatory risk assessments is challenging. The assessment of Endocrine Disruptors (EDs) relies largely on standardized animal studies, where both the methodology and measured endpoints are validated and familiar to most risk assessors. In transcriptomics, advanced computational tools are used to analyze the data, which would require certain expertise to evaluate. Moreover, there are uncertainties regarding the toxicological relevance of the new type of data that is generated, and if it can be reliably linked to adverse effects in an organism. This study aims to advance the integration of transcriptomics data in the identification of EDs relevant for human health by applying Adverse Outcome Pathways (AOPs). Additionally, the study investigates challenges associated with using these novel methodologies and attempts to identify future research directions in the field. Cadmium and PCB126 were chosen as model substances since they are suspected to be endocrine disruptors but are currently not identified as such in the EU.

Zebrafish embryos were exposed to cadmium and PCB126 for 96 hours, until 120hpf, followed by RNA-seq to capture the transcriptomic response of exposure to the chemicals. Data analysis is mainly conducted with different Bioconductor packages using the R programming language. Moreover, AOPs relevant to the Estrogen, Androgen, Thyroid, and Steroidogenesis (EATS) modalities were extracted from the AOPWiki to construct an EATS-related AOP network. Transcriptomics data is then linked to the AOP network, for example by matching Gene Ontology Biological Process (GO BP) terms from the transcriptomics data and those attached to specific key events in the AOP network. This will also be visualized in Cytoscape to identify possible Modes of Action (MoAs) of the compounds.

Several GO BP terms related to endocrine disruption were observed for both model substances. However, in the AOP network, there was a lack of GO BP terms and standardized terms in general. Therefore, GO BP terms from the transcriptomics were manually tagged as EATS, non-EATS, or irrelevant. Manual mapping of GO BP terms improved the results, emphasizing the importance of further harmonizing the development of AOPs to improve regulatory application. The application of AOP networks to integrate novel data seems promising, but certain challenges that hinder the process were identified.

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P19-04
Preclinical safety evaluation of endolysin-containing vaginal insert

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Bacterial vaginosis (BV) is a vaginal disorder, affecting up to 30% of women worldwide and is associated with reproductive and infectious complications. BV is characterized by a reduction in beneficial vaginal Lactobacillus species (spp.), and an increase in various anaerobic spp., particularly Gardnerella vaginalis. BioNTech is currently developing a recombinant phage-derived endolysin (termed BNT331), an enzyme protein which selectively lyses Gardnerella spp., as a vaginal insert for patients suffering from BV.

Non-clinical development of BNT331 followed the ICH S6(R1) guidance. Due to its two Gardnerella-specific functional domains (binding and hydrolytic), BNT331 has no target in healthy mammals including humans. The BNT331 non-clinical characterization comprised *in vitro* and ex vivo pharmacodynamics (PD) studies, establishing proof-of-concept *in vitro* and assessing the mode-of-action and efficacy of BNT331 in Gardnerella isolates and in biofilm. Secondary PD studies, addressing potential off-target activity and possible cytotoxicity of BNT331 were performed *in vitro* using human vagino-cervical derived HeLa and Ect1 cells, as well as human polymorphonuclear leukocytes and erythrocytes. The systemic pharmacokinetics (PK) profile after intravaginal application of BNT331 was characterized in PK/tolerability studies in rats, rabbits, and sheep. Local (vaginal) exposure was evaluated in sheep upon single and repeated dosing with BNT331 vaginal tablets.

The non-clinical toxicology program, assessing BNT331 safety, included *in vitro* studies, as well as *in vivo* studies in rats, rabbits, and sheep. Non-GLP studies to address PK and safety relevant parameters were performed in rats, rabbits, and sheep upon single or repeated intravaginal dosing with BNT331 drug product. A pivotal GLP-compliant 14-day repeated-dose toxicity study was conducted in rats. Additionally, a GLP-compliant 10-day local tolerability (vaginal irritation) study was performed in rabbits in accordance with the relevant FDA Guidance.

This presentation will focus on the preclinical safety evaluation of BNT331, I will also discuss the relevance of the existing animal models on vaginal tolerability.

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P19-05
Framework for classifying chemicals for repeat dose toxicity using NAMs

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EPAA's 'NAM Designathon 2023' challenge for human toxicity sought to identify better classification systems capable of categorising chemicals for risk assessment based on their intrinsic toxicodynamic (TD) and toxicokinetic properties (TK) [1]. The aim of the challenge is to explore the use of non-animal methods (NAMs) to design a future classification system for systemic toxicity. The chemicals requiring assessment are identified by three levels of concern: low concern being those that could be used without restriction; medium concern requiring assessment in order to establish safe use levels; and high concern being candidates requiring risk management [2].

In this work, we propose a NAMs based classification system developed by ECETOC's Staged Assessment Task Force integrating an evidence-based approach from three different workstreams:

- *In silico* predictions
- *In vitro* bioavailability (PBPK modelling and TK)
- *In vitro* bioactivity (TD)

The first stage employed an *in silico* approach, covering several toxicity endpoints across various (Q)SAR *in silico* models. The prediction is followed up by an expert review that guides the evaluation process and serves as a starting point for additional validation.

TK properties were analysed by employing a human PBPK model to evaluate accumulation concern levels, simulating 14-day plasma C_{max} predictions for a standard dose level with httk, PKSim and GastroPlus© models [3].

In vitro TD data obtained from ToxCast analysis incorporates both potency and severity. Potency makes use of dose response AC_{50} values, with additional analysis of extracted data based on known levels of assay toxicity. Severity categorization is based on known levels of toxicity for the endpoint.

Following *in silico*, TD and TK assessments, a weight of evidence approach is used to determine whether there is sufficient evidence to move away from an initial hypothesis that every chemical is of high concern [4].

12 chemicals have been assessed through the framework and the basic concept put forward by the EPAA has been shown to be workable. Our future efforts aim to expand the number of chemicals and investigate whether we could develop a tiered assessmente process making use of a Bayesian approach for information evaluation.

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P19-06
Hack to save lives and avoid animal suffering – Artificial Intelligence (AI) in toxicology – a potential driver for reducing/replacing laboratory animals in the future

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The ONTOX project will deliver a strategy to create innovative New Approach Methodologies (NAMs) in order to predict systemic repeated dose toxicity effects of any type of chemical that, upon combination with tailored exposure assessment, will enable human risk assessment. The project focuses on six specific NAMs addressing adversities in the liver, kidneys and developing brain. The NAMs will consist of an ontology-driven and artificial intelligence-based computational system, fed by available physiological human data and targeted vitro and in silico testing.

In March 2023, the first ONTOX stakeholder network meeting entitled: "Digging Under the Surface of ONTOX Together with the Stakeholders" took place. The discussion centred around identifying specific challenges, barriers, and drivers in relation to the implementation of NAMs and probabilistic risk assessment (ProbRA), in order to address the issues and rank them according to their level of difficulty. The participants identified several issues which should be addressed on the way towards full implementation of NAMs and ProbRA in chemical risk assessment. It was also concluded that there is a continued need for stakeholder engagement, including the organisation of a 'hackathon' to tackle challenges. One of the meeting outcomes was a list of recommendations for issues to be addressed during such a hackathon. Based on this list four challenges were formulated: 1) How to drive the use of AI in chemical risk assessment? 2) To predict or protect? 3) How can we secure human health and environmental protection at the same time? and 4) How can we facilitate the transition from animal tests to full implementation of human-relevant methods?

ONTOX hosted a two-and-a-half day Hackathon in Utrecht (NL) where a variety of people with different professional background were invited, to ensure that different perspectives are included in the discussions. More experienced professionals are included, a large number of early career scientists as well as students. This enabled a creative 'out-of-the-box' approach to overcoming the challenges, benefitting considerably from the available interdisciplinary expertise.

The respective issue were introduced to the participants by issue-owners. Together with appointed experts, covering different areas of expertise, they were available on consultant basis for the participants during the hackathon. Professional coaches guided the participants through the discussions as well as their preparation of the pitches presenting their solution. A jury monitored the process in the teams as well as the pitches to facilitate the final award of winning proposals. Jury members, issue owners, and experts covered regulatory authorities, industry, academia, and NGO to ensure all perspectives needed. The outcome of the hackathon as well as ONTOX experiences with such an event to find solutions on wicked questions will be presented.

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P19-07
An *in vitro* inhalation approach to discriminate respiratory effects from hair-straightener products

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Aerosols are generated during the heating procedure of hair straightener products. Professional hairdressers may experience discomfort and respiratory effects that may vary depending on the specific product used. However, it is challenging to establish correlations between the composition or application method of the products and their local respiratory effects due to a lack of experimental methods. To address this, an animal-free *in vitro* inhalation approach was developed to generate experimental data and investigate any potential biological effects based on product characteristics.

An *in vitro* inhalation model with air-liquid interface (ALI) cultures derived from A549 human lung alveolar epithelial cells was applied. Using the P.R.I.T.® ExpoCube® for exposure, viability (WST-1), mitochondrial membrane potential (MMP) and cellular stress (live fluorescence stains), and IL-8 release (ELISA) were assessed. The experimental setup was designed to accurately generate aerosols that occur during hair straightener use under real conditions and effectively deliver them to the ALI cultures. Three different hair straighteners (TM1, TM2, TM3) were tested in this study.

The test materials used in the study showed notable differences in the amount of aerosol released during use. TM1, associated with respiratory effects, produced the highest concentrations of aerosol compared with the two others. Cell viability was minimally affected only by TM3 at higher dosages, while mitochondrial membrane potential remained undisturbed for all three test materials. However, cellular stress and IL-8 release increased significantly in response to aerosols from certain test materials, but not all. Whereas TM1 significantly induced IL-8 release in a dose-responsive way (up to 160% of control), cellular stress was significantly and dose-responsive induced by TM3 (130% of control). Additionally, TM2, which was not associated with respiratory effects in real-world applications, did not induce any effects in the *in vitro* experiments.In conclusion, the approach used in this study shows great promise for investigating the potential biological effects of inhaling aerosols from actual hair straightener products. The efficient aerosol generation, sampling, and cell exposure technologies employed allowed for the establishment of dose-response relationships *in vitro*, serving as the foundation for evaluating potential biological effects. The successful correlation between *in vitro* and real-life effects observed with the three test materials suggests that the *in vitro* data has promising predictive value for *in vivo* scenarios. Expanding the scope of this approach to include more test materials and conducting systematic studies on hair straighteners with different compositions or application procedures could not only enhance its relevance and provide insights into toxicological mechanisms but also contribute to the development of "safe-by-design" cosmetic products.

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P19-08
Machine learning-driven oral-to-inhalation extrapolation for chemical toxicity value prediction

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Reference concentrations (RfCs) have been widely used to assess non-cancer human health risks from exposure to chemicals. RfCs can be generally derived from a NO(A)EC/LO(A)EC/BMC(L) of animal inhalation toxicity tests, with uncertainty factors defined by toxicologist expert judgement to reflect limitations of the data used. However, inhalation studies are conducted less frequently than oral toxicological studies. To avoid additional animal tests, in line with 3R principles, oral-to-inhalation extrapolation can be an alternative way to predict inhalation toxicity values of chemicals. To date, several data-driven approaches have been used to estimate oral-to-inhalation extrapolation factors from comprehensive animal tests, suggesting that substantial oral toxicity studies can be a key value to reasonably estimate inhalation toxicity for chemicals which have no inhalation toxicity results.

In this study, we applied a machine learning (ML) algorithm to predict RfCs, using reference doses (RfDs) as a key parameter for modeling, with several chemical and biological descriptors. We collected toxicity values from publicly available and peer-reviewed sources (e.g.,

U.S. EPA (IRIS, PPRTV, and Health Effects Assessment Summary Tables), California EPA, and Agency for Toxic Substances and Disease Registry). If a value was available from more than one source, we selected it according to the EPA Superfund program hierarchy. Predictive regression models were built in the KNIME Analytics platform. Chemical fingerprint and physical properties were calculated using RDKit as chemical descriptors, and biological activities obtained in TOX21 program were used as biological descriptors. Gradient boosted tree was used as a ML algorithm to efficiently deal with missing data in the biological descriptors, and predictive performance was calculated with leave-one-out cross-validation approach.The correlation coefficient (r2) between RfCs and RfDs was 0.4, suggesting weak correlation between them. We developed RfC prediction models, covering all combinations of each descriptor, and found that models built with RfDs as variables showed higher predictive performance than models built without RfDs. The best model showed r2=0.6, coefficient of determination (R2)=0.6, and mean absolute logarithmic error (MALE)=1.0. Considering that even the best model built without RfDs showed lower performance (r2=0.4, R2=0.3, and MALE=1.2), our results suggest that ML-based modeling approach for oral-to-inhalation extrapolation with chemical and/or biological descriptors has a potential to assess chemical inhalation toxicity without relevant animal inhalation toxicity tests. In the presentation, we will also provide details of the chemical space of the developed model and comparisons with publicly available high-throughput screening *in vitro* assay data in order to discuss future utilization and improvement of our approach.

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P19-09

Assessment of the health risks associated with exposure to carcinogenic substances in the workplace, illustrated through the examples of isoprene and furan

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The primary objective of the project is to develop guidelines for estimating the health risk associated with carcinogenic substances in the occupational environment based on scientific data. This involves assessing the cancer risk utilizing a statistical program and a selected mathematical model.

Complete elimination of exposure to carcinogens is not feasible; hence, it is imperative to minimize the risks arising from occupational exposure. In Poland, the Interdepartmental Commission for the Highest Allowable Concentrations and Intensities of Harmful Factors for Health in the Workplace has adopted the range of 10^(-4) to 10^(-3) for carcinogenic factors. This signifies an accepted level of occupational risk resulting from exposure to carcinogens, equating to 1 additional case of cancer per 10,000 or 1,000 individuals in population terms.

In 2023, two carcinogenic substances were selected for research, isoprene which is the subject of the Risk Assessment Committee's (RAC) work at the European Chemicals Agency (ECHA) to determine the Binding Occupational Exposure Limit (BOELV). Additionally, furan, for which Poland established the Occupational Exposure Limit (OEL) for the first time at a level of 0.05 mg/m^3.

To build a dose-response relationship (dose – risk of cancer), the two-stage model of cancerogenesis is employed. This model postulates two critical events in carcinogenesis that are specific, irreversible, and hereditary at the cellular level. The model incorporates three cell compartments: normal stem cells, intermediate cells altered by one genetic event, and malignant cells altered by two genetic events. The size of each compartment is influenced by cell birth, death, and differentiation processes, as well as the rates of transition between cell compartments. Experimental data involving animal studies were selected as the basis for modeling the target tumor resulting from the carcinogenic mechanism of the investigated substance. Calculations were based on the results of two-year, lifelong studies conducted on rodents (rats or mice). Standardization of various experimental dosing schemes, data, and toxicokinetic modeling was performed, along with interspecies scaling and extrapolation of the route of administration and averaged exposure throughout the lifespan.

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P19-10

Evaluating reliability and relevance of *in vitro* toxicity studies on micro- and nanoplastics

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As a result of their ubiquitous presence, there is a growing concern over potential human health impact of micro- and nanoplastics (MNPLs). This has resulted also in regulatory measures to limit human exposure. Despite a rapidly increasing number of MNPLs toxicity studies, a systematic and comprehensive analysis of the regulatory relevance and reliability of the data generated is lacking at present. Several studies suggest that insufficient characterization (e.g., particle sizes, agglomeration, release of additives), and documentation of exposure conditions are main weaknesses of published MNPL toxicity studies for regulatory decision-making. Therefore, it is crucial to increase the overall transparency on how the MNPLs toxicity studies are conducted, and to have tailored MNPL criteria to determine the reporting- and methodological quality of MNPL toxicity studies.

Different tools exist that can support transparency and harmonization in selection of data for regulatory use. This includes the framework suggested by the Science in Risk Assessment and Policy (SciRAP) initiative. This framework is focused on evaluating reliability and relevance of (eco)toxicity data for hazard and risk assessment of chemicals and engineered nanomaterials. Hence, it is not tailored specifically towards MNPLs. At the same time, existing MNPL specific toxicity screening assessment tools, do not account for certain key aspects (e.g. reference materials, true to live materials, controls for differentiating particle or material related toxicity). Recognizing that MNPL research is in its infancy, revisions and adaptation of existing tools based on latest learnings is key. Thus, there is a need to update such tools in relation to latest development of MNPL research.

In this study, we build on the existing SciRAPnano *in vitro* tool, incorporating latest findings regarding environmental relevant MNPL-specific criteria identified via a literature review. Among these criteria are pristine MNPL material characterization, adequate description of the sample preparation methods, and behavior of MNPL during and after testing. The tool is in the form of an excel sheet with pre-specified criteria for evaluating reliability, divided into reporting quality (RQ) and methodological quality (MQ), as well as relevance. Several RQ and MQ criteria are specified in the categories 'Test item and controls', 'Physicochemical properties of the test item', 'Test system', 'Administration of test item', 'Data collection and analysis', and 'Funding and competing interest' (RQ only). Each RQ and MQ criterion is evaluated as fulfilled, partially fulfilled, not fulfilled, or not determined. Relevance items are judged as directly relevant, indirectly relevant, and not relevant. The output of the SciRAP evaluation is a colour profile that provides a qualitative overview of the reliability and relevance. To test the applicability of the adapted tool, a case study was conducted on *in vitro* MNPL studies.

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P19-11
Establishing scientific confidence in a NAM toolbox and workflow using a flexible framework

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In recent years significant progress has been made in the development, evaluation, and application of new approach methods (NAMS) for Next Generation Risk Assessment of systemic safety which is increasing confidence their use for making robust safety decisions. However, it is important to go beyond this and evidence areas such as technical characterization of decision frameworks and their component NAMs, to establish scientific confidence for regulatory purposes. In a paper by Van der Zalm *et al.*, (2022) a framework for establishing confidence in NAMs was proposed, comprising five elements (fitness for purpose, human biological relevance, technical characterization, data integrity and transparency, and independent review). This flexible approach was applied to the components of the systemic toolbox and workflow described in Middleton *et al.* (2022). The toolbox – intended to be used as a tier one approach within an integrated approach to testing and assessment for cosmetic safety assessments – includes physiologically based kinetic (PBK) models to estimate human plasma C_{max} , and 3 bioactivity platforms, comprising high-throughput transcriptomics, a cell stress panel, and *in vitro* pharmacological profiling, from which points of departure (PoD) are estimated and a bioactivity exposure ratio defined.

Taking the flexible approach outlined in van der Zalm *et al.*, we mapped current evidence against the expanded framework created specifically for the toolbox and workflow, totalling 19 topics e.g. predictive capacity, variability and equivalence. Results included a protectiveness metric, where performance is calculated against pre-defined chemical exposure benchmarks. Depending on which level of PBK was used to parameterise the model, the toolbox is protective of 93% (*in silico*), 93% (*in vitro*) and 98% (clinical) of high-risk benchmark chemical exposures. The utility of the approach, i.e. correctly identifying low-risk benchmark chemical exposures as such, was 8%, 24% and 0% respectively. Giving total balanced accuracy of 51%, 59%, 49% respectively for this tier 1 approach. Improving the utility of the toolbox is a priority for future versions. To consider applicability domain, coverage of chemotypes was calculated. Overall, the 38 substances tested in the toolbox are structurally diverse when compared with cosmetic structures. Under the area of reliability, for example, a preliminary dataset for chemical reproducibility of global PoDs in the cell stress panel assay, ranged between <3-fold to 9.4-fold depending on the chemical, however correlation of global PoDs between laboratories was high (0.97).

It is possible to expand upon a flexible framework to establish confidence and evidence whether our NAM workflow is fit for purpose, robust, relevant and reliable. The framework for scientific confidence provides a scaffold to easily identify where evidence may be lacking, so that strategies can be implemented to fully meet these principles.

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P19-12
Impact and implication of causal terminology in adverse outcome pathways

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To meet the need of next-generation risk assessment under the 3R principles, adverse outcome pathways (AOPs) facilitate the extrapolation of evidence at lower biological levels for the prediction of adverse outcomes at higher biological levels. Such extrapolation depends on the characterization of the relationships between key measurable events across biological levels, which are defined as key event relationships (KERs). While non-animal evidence becomes more preferable in a 3R setting, the inference of causality in KERs remains one of the key questions of toxicology. However, the nature of KER has been prescribed inconsistently throughout AOP documentations. Some instances require KERs to be strictly causal, while others relax the requirement to be causal and/or predictive. In this work, we will demonstrate that the causal definition on KER could be a misuse of terminology. The inference of KER is empirical, despite the involvement of experimental data. Hence it faces the fundamental challenge to claim causality from empirical evidence. Based on the KER evaluation protocol of empirical evidence in the AOP handbook, we will discuss several alternative strategies to unify the causal terminology of KER and evaluate the strength and weakness of these alternatives.

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P19-13
Occupational standards and its endpoints for chemicals in Poland 2023

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Purpose: In 2023, the Expert Group of Chemical Agents (GECA), operating at the Nofer Institute of Occupational Medicine in Poland, proposed Maximum Admissible Concentrations (MAC) values for 10 chemical agents in the workplace environment. The objective of the study is to present these new OEL values along with their corresponding endpoints.

Methods: In Poland, the Intersectoral Commission for Setting OEL Values, established in 1983 by the Minister of Health and Social Policy, independently makes regulatory decisions on permissible concentration levels of chemical substances in the workplace environment. The Commission comprises representatives from the Ministry of Health, the Ministry of Labour, as well as experts from commercial organizations, companies, and scientific research institutions.

An essential element of this process is the assessment of the health effects of chemical substances in the workplace atmosphere, conducted by a competent team of experts. The Group of Experts for Chemical Agents (GECA), which includes experts from various scientific fields such as toxicology, occupational medicine, work hygiene, epidemiology, and statistics, evaluates the health effects of exposure to chemical substances based on current scientific data available.

Results: The experts from GECA (Group of Experts for Chemical Agents) prepare documentation outlining proposed MAC values for chemicals, utilizing the latest literature data. These documents include information on the physical and chemical characteristics of the substances, their presence and applications, potential exposures, and biological activity. The biological activity section covers absorption routes, acute and chronic toxicity in humans, acute and chronic toxicity in animals, carcinogenic, mutagenic, teratogenic activity, and effects on reproduction. It assesses the health hazards associated with a specified agent in the workplace environment. Finally, the documentation outlines the basis for the proposed MAC values.

Determining MAC values involves utilizing published study results to establish either the NOAEL (No-Observed-Adverse-Effect-Level) or LOAEL (Lowest-Observed-Adverse-Effect-Level). Subsequently, the MAC value is calculated by applying uncertainty factors. These coeffi-

cients account for various factors influencing human susceptibility and potential uncertainties in the data, allowing for a more comprehensive and conservative approach in establishing MAC values, considering various sources of uncertainty in the available data.

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P19-15
Comparison of toxicity of BPA alternatives in *Caenorhabditis elegans*

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Bisphenol A (BPA) is a plasticizer used in the manufacture of high-strength technical plastics such as polycarbonate (PC), polyacrylsulfone (PASF), and epoxy resins. Recently, printing paper has been coated with BPA, so even frequently used receipt paper may be exposed to BPA. Many studies have identified BPA as an endocrine disruptor, causing reproductive issues, early maturity, and obesity, and other compounds have been created to replace it. Many studies have shown that BPA causes endocrine disruption, and numerous BPA alternatives have been created to replace it. However, information regarding BPA alternatives is currently insufficient. We selected bisphenol AF (BPAF), bisphenol B (BPB), bisphenol C (BPC), bisphenol TMC (BPTMC), bisphenol AP (BPAP), bisphenol C-dichloride (BPC2), bisphenol P (BPP), tetrabromobisphenol A (TBBPA), and bisphenol Z (BPZ) as novel BPA alternatives and examined their harmful effects in Caenorhabditis elegans (C. elegans). Synchronized C. elegans embryos and L1 stages were exposed to BPA alternatives for 24 hours before lethality was determined. C. elegans was synchronized to the L4 stage and exposed to BPA alternatives to evaluate their influence on development and reproduction. In addition, the synchronized L4 was subjected to BPA alternatives to test their influence on lifespan. The final concentration of BPA alternatives in the Lethality assay was 1mM, and in the development and reproduction assays, 0.1mM BPAF, 0.5mM BPB, BPC, and others were used at the same concentration. In both embryonic and L1 lethality analyses, BPAF and BPTMC had a higher mortality rate than BPA at 100%, and the remaining substitutes showed toxicity in the order of BPB>BPC,>BPAP>BPC2>BPP>TBPB=BPZ. In the development assay, it was proven that 0.1mM BPAF lowered adulthood compared to other test groups of 1mM, and 1mM BPC2 delayed growth to a level comparable to BPA. The reproduction assay revealed that the BPB, BPC, BIPAP, and BPC2 exposure groups had lower reproduction numbers than the BPA exposure group. The lifespan assay demonstrated that bisphenol alternatives diminish lifetime to the same or greater extent than BPA. In summary, when the health impacts were indirectly validated using C. elegans, it was found that bisphenol alternatives were more toxic than BPA and lead, demonstrating that the alternatives were as hazardous. To determine the proper usage and administration of bisphenol alternatives in the future, we intend to carry out a thorough investigation into the molecular impacts of these substances based on the findings of this study.

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P19-16
Survey and risk assessment of pesticides in cut flowers from non-EU countries

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Cut flowers sold to consumers in Denmark are imported from countries outside the EU and may contain pesticides that are not approved for use within the EU, leading to potential consumer exposure to those pesticides. The objective of this study is to determine to which extent pesticides occur in cut flowers imported from non-EU countries and whether they pose a risk to consumer health.

The study comprises a survey of pesticides in popular flower species in Denmark, chemical analyses for pesticides in cut flowers purchased in Denmark, and a risk assessment.

For the survey of pesticides in cut flowers, local producers of roses, chrysanthemums and carnations were interviewed in Columbia, Ecuador and Kenya. Based on the producers' information, significant fractions of pesticides used by the producers are not approved for use within the EU (fractions of approved pesticides vary between 14% and 72% within the single production sites).

Samples from 60 single-species flower bouquets were analysed using GC/LC-MS-method covering 90 pesticide residues, including some metabolites and/or degradation products. The pesticides carbendazim, propamocarb and fipronil were detected most frequently in the analysed flowers with detection fractions of 65%, 57% and 50%, respectively. The five pesticides with the highest maximum concentrations were carbendazim, captan/THPI, propamocarb, formetanate and iprodione (maximum concentrations between 60 – 106 mg/kg).

The ten most hazardous pesticides were prioritized for the risk assessment based on the hazard properties according to the CLP Regulation (EC) No 1272/2008, low health-based reference values, approval-status under the Pesticides Regulation (EC) No 1107/2009, as well as high maximum concentrations and high detection frequency in the chemical analyses.

Human health reference values were derived from the European Food Safety Authority's peer reviews on pesticides or other health assessments of public authorities. Dermal and inhalation exposure was estimated for adult consumers regularly handling cut flowers for decoration in their homes following the methodology of the ECHA guidance on consumer exposure.

For the human health risk characterisation, the health-based reference values were compared with the exposure estimates and risk characterisation ratios (RCR) were calculated for nine out of the ten pesticides. All RCR were well below 1, meaning the risk can be assumed to be controlled. For one substance, chlorpyrifos, a RCR could not be calculated due to lack of hazard data. Available data on hazardous effects and dermal uptake suggest a low risk based on a qualitative consideration. However, a risk from chlorpyrifos exposure in cut flowers cannot be excluded.

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P19-17
Use of diverse human skin models in *in vitro* skin permeation testing of dermatological ingredients

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The prevalence and frequency of use of dermatological products varies across consumers, including by sex, age, race, and skin type. Skin permeation is a key consideration in the safety assessment of topical drugs and cosmetic products; however, the influence of skin pigmentation on the amount and rate at which chemicals penetrate the skin

barrier remains poorly understood [1]. Previously, our group established a standardized method to evaluate the dermal penetration of chemicals *in vitro* [2]. This methodology has now been expanded to evaluate the permeation of select dermatological ingredients through diverse human skin models. Dermatomed excised human skin (EHS; BioIVT, LLC) was obtained from age-matched human female donors undergoing abdominoplasty. EHS was grouped based on donor skin phototype as determined by the Fitzpatrick scale (FS), with one group consisting of skin from donors with FS I or II (light pigmentation) and one group from donors with FS V or VI (heavy pigmentation). A reconstructed human pigmented epidermis model (RHPE; MelanoDerm, MatTek) consisting of keratinocytes and melanocytes derived from Caucasian or African American donors was also tested. The melanin content, individual typology angle, and transepidermal water loss of the skin models were quantified; histological assessments were also performed. For the *in vitro* permeation testing, EHS and RHPE samples were mounted on flow-through diffusion cells (PermeGear, Inc.) and a finite dose of [^{14}C]-caffeine, [^{14}C]-salicylic acid, or [^{14}C]-testosterone was applied to the apical skin surface. Receptor fluid was collected over 24 h post-dosing. The amount of compound that permeated through the skin, along with that remaining on top of the skin or retained in the skin 24 h post-dosing, was determined by scintillation counting. The model characterization showed that the melanin content in EHS from donors with FS I/II was lower versus the FS V/VI donor group and was inversely proportional to the individual typology angles. A similar trend was observed in RHPE incorporating Caucasian-derived melanocytes versus African American-derived melanocytes. Differences in melanin content and distribution between the pigmentation groups were also observed by microscopic evaluation of hematoxylin and eosin- and Fontana-Masson-stained tissue sections. The mean transepidermal water loss and the permeation profiles of each compound tested were similar across pigmentation groups within each human skin model. Capturing the consumer diversity in dermatological product safety assessments is critical to avoid disproportionate adverse health effects in certain populations. Utilizing the comprehensive methodology established here can help discern potential skin pigmentation-dependent differences in dermal absorption.

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P19-18
Consumer risk assessment of new fungicide fluoxapiprolin residues in Ukraine

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Fluoxapiprolin is a new active ingredient (a.i.) of pesticides from the chemical class of piperidinyl thiazole isoxazoline. Fluoxapiprolin demonstrates biological activity against plant pathogenic oomycetes with mechanisms of action involving oxysterol binding protein inhibition. It is proposed to control late blight in tomato and potato crops.

Aim: The determination and risk assessment of fluoxapiprolin residues after treatment with tomatoes and potatoes.

Material and methods: Fluoxapiprolin residue decline studies were conducted in two main agro-climatic zones of Ukraine during fluoxapiprolin-based fungicides field trials in 2022–2023. The recommended manufacturer maximum application rates were 15 g a.i./ha for tomatoes and 20 g a.i./ha for potatoes with up to 3 applications. The actual quantity of Fluoxapiprolin residues in treated tomatoes and potatoes was assessed. Residues in treated crops were analyzed by high-performance liquid chromatography (HPLC/DAD). The limit of quantitation of Fluoxapiprolin (LOQ) was 0,01 mg/kg. The possible daily intake of fluoxapiprolin, considering our results, was calculated and estimated.

Results: The obtained data of field trials showed that fluoxapiprolin residues in the harvest of tomatoes were below limits of quantitation (LOQ of 0.01 mg/kg). Fungicide residues in potatoes were not detectable in all studied samples, including during harvest time. The half-life (DT50) values of fluoxapiprolin degradation in tomatoes and potatoes were calculated. The possible daily intake of fluoxapiprolin was significantly lower than the allowable level. Based on the conducted research, themaximum residue limits (MRLs) of fluoxapiprolin for safety use in Ukraine were set and recommended: tomatoes and potatoes – 0.01 mg/kg and values of pre-harvest interval (PHI): potatoes – 14 days, tomatoes – 35 days. In the European Union and the United States the MRLs for fluoxapiprolin have not yet been established, nevertheless in New Zealand set the MRLs: tomatoes – 0.09 mg/kg and potatoes – 0.01 mg/kg (PHI – 7 days).

Conclusions: Dietary intake of fluoxapiprolin residues is unlikely to present public health concerns.

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P19-19
Evaluation of a Next Generation Risk Assessment framework for Developmental and Reproductive Toxicity

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Background and Purpose: Encouraged by the successful application of New Approach Methodologies (NAMs) in an exposure-driven Next Generation Risk Assessment (NGRA) approach for systemic toxicity (Middleton *et al.*, 2022), we developed a NGRA framework for Developmental and Reproductive Toxicity (DART). To determine if this framework is sufficiently protective for consumer safety assessments, a two-step evaluation was performed. Firstly, the biological coverage of the framework was evaluated (Rajagopal *et al.* 2022). In the second step of the evaluation, 37 benchmark compounds were selected for assessment using the proposed framework.

Methods: NAMs utilised in this framework included DART-specific in silico predictions, *in vitro* physiologically based kinetic (PBK) modelling, a cell stress panel, high-throughput transcriptomics, *in vitro* pharmacological profiling as well as ReproTracker® and the devTOX quickPredict™ assay. Points of departure for the 37 benchmark substances were compared to exposure estimates for 76 human exposure scenarios (including plasma Cmax for healthy adult, pregnancy, and fetal exposure), obtained from clinical studies where available, or by using PBK modelling if not, to calculate a bioactivity-exposure ratio (BER). Each exposure scenario was classified as high or low risk depending on DART relevant data from authoritative sources (e.g. FDA label, SCCS opinion, etc.). The BERs together with in silico predictions were compared with the literature derived classification for DART risk for each exposure scenario to evaluate the approach. A larger BER indicates lower risk, and conceptually a BER >1 indicates that biological activity (which could lead to adversity) would not be expected at human relevant exposures.

Results: All but one of the human exposure scenarios classified as high-risk for DART (27 total), were identified as having a BER <1. This

would conceptually indicate uncertain risk and would trigger the requirement for additional assessment to understand potential adversity. Pharmaceutical use of warfarin (5 mg/day) was the only high-risk exposure scenario with a BER >1 (5) which could provide a false reassurance of safety. Most low-risk exposure scenarios were identified as having a BER around 1 or higher. Exceptions here include exposures to compounds with well-known systemic bioactivity such as retinol and caffeine where even "normal" dietary intake generally considered as low risk was flagged by the DART NGRA framework as uncertain risk. In general, the approach of using any bioactivity *in vitro* as a surrogate for adversity or toxicity is a conservative approach. In summary, a first evaluation of the protectiveness of this framework using benchmark compounds with known outcomes for DART, at specific human-relevant concentrations, shows that the framework is a good starting point in building a fit-for-purpose and protective NGRA approach for DART risk assessment.

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P19-20
Eye damage: the use of reconstructed human cornea to classify biopesticide

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Biopesticides are defined as types of pesticides derived from natural materials, among them bacteria, which a microorganism consists in the active ingredient, often not inactivated [1]. They tend to present fewer risks than conventional pesticides [1] and requires much less data to register it, although it is necessary to predict eye hazard potential and due to their characteristic, makes the use of alternative methods a challenge for such product. Significant progress has been made in replacement of the regulatory *in vivo* Draize rabbit eye test. The OECD 492B guideline published on June 30th, 2022, describes a method capable to classify the three main categories of UN GHS using reconstructed Human Cornea-like Epithelium (HCE) [2,3]. The aim is to verify the efficiency of HCE to identify eye hazard for a microbial pesticide (*Bacillus subtilis*) according to OECD 492B [2]. Some care must be taken when handling the biopesticide to avoid cross contamination or loss of effectiveness of the product. The test item did not demonstrate the ability to directly reduce the vital dye MTT and interfere with colour, then it was not necessarily additional controls. The results were within the acceptance criteria. The negative and positive controls showed the viability as expected. The viability of Test item obtained (mean±difference of viability) were 89.66%±7.46 and 87.80%±3.34 for 30 and 120 minutes of exposure, respectively. The biopesticide was classified according to UN GHS Category as "No Category" corroborating with *in vivo* classification tests. According to results, the HCE was extremely efficient and safe to classify the biological product if proper precautions are taken to obtain quality results. Therefore, OECD 492B could be consider an alternative to predict eye hazard potential for biopesticides.

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P19-21
Hazard characterization and quantification of health risk from chronic exposure to metofluthrin through contamination of the air environment of human life

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Metofluthrin is an insecticide from the class of synthetic pyrethroids, which is used in biocidal products to kill mosquitoes and midges in the open air. Its feature is the ability to easily evaporate, which carries the potential danger of air pollution with exposure doses that can have a negative effect on a person when entering the body by inhalation.

The aim of the investigation is toanalyze the criteria for the danger of the insecticide metofluthrin and assess the risk to health caused by chronic inhalation exposure to the substance at the level of the maximum possible concentrations in the air.

An expert-analytical study of scientific information on the toxicological properties of metofluthrin was conducted and the key effects of its negative impact on the body were determined. To characterize the inhalation hazard, the threshold of acute and chronic action (Limac, Limch), the zone of acute and chronic action (Zac, Zch), and the coefficient of probable inhalation poisoning (CPIP) were calculated. The assessment of the risk of developing carcinogenic effects from chronic inhalation exposure to metofluthrin was carried out according to the system of criteria recommended by the US EPA, using proven international approaches.

The results obtained in the study indicate that the main criteria for the danger of metofluthrin are acute inhalation toxicity and Zch. The coefficient of carcinogenic potential is 1.62×10^{-2} $(mg/kg\times day)^{-1}$, based on the increased incidence of liver tumors in rats.It has been established that the oncogenic potential of metofluthrin is non-genotoxic. The basis of the hepatocarcinogenesis in rats is the activation of the constitutive androstane receptor (CAR), which leads to the induction of cytochrome P450 enzymes (the CYP2B group), proliferation of hepatocytes and changes in the liver. The calculated individual carcinogenic risk under different scenarios of inhalation exposure to metofluthrin at a concentration of 1,0 mg/m^3 is $4,1\times10^{-4}$ and is estimated as average, which is acceptable for industrial conditions. The risk level from exposure to a concentration of 0,14 mg/m^3 is $5,7\times10^{-5}$ and is classified as low – an acceptable level for the population.

Conclusions: The likelihood of developing negative consequences for public health from chronic inhalation exposure to metofluthrin, which was assessed by quantitative risk indicators, is low.Reducing the risk to minimum values ($<10^{-6}$) is achieved by establishing the "dose-time-effect" relationship and developing appropriate medical-sanitary measures.

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P19-22
Development of an IATA for antiandrogen endocrine disruption

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Androgens regulate reproductive function, behaviour, and male secondary sexual characteristics and are critical during development, puberty, and adulthood. They mediate their biological effects via binding to and activation of Androgen Receptors (ARs), initiating downstream gene expression. Antiandrogenic effects of chemicals, and specifically effects on male reproductive function, have long been an issue of concern for both human health and environmental species. Antiandrogens may disrupt the androgen axis by affecting levels of circulating androgens or by interfering with the ARs (expression levels, competitive binding, deregulation of key co-regulators, and other essential transcription factors). Effects on either hormone levels or the AR, result in altered expression of androgen dependent genes and proteins. The consequences can be a variety of adverse health outcomes, that may be irreversible if disruption occurs during development. The most well understood of these are decreased anogenital distance (AGD), cryptorchidism, hypospadias and poor sperm quality in male mammals, and effects on male secondary sex characteristics in fish. Androgens also regulate sexual development and reproductive function in female vertebrates but adverse effects of antiandrogenic chemicals in females are less understood.

The aim of this work is to develop an Integrated Approach to Testing and Assessment (IATA) for identifying chemicals that cause adverse health effects in both humans and environmental species via an antiandrogenic mode of action. The activity is an international collaboration within WP 6 of the PARC partnership. In the first step of the IATA development, a mechanistic model has been created that describes key mechanisms and effects involved in antiandrogen disruption on different levels of biological organisation, from molecular initiating events (MIE) via key events (KEs) to adverse outcomes (AO) on the organism or population levels. The model was based on relevant Adverse Outcome Pathways (AOPs) available in the AOP wiki (https://aopwiki.org/), as well as on expert knowledge and literature searches. In subsequent steps, *in vivo*, *in vitro* and *in silico* test methods will be identified and mapped onto the model. Both standardized regulatory tests and assays and relevant non-standard methods will be described and included. Case studies will be conducted at different stages to evaluate and refine the IATA. The work incorporates the latest relevant scientific research and regulatory frameworks and developments, and promotes the use of mechanistic information and implementation of New Approach Methodologies (NAMs), to better address chemical hazard and risk assessment needs.

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P19-23
A new approach of well-being assessment in the workplace: an integrated health promotion program for night-shift workers

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Workplace Health Promotion (WHP) is crucial for enhancing employee well-being and productivity [1]. Following the principles of the Total Worker Health trademarked approach, this study employs a comprehensive range of biological assessments and validated questionnaires [2]. The aim is to integrate preventive and protective measures against workplace hazards with the promotion of employee health and well-being [3,4]. Over a 2-year period, 82 maritime terminal workers, with a majority (72%) working night shifts, participated in the initiative. With explicit consent, extensive socio-demographic, lifestyle, health, and occupational data, including night shift involvement, were meticulously recorded. The program included various biological evaluations, such as blood and saliva sampling, electrocardiography, alongside administration of validated lifestyle and psychodiagnostics questionnaires. Monthly telephonic follow-ups tracked key health parameters, followed by a comprehensive reassessment after 12 months. Baseline analyses revealed high adherence to the Mediterranean diet (96%) and satisfactory physical activity levels (88%). However, 18% were obese, and almost half had a medium cardiovascular risk index (CVRI). Notably, non-night shift workers exhibited significantly higher CVRI (68% vs. 35%). Also, despite poor sleep quality in 49% of workers, no significant correlation with salivary melatonin levels was observed, although higher levels were found among night shift workers. Evaluation of work-related stress indicated low levels, with all participants displaying an E/R ratio <1. While 12% exhibited overcommitment, no disparities were noted across shifts. Moreover, findings from the Work Ability Index mirrored those of the ERI, with 96% perceiving good or excellent work ability, regardless of shift patterns. Although salivary cortisol levels were within the normal range, elevated alpha-amylase levels were noted among non-night shift workers. Monthly monitoring revealed challenges in weight and smoking cessation interventions. However, significant improvements were observed in perceived well-being (20%), physical activity levels, and sleep quality (20%) at the 12-month follow-up. In summary, these findings, particularly the notable improvements in perceived well-being and sleep quality observed after 12 months of program implementation, highlight the evolving nature of WHP. This underscores the need for holistic strategies that comprehensively support employees' well-being over the course of their careers [5]. In conclusion, the incorporation of biological monitoring and questionnaires emerges as an innovative assessment strategy within WHP, enabling customized interventions and yielding enhanced outcomes.

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P19-24
Risk modeling of mixtures of endocrine disrupting chemicals relevant to human exposure, using zebrafish (Danio rerio) embryo as model organism – the RiskMix project

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Between 2019–2024, the FORMAS funded project RiskMix addressed different aspects of potential health effects posed by exposure to complex mixtures of anthropogenic chemicals, focusing on the general background exposure. Here we will present an overview of the projects' achievements, such as that we:

- Obtained an updated overview regarding the chemical exposure reaching beyond current knowledge from monitoring programmes of already regulated chemicals. [1]
- Used so-called market data in combination with *in silico* tools to facilitate the search for key toxicants released from our products. [2]
- Established novel computational models to assess chemicals potential effect on the thyroid hormone pathways avoiding animal tests. [3]
- Developed and validated chemical analytical methods to facilitate the analysis of currently used phenolic endocrine disrupting compounds. (manuscript in preparation)
- Set-up of a chemical group mixture assessment approach based on established exposure, using a several biological test systems (*in vivo and in vitro*) to facilitate a multidimensional risk assessment whilst keeping down the workload. (manuscripts in preparation)
- Proposed a novel 5-scale relevance level matrix for derived effect levels used in mixture risk assessments. [4]
- Derived novel effect levels at a high relevance level for PFAS exposure, used in mixture risk assessments. [4]
- Established several physiological based kinetic models for single and chemicals in combinations, drawing the conclusion that no mixture effect could be observed at environmental relevant levels. Higher exposure levels did affect the uptake competitively, which is important information for the design of mixture studies. [5,6]

Effects have been observed already at 10x human blood concentration (HBC) in zebrafish embryo toxicity test and *in vitro* cell based assays on the androgen (anti-) and estrogen systems. On the adipogenesis, the exposure to 100x HBC induces an increase in the number of cells that are recruited to become adipocytes and also could contribute to the development of obesity. Data is being evaluated from metabolomics and transcriptomics studies on the exposed zebrafish embryos, and we have indications of altered lipid metabolism at levels <<1 HBC.

The project delivered knowledge important for the inclusion of chemical mixtures as an entity in REACH chemical management strategy, e.g. addressing the implementation of a sufficiently protective mixture assessment factor.

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P19-26
Moving towards a framework for *in vitro* risk assessment and determination of human relevance of rodent liver-mediated thyroid toxicity

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Liver-mediated (indirect) thyroid toxicity is a common observation in rodents following repeated exposure to exogenous substances. Determining whether such toxicity is human relevant has generally relied on the conduct of extensive mechanistic *in vivo* investigations in rodents, to establish the primary molecular initiating event (MIE) and mode of action (MoA), coupled with limited *in vitro* evaluations to eliminate other potential MIEs. New Approach Methods (NAMs) offer the possibility to explore toxicities using *in vitro* (usually human based), *in silico* and *in chemico* models and thus to significantly reduce animal use. Furthermore, NAMs are intrinsic to the development of exposure-driven Next Generation Risk Assessment (NGRA). However, to ensure a robust NGRA, quantitative knowledge concerning the Adverse Outcome Pathway (AOP) for the toxicity in question is required. Although rodent indirect thyroid toxicity has been widely investigated, data to develop a quantitative AOP are sparse.

To address this deficit, short-term *in vivo* toxicodynamic (TD) (mRNA/enzyme activity, thyroid hormone measurements and liver/thyroid histopathology following 7-day treatment) and toxicokinetic (TK) (plasma concentrations and TK evaluations after a single dose) studies were performed in male Wistar rats using two reference compounds that induce thyroid toxicity via induction of hepatic enzymes, phenobarbital (PB) and pregneneolone-16α-carbonitrile (PCN). To confirm the absence of a direct effect on the thyroid both compounds were evaluated in an *in vitro* 3D rat thyroid model at several concentrations. Finally, to determine quantitative and/or qualitative species differences for this MoA, both compounds were assessed *in vitro* by measuring UGT-T4 enzyme activity and T4 clearance in rat and human hepatocytes.

Dose responses for both compounds were established for each of the parameters measured in the *in vivo* rat TD study allowing an estimation of the point of departure for each of the key events (KEs) associated with liver-mediated thyroid toxicity as well as quantification of the KE relationships. Neither PB nor PCN impacted T4/T3 secretion in the 3D rat thyroid model in contrast to the reference thyroid toxicant, propylthiouracil; thus, confirming the absence of a direct thyroid effect for both test compounds. Finally, concentration-related increases in UGT-T4/T4 clearance were observed *invitro* in rat but not human hepat-

ocytes. These *in vitro* data demonstrated marked species differences with respect to the critical KE in the MoA, namely increased T4 clearance. In addition, the rat-specific data allowed for quantitative *in vitro/in vivo* comparisons.

Modelling of the rodent (*in vivo/in vitro*) and human data is on-going to develop a framework that could eventually be used for an *in vitro* risk assessment of rodent liver-mediated thyroid toxicity.

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P19-27

Effect of per- and polyfluoroalkyl substances (PFAS) on neurodevelopment: evaluation based on the TRAEC strategy in a systematic review context

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Objectives: Although emerging evidence to the association between per- and polyfluoroalkyl substances (PFAS) and neurodevelopment have been investigated, there is no consensus on the effect of PFAS on neurodevelopment. The novelty Targeted Risk Assessment of Environmental Chemicals (TRAEC) strategy in a systematic review context was performed to assess the association between PFAS and neurodevelopment risk.

Methods: The studies from five online databases were analyzed the effect of PFAS on neurodevelopment. The potential neurodevelopment risk of PFAS was evaluated by the TRAEC strategy, which was conducted on a comprehensive scoring system with reliability, correlation, outcome fitness and integrity.

Results: There were 2597 papers were identified according to search strategies, and the remaining 30 literatures were included the present study to proceed following risk assessment. The overall risk-score was 4.4 for assessment between PFAS exposure and neurodevelopment based on the comprehensive evaluation with TRAEC strategy, illustrating that the effect of PFAS exposure on neurodevelopment was at a mid-level risk. The population-attributable risk (PAR) was 14.5% for mixed PFAS exposure. *In vivo*, the significant effect between short-chain PFAS exposure and assessment of neurodevelopment with a 6.67 risk-score. Four criteria-based tools for health risk assessment (ToxR-Tool, SciRAP, OHAT and IRIS) were employed to illustrate the robustness and reliability of our results in the present study.

Conclusions: There was mid-risk effect of PFAS exposure on neurodevelopment. In addition, The TRAEC strategy provided a scientific and structured method for risk assessment between PFAS and neurodevelopment, promoting the consistency and validation in study evaluation.

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P19-28

Exposure data to cosmetic products of consumers presenting atopic dermatitis

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Atopic dermatitis (AD) is a chronic inflammatory dermatosis characterized by flare-ups and affecting worldwide 20% of children and 10% of adults. The main cutaneous manifestations are xerosis, erythema, pruritus and vesicles. Emollient application can reduce these symptoms and prevent flare-ups. Therefore, people presenting atopic dermatitis can apply a large quantity of emollient. Prior to marketing a cosmetic product, a safety assessment based on realistic exposure data must be performed. However, few data are available in literature for patients suffering from AD. Thus, the aim of the study was to collect exposure data and information about cosmetic product consumption habits of patients presenting atopic dermatitis.

204 subjects with a SCORAD over 10 were recruited. The panel was composed of 100 children from 2 to 11 years old and 104 adults from 20 to 57 years old. A survey was performed to collect Information on subjects' AD, such as body areas affected, SCORAD, AD treatment. Frequencies of use of cosmetic products during and outside AD flare-ups were collected for 12 products for children and 18 products for adults. Quantities of 2 cosmetic products, a cleansing oil and a lipid-replenishing balm, applied by the participants for 7 days were also obtained. The products were supplied to the panel and weighed before the first use and after the last one.

Among the adult panel, subjects were presenting a SCORAD from 11 to 85 with a mean value of 36.57. 31.73% of adults received a treatment for their AD. The most affected body areas were legs, arms, hands and face. 92.31% of adults reported skin dryness during AD flare-ups, 87.50% discomfort sensations and 85.58% itching. 65.38% of adult participants indicated a modification in their cosmetic products consumption during atopic dermatitis flare-ups. 30.77% of them increased the applied quantity of some products and 37.50% changed the types of cosmetics used.

14 of the 18 investigated products were used by the adult panel with a higher frequency during AD flare-ups than outside flare-ups. For example, the mean frequency of use of cleansing oil was 1.35 times/day during flare-ups versus 1.07 times/day outside, it was 1.58 versus 1.18 for barrier cream and 1.73 versus 1.49 for face cream. The percentage of users were also higher during AD flare-ups for 12 products. The prevalence of use was 83.65% for lip balm during flare-ups versus 75.96% outside, 56.73% versus 35.58% for the repairing cream and 27.88% versus 18.27% for the scalp soothing lotion.

During the application week of 2 cosmetic products, the mean quantity applied was 15.04 g/day (P90=27.79 g/day) for cleansing oil and 9.75 g/day (P90=20.49 g/day) for the lipid-replenishing balm.

Thanks to this study, accurate consumption and exposure data to cosmetic products for adult and child populations presenting atopic dermatitis will be available. All information collected will help to perform more suitable safety assessment of cosmetic products.

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P19-29

Trifloxystrobin – combination of *in vivo* and *in vitro* approaches to address Parkinson disease concerns

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Trifloxystrobin (TFS) is an established fungicide widely used in food and feed crops. As a member of the strobilurin fungicide class it acts as disruptor of mitochondrial respiration by targeting mitochondrial complex III in fungi. Even though the available robust *in vivo* database for TFS showed no indication of neurotoxicity there is increasing concern mainly due the assumed relationship between mitochondrial complex I inhibitors and Parkinson disease and recent *in vitro* investigations with strobilurins in neuronal cell models.

Exposure to the target tissues, in this case the brain, is a pre-requisite for the elicitation of a toxic effect *in vivo*. Therefore, the goal of this project was the development of a human PBPK model to predict potential brain exposure level of TFS and its major carboxylic acid metabolite M05. A combination of *in vivo* and *in vitro* methods was used to inform the different model parameters.

Stability investigations under acidic and basic conditions in *in vitro* gastrointestinal fluids and Caco-2 cells indicate that transformation of TFS already take place during the passage through the intestine. Additional studies on the absorption using a human gut-model, revealed low *in vitro* intestinal absorption indicating low oral bioavailability. Incubations with rat and human microsomes revealed a complete metabolization after one hour, further supporting an extensive degradation of parent TFS before entering the systemic circulation. Prediction of blood brain barrier (BBB) permeability is a key factor in central nervous system exposure estimation, given that a functional BBB prevents over 98% of chemicals from penetration. Initial investigations in a human *in vitro* BBB model showed that neither TFS nor its major transformation products were able to enter the brain compartment. Confirmation of these *in vitro* observations was aimed for by conduct of a rat *in vivo* TK study with focus on brain tissue levels.

Based on a set of *in vitro* investigations, it could be shown that both the inhibitory potential on cellular respiration and the cytotoxicity is related to the stereoisomeric form of TFS as well as to the presence of the toxophore (methoxyacrylate). Comparison of available cytotoxicity results in different *in vitro* test systems also showed that there seems to be no increased sensitivity of neuronal cells compared to other mammalian cell types. *Invitro* TFS was cytotoxic at similar concentrations in the primary hepatocytes and the undifferentiated dopaminergic neuronal cells (SH-SY5Y) and less cytotoxic in the cholinergic differentiated SH-SY5Y. M05 showed no cytotoxicity up to 100 µM in all cell models tested.

Thus, the extensive and quick metabolism of TFS, leading to the generation of M05 lacking the toxophore, and its low brain penetration is supporting the conclusion that there is a very low probability that its biological activity (inhibition of mitochondrial complex III) translates into a human Parkinson disease risk

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P19b | Risk Prediction and Assessment/ Risk assessment using New Approach Methodologies

P19-30
Integrated Risk Assessment for Pyrethroids: A "Proof of Concept" approach for toxicokinetics supported by NAM-based toxicodynamics

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Pyrethroids, a widely used insecticide with structural similarities, are widely used as active ingredients in various products, including plant protection products, household insecticides, and even some pharmaceuticals. While human biomonitoring data is providing actual exposure levels, translating this exposure estimations into actual risk remains complex. This complexity is due to multiple exposure routes and the presence of common urinary markers for pyrethroids with varying potencies for neurotoxic and other health effects. Therefore, the retrospective risk assessment should consider the aggregate exposure of each pyrethroid from different sources, and the combined effect of different pyrethroids. This study explores the potential of TKs and NAM-based mechanistic TDs to address this challenge. The EFSA's TKPlate contains multiple generic physiologically based kinetic models including physiological parameters that describe general population with most of the chemical parameters required for the simulation. Using an embedded QIVIVE tool, the required clearance values can be aggregated, while also accounting for population variability and polymorphism. The TKPlate has been used to model the pyrethroids' internal concentrations using two complementary input sources, human biomonitoring and probabilistic external exposure estimations adapted from regulatory assessments. Since current regulatory assessment focus on worst-case assumptions, this adaptation is needed and should be replaced by a full distribution of each parameter to get realistic estimations that include population variability. In parallel, we have explored the capacity of ToxCast's high-throughput bioactivity data to inform on the similarities and differences in the bioactivity/potency of the different pyrethroids. For this, the available *in vitro* information has been extracted for conducting a direct comparison of pyrethroid bioactivity profiles. Then, the profiles have been analyzed alongside estimated internal exposure levels and compared with the related information from the available *in vivo* studies. The integration of both lines of evidence provides information on the relative contribution of TK and bioactivity as explanatory factors for the different toxicity potencies observed for pyrethroids *in vivo*. In addition, the comparison of the bioactivity profiles offers additional value for defining the problem formulation of the integrated (aggregate and combined) higher tier retrospective risk assessment of measured exposure levels and paves the way for further exploration of ToxCast data in evaluating the safety of complex chemical mixtures.

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P19-31
Comaparison of TiO_2 particle size distribution in artificial digestion solutions using single-particle ICP-MS

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It is important to assess the dispersion state of the nanoparticles and their mode of presence in the digestive system for risk assessment of nanoparticles exposure via food products. It is known that in titanium dioxide, which is known to be a food additive, some percentage of nanoparticles as a primary size present at the product level. However, the state of presence of nano-sized particles just before absorption is unclear. Recently, we have investigated the particle size distribution of titanium dioxide in artificial digestive juices using dynamic light scattering (DLS) method. In general, it is recommended to use several methods to analyze the physical properties of nanoparticles. The purpose of this study was to determine the status of nanoparticle distribution using single particle ICP-MS (spICP-MS).

As for the test materials, seven titanium dioxide samples for food additives and five samples for industrial use were used. The sample

preparation for the spICP-MS analysis was conducted according to the previously reported method[1] using the continuous artificial digestion solution. As additional dispersion method, the first fluid and the second fluid in the disintegration and dissolution test in the Japanese Pharmacopoeia were used as gastric and intestinal artificial fluid respectively. The samples for spICP-MS analyzing were dispersed in an ultrasonic disperser for 15 min or were dispersed by simple inverting with hand.

Treatment with artificial saliva and gastric juice tended to increase the average particle size. On the other hand, treatment with artificial intestinal fluid tended to decrease the average particle size, especially when dispersion by ultrasonic treatment was not performed. Similar results were obtained when titanium dioxide was used for industrial applications with a large proportion of small primary particles. Generally, when the isoelectric point of the particles is close to the pH of the solvent, electrostatic repulsion does not work and they tend to aggregate. Since the isoelectric point of TiO_2 is around pH 6, it is considered that it tends to aggregate in the artificial intestinal fluid. Therfore, the average particle size decreased due to the sedimentation of agglomerated particles. However, the degree of changes in the average particle size depending on different type of fluids was smaller than the width of the particle size distribution.

Conclusively, there were more than 10% of the particles below 500 nm were below 250 nm in the continuous artificial digestion solution for all the samples examined. It was judged that all samples were necessary to evaluate the safety as a nanomaterial based on the EFSA technical guidance criteria. Although similar results obtained when DLS methods was used for analyzing, it was considered that it was necessary to evaluate the aggregation and sedimentation of particles.

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P19-32
Advancing the application of New Approach Methodologies (NAMs) in systemic toxicity assessment of cosmetic ingredients: insights from cosmetics Europe long range science strategy (2016–2022) case studies

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The Cosmetics Europe Long Range Science Strategy (2016–2022) has performed a series of 15 read across and ab initio Next Generation Case Studies to further the application of New Approach Methodologies (NAMs) in systemic toxicity assessment of cosmetic ingredients. We selected a mixture of cosmetic-relevant case studies including substances as Benzyl salicylate, Genistein, homosalate, 2-ethylhexysalicylate, benzoic acid salts and esters, avobenzone, benzophenone-4 and octocrylene.

The 10-step read-across framework was applied to read across case studies, with a focus on building confidence in the use of different NAM approaches for the selection of appropriate analogues from a chemistry (e.g. Matched-Molecular Pair Approach, Quantitative Similarity Scoring), toxicokinetics (e.g. *in vitro* metabolism) and Mode of Action/bioactivity (e.g. toxicogenomics and Connectivity Mapping, ToxCast, Pharmacology Profiling, CALUX data) point of view. For the ab initio case studies, the workflow was inspired by the SEURAT-1 framework by integrating NAM approaches for estimating internal exposure and estimating levels of bioactivity. Key ADME parameters were measured *in vitro* (e.g. skin absorption, hepatic clearance and plasma protein binding) and these were then used in physiologically-based kinetic modelling to estimate internal exposures. The bioactivity/toxicodynamic tier 1 testing included assays such as cell stress panels, toxicogenomics, and pharmacology profiling. For a subset of case studies, further testing was necessary based on hypothesis identified at tier 1 or due to uncertainties in the assessment. These included follow-ups with developmental toxicity and renal toxicity assays. PoDs were compared with internal exposure metrics to derive margins of internal exposure or bioactivity-exposure ratios.

The various case study achievements are presented here, which have increased confidence in read across and ab initio assessments. The conclusions drawn and the remaining challenges identified have paved the way for various projects under the International Collaboration for Cosmetic Safety (ICCS), established in 2023. This global organization aims to foster confidence in NAMs, leading to international consensus that animals are no longer necessary for the safety assessment or registration of cosmetic ingredients. In conclusion, it could be demonstrated in many cases that NGRA approaches enable robust safety assessments that are at least as protective as traditional approaches.

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P19-33
Per- and Polyfluoroalkyl Substances (PFAS) in food: Background exposure in the Swedish population

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Background and aim: Diet and drinking water have been identified as major sources of PFAS exposure in humans. To accurately estimate exposure in the Swedish population, knowledge of background levels in foods are crucial. The study aims to increase knowledge of levels of PFOA, PFNA, PFHxS, and PFOS (PFAS4) in foods commonly consumed by the majority of Swedish consumers, to enable updated exposure estimations in small children, adolescents, and adults.

Material and methods: Food samples from the Swedish Market Basket 2022, including 16 food groups, were analysed for PFAS. Exposure estimations in the Swedish population were performed using consumption data from three Swedish dietary surveys: Riksmaten adults (2010–11), Riksmaten adolescents (2016–17), and Riksmaten small children (2021–2023). Exposure scenarios for drinking water (4 ng PFAS4/L) and fish consumption (2–3 portions/week) were used. A drinking water consumption of 2 (adults, adolescents), 1.6 (4-year-olds), and 1.2 (1.5-year-olds) L per day was used, and a portion size of 150 (adults), 100 (adolescents), and 50 (small children) g for fish. All results are presented as lower bound.

Results: Detectable levels of PFAS4 were found in 3 of the 16 food groups, in lean fish (PFOA, PFNA, PFOS), fatty fish (PFOS), and eggs (PFOS). The mean PFAS4 concentration was 0.30 ng/g in lean fish, 0.08 ng/g in fatty fish, and 0.05 ng/g in eggs. The median exposure of PFAS4 from food in adult women and men was 0.12 and 0.10 ng/kg body weight (BW)/day (75th percentile (p75), 0.22, 0.21), respectively. In 4-year-olds and adolescents, it was lower, medians 0.03–0.08 ng/kg BW/day (p75, 0.13–0.42), and in 1.5-year-olds, slightly higher, median 0.11 ng/kg BW/day (p75, 0.46). The proportion exceeding the TDI by EFSA (0.63 ng/kg BW/day) was decreasing by increasing age. In the

small children 16–19% exceeded the TDI, whereas 1–4% of the adults and adolescents. When drinking water was added, the median exposure rose to 0.24 and 0.20 ng/kg BW/day for women and men, respectively, and to 0.16–0.27 ng/kg BW/day for adolescents. In 1.5-year-olds, median exposure was 0.55 ng/kg BW/day and in 4-year-olds 0.47 ng/kg BW/day. TDI exceeded in 3–11% of adults and adolescents and 33–41% of small children. Consuming fish 2–3 times/week, including drinking water, raised median exposure to 0.31 and 0.26 ng/kg BW/day for women and men, respectively, and to 0.25–0.39 ng/kg BW/day in adolescents. The median exposure was 0.83 and 0.64 ng/kg BW/day in 1.5- and 4-year-olds, in turn.

Conclusion: The highest exposure to PFAS4 was seen in 1.5-year-olds, and the lowest in adolescents. The median exposure in the scenario of fish consumption 2–3 times/week and drinking water included was below the TDI, except for the small children. The results stress the importance of updating levels in food in order to obtain accurate exposure estimations, as background exposure to PFAS4 is decreasing.

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P19-34
Evaluating the risk of adverse health effects from the PFAS exposure in the Swedish population

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Per- and polyfluoroalkyl substances (PFAS) are a group of anthropogenic chemicals used because of their unique combination of both hydrophobic and heat-resistant properties. PFAS have been found in high levels both in the environment and in humans. Based on previous results, PFAS were identified as the risk drivers of the known chemical mixture in human blood [1]. The aim of this study was to establish the PFASs blood levels and assess the risk of different adverse health effects.

This study analyzed PFAS in 60 presumably healthy blood donors from Stockholm using a previously described method [2]. A targeted method including 42 PFAS analytes and a suspect screening of over 300 molecular features were used. For the mixture risk assessment (MRA), 7 novel PFAS effect levels were derived and 5 were found in literature. Epidemiological studies with at least 750 individuals were used to derive the new effect levels. In total, 12 MRAs were conducted including effects related to the immune system, kidney, thyroid, development, reproduction and blood lipids. Additionally, a grading system for the effect levels was established, with a five-level scoring system called relevance levels (RL). The RL are based on data quality and methodology used for deriving the effect level. RL 1 are effect levels derived as human biomonitoring guidance values where a thorough literature search of all available data has been done. RL 2 and 3 are effect levels derived using epidemiological studies where a statistically significant association has been found. RL 4 are effect levels derived using external reference values and RL 5 are directly derived from animal studies.

The entire studied population exceeded the risk threshold related to thyroid disruption, kidney disruption and lipid metabolism disruption, suggesting that the risk of these effect cannot be excluded for the Swedish population. For the effect levels used in this study, 8 of 12 MRAs were categorized with strong relevance (RL 1 or 2). During the suspect screening, 20 features in the samples were found. Three H-PFCAs and PFECHS could be identified at confidence level 1b using reference standards. The result from this study shows that a risk for adverse health effects from PFAS cannot be excluded for the Swedish population.

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P19-35
Bronchoalveolar lavage fluid analysis method for the inhalation toxicity studies of nanomaterials

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The safety of nanomaterials has become a global agenda due to the rapid industrialization of nanotechnology, leading to a heightened focus on Environmental, Health, and Safety (EHS) studies and regulations. Consequently, the analysis of bronchoalveolar lavage fluids (BALF) and lung burden in inhalation toxicity studies of nanomaterials has become more important. However, the absence of standardization in BALF analysis methods has led to potential laboratory discrepancies. Therefore, we evaluated various collection and analysis methods in BALF using nickel oxide nanoparticles, zinc oxide nanoparticles, and graphene oxide nanoparticles. The process of BALF collection had crucial factors, including the number of collection times, flushing, and massage. The total cells, lactate dehydrogenase (LDH), and total protein in inflammation analysis of BALFs decreased as the number of lavage fluid collection times increased regardless of nanoparticle instillation. Moreover, a group that did flush and massage (wFwM) showed an increase in total cells, LDH, and total protein compared to the other group that did not flush and massage (woFwoM). However, the proportion of inflammatory cells in the wFwM group gradually decreased as the number of collected lavage fluid increased. These results could be interpreted overly in analyzing lung inflammation caused by nanoparticles. The PBS with fetal bovine serum (FBS) was excellent as the re-suspension manner for cells after centrifugation of BALF, and the 4°C was better than 37°C as storage temperature because other manner showed cell death with the frustrated cell membrane. Lastly, we compared the manual and automatic methods of counting total cells using a hemocytometer and Nucleocounter. When the number of total cells was high, the manual method tended to count far more cells than the automatic method. These results can contribute to the standardization of processes by providing a method for collecting and analyzing BALF, thereby enhancing the reliability and comparability of research in this field.

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P19-36
Dietary exposure assessment to plasticisers migrating from food contact materials

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In 2020, EFSA was requested by the European Commission to re-evaluate the risks to public health related to the presence of plasticisers such as phthalates, structurally similar substances and replacement substances, as a consequence of migration from food contact materials (FCMs). After identifying and prioritising those plasticisers that may warrant further data collection and eventual risk assessment [1], EFSA established a protocol for assessing the exposure of EU consumers to the prioritised plasticisers [2]. This work was undertaken in close collaboration with the European Chemicals Agency (ECHA). The protocol outlines the approach for assessing dietary exposure to the plasticisers, with the aim of addressing the relative contribution from FCMs to dietary exposure, considering data on migration from FCMs and eventual comparison of these contributions with the overall exposure of EU consumers. For that purpose, three key questions have been defined: i) what is the total dietary exposure, ii) what is the dietary exposure coming from FCMs, and iii) what is the overall exposure (dietary and non-dietary) to the plasticisers in different population groups and age classes in the EU. Furthermore, from 2022 EFSA established calls for data on occurrence of the prioritised substances in food and in FCMs with the aim to acquire evidence to support dietary/non-dietary exposure and eventually risk assessment. In this contribution, key methodological points of the protocol will be presented with a focus on dietary exposure assessment.

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P19-37
How to manage 2-MCPD, 3-MCPD and glycidol in cosmetic products?

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2-monochloropropane1,3-diol (2-MCPD), 3-monochloropropane1,2-diol (3-MCPD), glycidol and their fatty acids esters are impurities of safety concern initially described in food. These impurities are unintentionally formed in vegetable oils, fats and certain processed foods. Two of them are regulated in food for adults (EU 2023/915) with maximum acceptable levels in vegetable oils at 1250 µg/kg for 3-MCPD and 1000 µg/kg for glycidol.

Because vegetable oils and fats are widely used in cosmetic products, we wondered whether these impurities could be at risk for consumers.

76 raw materials (RM) likely to contain these impurities were identified and dosages were carried out using gas chromatography-mass spectrometry in our cosmetic RM.

To characterize the hazards for each impurity, a bibliographic search was first conducted.

Secondly, a read-across analysis using 3 softwares, COSMOS, GENRa and QSAR Toolbox was performed between 2-MCPD and 3-MCPD. Skin prediction absorption was also realized using ISafeRat® Dermal Absorption model which is a mathematical tool based on Quantitative Structure Activity Relationships.

Then, *in vitro* studies were conducted to confirm data on skin absorption (OECD 428) as well as cutaneous metabolism to refine consumers' systemic exposure. In addition, a modified Ames test (*S. typhimurium* and *E. coli*) was carried out for 2-MCPD.

Finally, margin of safety calculations were performed to determine safe maximum levels for these 3 impurities.

Among the 76 RM dosed, 50 contained at least one of the 3 impurities. 2-MCPD was detected from 16 to 19067 µg/kg, 3-MCPD from 14 to 7999 µg/kg and glycidol from 20 to 949722 µg/kg.

2-MCPD toxicity is not totally described particularly for genotoxicity, for which only equivocal results are available. 3-MCPD has renal and male fertility toxicity in rodents and is considered as probably carcinogenic to humans according to International Agency for Research on Cancer classification. The Tolerable Daily Intake of 2 µg/kg pc/d was selected as Point of Departure (PoD). Glycidol is a genotoxic carcinogen. The low concern intake of 0.24 µg/kg pc/d was selected as PoD.

Read-across between 2-MCPD and 3-MCPD was not relevant due to non-comparable metabolism and toxicity according to the 3 softwares used. The skin absorption of 2-MCPD, 3-MCPD and glycidol predicted by iSafeRat® was 85.8%, 88.8% and 15.8% respectively.

The preliminary results available from the *in vitro* studies showed that 2-MCPD is mutagenic in *S. Typhimurium* TA 98 with metabolic activation at 1250 µg/plate. Moreover, glycidyl palmitate is poorly absorbed through the skin and completely metabolized to glycidol. Final results are pending to confirm these observations.

Overall, these results led to determine relevant PoD and exposure to calculate maximum safe level of 2-MCPD, 3-MCPD and glycidol in cosmetic product for adults.

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P19-38
In vitro assessment of tobacco free nicotine pouches reveals marked reductions in toxicity when compared to cigarettes

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Tobacco-free nicotine pouches (TFNPs) are an emerging category of nicotine-containing oral products. These products do not contain tobacco leaves, do not undergo combustion, and nicotine delivery is through the gum rather than inhalation. As TFNPs don't contain or burn tobacco, research demonstrates that they contain fewer and substantially lower levels of the harmful chemicals found in cigarette smoke. Studies have shown that the reduced level of toxicants translates to reduced *in vitro* biological activity compared to cigarette smoke extracts.

Twenty-one TFNPs, of varying designs (nicotine content, flavours, and pouch sizes) were assessed in the *In vitro* Micronucleus (IVM), Bacterial Reverse Mutation Test (Ames) and Neutral red assays (NRU).

The studies were performed in general compliance with OECD Guidelines (Test guideline No. 487 for IVM, Test guideline No. 471 for Ames) and ISO guidelines (ISO 10993 for the extraction of OND products). NRU tests were performed using BEASB-2B human bronchial epithelium and HepG2 human hepatoma cells, IVM tests were performed using V79 hamster lung fibroblasts, and Ames performed with the inclusion of five strains: TA98, TA100, TA102, TA1535 and TA1537. In each test, the cell lines were treated with the pouch extracts, and

in the IVM and Ames tests the exposure was carried out in both the absence and presence of metabolic activation with S9.

None of the TFNP extracts induced dose-dependent, reproducible, or statistically significant increases in micronucleus frequencies and therefore they did not meet the criteria to be classified as genotoxic under the test conditions.

Likewise, in the Ames Test, none of the TFNP extracts demonstrated evidence of causing reproducible, dose-dependent, or statistically significant increases in the number of revertants with or without S9 (metabolic activation) mix and were therefore classified as not mutagenic under the test conditions.

In contrast, the 1R6F reference cigarette was classed as genotoxic and mutagenic in TA98 and TA100 (+/-S9) and TA1537 (+S9).

The cytotoxic effects of all extracts were compared to the cytotoxic effects of 1R6F reference cigarette Total Particulate Matter (TPM). Based on the NRU results, the EC20 (Effective Concentration at which cytotoxic effects were seen based on growth inhibition of 20%) values obtained for the TFNP extracts were 32–618 times less cytotoxic than the levels for cytotoxicity obtained for the 1R6F cigarette TPM. Likewise based on the EC50 values obtained the TFNP extracts were 50–260 times less cytotoxic than the levels for cytotoxicity obtained for the 1R6F cigarette TPM. Not all TFNP extracts induced EC50 values.

These results add to the growing body of literature showing that reduced toxicant levels result in marked reductions of *in vitro* activity, solidifying the TFNP's tobacco harm reduction potential for adult smokers.

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P19-40

Risk assessment of AFB1 due to CYP3A4-mediated biotransformation with a highly sensitive human intestinal model

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Background: Exposure of humans to aflatoxin B1 (AFB1) is a major concern because of its potent carcinogenic properties. The biotransformation of AFB1 in humans primarily involves CYP3A4, a predominant metabolizing enzyme that is highly expressed in the intestine and liver. Considering that the human intestine is the major site of absorption and metabolism for xenobiotics, the gastrointestinal tract is possibly the first organ targeted by AFB1 toxicity. For this reason, investigating the risk of AFB1 exposure to the human intestine is essential. However, the intestinal risk assessment of AFB1 remains unclear due to the lack of CYP3A4 expression in current intestinal models. To address this issue, a doxycycline-inducible CYP3A4-expressing Caco-2 cell line was established in the Mizuguchi Lab (Sci Rep, 11, 11670, 2021). These cells exhibit CYP3A4 activity comparable to that in the adult human intestine.

Objectives: This study aims to investigate the effects of AFB1 on the gastrointestinal tract mediated by intestinal CYP3A4 by using the CYP3A4-expressing Caco-2 cells.

Methods: Cytotoxicity, barrier integrity, and epithelial permeability of different-sized dextrans were evaluated under treatment with different concentrations of AFB1.

Results: The cellular toxicity of AFB1 in induced Caco-2 cells was obviously higher than in cells without induction. When exposed to 16 μM AFB1, the barrier integrity was compromised, whereas it remained unchanged in cells that were not induced. Furthermore, there was a significant increase in the paracellular transport of 4- and 20-kDa dextran in the induced Caco-2 cells, which was 5.4 and 5.2 times greater, respectively, compared to the uninduced cells.

Conclusions: The biotransformation of AFB1 by intestinal CYP3A4 could pose a risk for the temporary dysfunction of the intestinal barrier, leading to the paracellular permeation of other macromolecules from the gastrointestinal tract into the body. In addition, the doxycycline-inducible CYP3A4-expressing Caco-2 cell line will be an innovative approach for investigating the safety of food-associated chemicals and their potential effects on intestinal health.

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P19-41

The SENS-IS *in vitro* assay to predict and classify the skin sensitization potency of finished cosmetic products

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Skin sensitization is a major concern for the cosmetic industry due to its potential adverse effects on human skin. Therefore, the identification and evaluation of potential skin sensitizers is essential to ensure consumer safety. Recently, there has been significant progress in the development of *in vitro* tests that follow the Adverse Outcome Pathway (AOP) for skin sensitization. Some of these tests have been incorporated into Defined Approaches (DAs) validated by OECD Test Guideline 497 and can be used in the Next Generation Safety Assessment (NGRA) [1].

However, there remains a gap in the sensitization prediction for finished products. Currently, safety assessments rely solely on the assessment of individual ingredients, lacking an alternative method to directly confirm the skin sensitizing potential of formulations and finished products.

The SENS-IS assay uses a genomic signature to discriminate between non sensitizers from sensitizers. It has been shown to accurately assess both skin sensitization hazard and potency by detecting the minimal positive dose tested with high accuracy, comparable to data from the LLNA [2]. Based on its capacity to address a wide domain of application [3], the ability of the SENS-IS assay in sensitization prediction of formula was assessed.

15 cosmetic products were selected from the global market, containing or not known sensitizers and representing a wide range of product categories. The exposure time was set at 15 minutes, and the fold change in gene expression was adapted to 1.3, giving an overall accuracy between 80 and 90%.

Then, a mathematical model was developed to express the results using the full amplitude of overexpression of genes associated to skin sensitization. This led to the assignment of a score-based result for each product and a construction of a sensitization response scale, representing the sensitization potency.

The results of the SENS-IS assay for a finished product were compared with those expected for its known sensitizing ingredients, using the traditional quantitative risk assessment (QRA) approach [4] and new multiple regression models [5].

In conclusion, the SENS-IS assay demonstrates its suitability for the detection and classification of the sensitization of finished cosmetic products. The score-based classification could be used to validate the sensitization potential of a cosmetic product as a complement to traditional ingredients assessment.

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P19-42
Steroidogenesis assay (OECD TG 456) to fill data gap on BPA alternatives and on the natural mycotoxins Enniatins and Beuvericin: preliminary results from two PARC projects

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Identification and characterization of endocrine disrupting (ED) activity of compounds is of high relevance for risk assessment. In the frame of the European Partnership for the Assessment of Risks from Chemicals (PARC), the 5.1.1 activities aim to fill data gaps for human relevance on prioritized compounds. In particular, the following chemicals are under study: the natural mycotoxins Enniatins (ENNA, ENNA1, ENNB, ENNB1) and Beuvericin (BEA) in 5.1.1a, and alternatives of BPA (BPE, BPP, BPZ, BPAP, BPS-MAE, TCBPA, TBBPA) in 5.1.1b.

Within these projects, we are assessing ED activity of the selected compounds by performing the steroidogenesis assay, a validated NAM (OECD TG 456) implying the human H295R adreno-carcinoma cell line which expresses all the enzymes of the steroidogenic pathway. We tested the compounds in a range of 7 ten-fold spanned concentrations (10 pM to 10 μM for Enniatins; 100 pM to 100 μM for BPA alternatives) to firstly assess cytotoxicity and exclude concentrations which decrease vitality >20%. Conditioned medium of each eligible concentration was measured by commercial ELISA kits for 17β-estradiol and testosterone levels.

In addition to what is requested by the TG, we are also analysing by HPLC-MS/MS these same hormones, as well as progesterone, aldosterone and cortisol, to obtain more comprehensive data on possible adverse effects on steroidogenesis exerted by considered compounds.Preliminary results clearly show that some of the BPA alternatives have estrogenic and anti-androgenic properties with a potency higher than BPA. Other analyses are in progress and will be presented at the congress.

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P19-43
Identification of chemical categories of E&Ls having PDEs below the proposed threshold of toxicological concern for non-genotoxic chemicals via intravenous (TTCiv)

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Risk management of E&Ls often requires the evaluation of chemicals without sufficient toxicity data via the intravenous (IV) route. To develop the threshold of toxicological concern for non-genotoxic chemicals via IV (TTCiv) that can be used for evaluation of E&Ls without sufficient toxicity data, we previously proposed route-to-route extrapolation from oral to IV route using the dose ratios based on IV to oral blood concentrations (C_{max}) of about 200 chemicals, and approx. 0.061 mg/day as TTCiv was identified by applying the 5 percentile value of the PDEs via IV (presented in a poster at EUROTOX 2023). Chemicals with PDEs below TTCiv included acrylonitrile, toluene 2,6-diisocyanate, Tinuvin 144, Tinuvin 320, glutaraldehyde, and tetramethylthiuram mono-/di-sulfide. These chemicals and their structural analogues may not be suitable for TTCiv application. In order to identify categories for whichTTCiv cannot be applied in E&Ls assessment, structural analogues of each of above-mentioned chemicals were collected and investigated their toxicological profiles from the toxicity databases.

For those chemicals with a PDE below the TTCiv, the substructures that might contribute to toxicity were investigated. As a result of the investigation excluding acrylonitrile and toluene 2,6-diisocyanate with genotoxic alert structures to be controlled under ICH M7, the identified analogues were categorized into: 2,6-di-tert-butyl-4-methylphenol, 1,2,2,6,6-pentamethylpiperidin-4-ol, phenolic benzotriazole, polyaldehyde structure, and disubstituted thiocarbamate.

The range of NO(A)EL for repeated dose toxicity of chemicals with each substructure listed in the OECD QSAR toolbox were 0.1–1,000 mg/kg/day for 2,6-di-tert-butyl-4-methylphenol or 1,2,2,6,6-pentamethylpiperidin-4-ol), 0.1–1,000 mg/kg/day for phenolic benzotriazole, 0.5–1,000 mg/kg/day for 4-methylmorpholine or 4-(methylthio) benzaldehyde), 4–300 mg/kg/day for polyaldehyde structure, and 0.8 – 1,000 mg/kg/day for disubstituted thiocarbamate. These NO(A)ELs might be derived based on the different toxicological interpretation. These NO(A)ELs ranged from low to high, and category refinement of chemicals with high toxicity was considered necessary.Further improvements of categories will be discussed, including detailed review of NO(A)ELs and refinement of the categories based on the mechanism of toxicity for each structural group. As a result of research, it will be possible to identify sub-categories that require individual evaluation of non-genotoxic chemicals and a read across approach may be applied. The evaluation scheme discussed in this study will enable efficient evaluation of E&Ls using relevant TTCs in the future.

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P19-44
First-pass effect in skin during tattooing – a short-term clinical study on exposure and kinetics of soluble tattoo ink ingredients

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Aim: Following the implementation of the REACH restriction, new limits have been introduced on substances in tattoo inks. However, these are based on *ex vivo*-experiments with pigments and might not accurately represent an *in vivo*-scenario. The aim of this study is therefore to determine the exposure, kinetics and metabolic profiles of soluble tracer substances that were added to the ink prior to tattooing.

Methods: Twenty-four subjects were tattooed with a design of their choice using black or red ink. The three tracer substances 4-aminobenzoic acid, 2-phenoxyethanol and potassium iodide were added to commercially available tattoo inks. 4-Aminobenzoic acid was selected due to the structural similarity to aryls with an amino group, 2-phenoxyethanol as a representative for preservatives used in tattoo inks and iodide to determine the amount of intradermally applied ink. Blood, urine, ink and the consumables used for tattooing were collected and subsequently analysed. The study period was 24 hours before and after start of tattooing. Liquid chromatography coupled to time-of-flight mass spectrometer (HPLC-QTOF) was used to determine 4-aminobenzoic acid, 2-phenoxyethanol and their metabolites. Iodine was quantified by elemental analysis using inductively-coupled plasma mass spectrometry (ICP-MS). All methods were validated beforehand. The peroral metabolic profile of PABA was determined in three subjects as comparison.

Results: The estimated amount of ink and systemically available amount of tracers showed large discrepancies. We conclude that a large amount of tracers were excreted via wound healing. The systemic exposure to tattoo inks in this study is about a magnitude lower then previously estimated. The metabolic profiles of 4-aminobenzoic acid showed increased *N*-acetylation compared to peroral administration which confirms a first-pass effect occurs in skin also occurs during tattooing.

Outlook: The obtained exposure data can contribute to an improved risk assessment and may translate into new limits for substances in tattoo inks. Furthermore, the metabolization data of 4-aminobenzoic acid upon tattooing might be transferable to substances of high concern, such as primary aromatic amines, which cannot be tested due to their hazard profile. The data will be used in a tattoo-specific physiologically based (pharmaco)kinetic model in future.

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P19-45
Data management for image-based characterisation of 2D nano-materials

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The ACCORDs project, funded through Horizon Europe, is pioneering a novel approach to investigate Graphene Family Materials (GFMs) through image analysis. Our aim is to unveil how these materials might influence health and the environment. To achieve this, we are developing a platform designed for the easy retrieval, access, sharing, and utilisation of GFM data and the coordination between biological and physico-chemical data formats. Integral to this platform is an OMERO-based library for image storage,[1] alongside data collection forms and image analysis tools. Efforts are underway to streamline the process for researchers to upload and disseminate their findings, manage information within a database and navigate the data with ease. Adhering to REMBI guidelines,[2] which set the standard for annotating biological images with metadata, we ensure our data collection is comprehensive and adheres to established best practices. Initially, we are employing thresholding and basic machine learning techniques for image segmentation, laying the groundwork for advanced analysis through deep learning to gain more profound insights. Upon completion, the project will deliver a comprehensive platform facilitating efficient data and image management concerning GFMs. This platform will enable the straightforward discovery and use of protocols and results, all organised in accordance with the FAIR principles – Findable, Accessible, Interoperable, and Reusable. [3] This initiative is poised to significantly impact materials science, enhancing our comprehension of the safety and environmental implications of 2D materials.

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P19-46
Risk assessment of chemicals in Dutch seaweed

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Aim: Seaweed refers to several species of macroscopic multicellular marine algae which can be found in nature on hard surfaces such as rocks. Seaweeds are harvested form the wild or cultivated in aquaculture. Europe identified the potential of farmed seafood as a source of protein for food and feed with a low-carbon footprint and highlighted the role of algae as an important source of alternative protein for a sustainable food system and global food security. Therefore, seaweed cultivation at sea is stimulated. Despite wide-ranging non-food applications, about 80 percent of seaweed production is for direct or indirect human consumption. When used as food, seaweed is consumed fresh, dried, defrosted, fermented, cooked, or as products from a combination of the aforementioned methods. Seaweeds can provide a significant amount of the recommended daily intake or adequate intake of essential minerals. In contrast, seaweed might contain contaminants which might pose a food safety risk. The main contaminants studied at the moment are iodine, arsenic and heavy metals. The presence of contaminants in seaweeds depends on the environment (wild or cultivated), seaweed species and age of seaweed during harvest. The aim of this study was to assess the risk of Dutch consumers after the consumption of potentially contaminated seaweed (Ulva and Saccharina) cultivated in the Netherlands.

Methods: The risk assessment of chemicals in Dutch seaweed is based on the method followed by the Codex Alimentarius and the working method of the European Food Safety Authority (EFSA). This method consists of four steps: hazard identification, hazard characterization, exposure assessment and risk characterization.

Results: The first preliminary results show that seaweed cultivated in the Netherlands (Ulva and Saccharina) contains iodine, arsenic, (heavy) metals, and other contaminants such as dioxins, PAHs and PFAS. Based on these first results it can be concluded, when assessing individual contaminants, that the presence of cadmium, mercury, nickel and PAH4 in fresh (raw) seaweed does not lead to a risk for the health of the consumer. However, daily consumption of fresh (raw) wet and dry seaweed grown in the Netherlands (Ulva and Saccharina) may lead to a risk to consumer health due to the presence of iodine, lead, and arsenic. The possible decreases in (heavy) metals and iodine due to the processing and/or preparation steps have not been taken into account in the risk assessment as the research on this topic is not conclusive at the moment.

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P19-47
Predicting mutagenicity using cell painting data

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Studying the mutagenicity of compounds is crucial for assessing risks to human health and the environment, ensuring chemical product safety across various industries, and maintaining regulatory compliance. This evaluation also helps to optimize drug design by eliminating high-risk candidates at early development stages.

Here, we want to present a study that aims to investigate the potential of predicting the chemical's mutagenicity using cell morphological feature perturbations through High Content-Image-Based data (Cell Painting). By integrating mutagenicity data from various sources with Cell Painting profiles, we seek to assess whether the detailed representation of cellular features captured can serve as predictive indicators of mutagenic potential.

Our study is based on two Cell Painting datasets (provided by the Broad Institute and US-EPA) for which we gathered 230 and 299 compounds respectively with mutagenic data, comprising detailed cellular image profiles capturing various morphological and textural features. Several methods of feature selection and compound selection were applied to obtain high-quality data sets. Then, compounds and features were clustered using the clusterMap algorithm (hierarchically-clustered heatmap) in order to provide a classification of mutagenic and non-mutagenic compounds while highlighting features that appear important in differentiating mutagenic and non-mutagenic compounds.

Our results demonstrate promising potential in explaining mutagenicity using Cell Painting data. Further analyses with the implementation of machine learning techniques such as Random Forest or Gradient boosting are underway to study the relevance of developing a classification model capable of predicting mutagenic compounds on the basis of high-content image data.

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P19-49
***In vitro* and PBTK models to assess the hepatic and extra-hepatic metabolism of propylene glycol ethers in the context of CNS toxicity**

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Several neurological disorders have been associated with occupational exposure to chemicals. Propylene glycol ethers (PGEs) are commonly used as mixtures of a non-toxic α-isomer and a β-isomer that is oxidized via the alcohol dehydrogenase (ADH) and the aldehyde dehydrogenase (ALDH) to a potential noxious acid metabolite. However, studies on the neurotoxicity of PGEs are rare. Knowing the rate of solvent metabolism is important for estimating metabolite exposure to the brain. Although the liver is the main organ for ADH- and ALDH-mediated metabolism, the activity of mitochondrial ALDH2 in the brain and the blood-brain barrier (BBB) has been described. Here, we demonstrated the presence and activity of the two enzymes ADH1 and ALDH2 in liver, BBB, and brain *in vitro* models. Additionally, we determined *in vitro* hepatic intrinsic clearance (CL_{int}) in a comparative approach using an established 3D HepaRG model and human liver subcellular fraction (S9) and integrated the parameter into a toxicokinetic (TK) model to compare the predicted metabolite urine concentration with data from an existing human exposure study.

Gene expression of ADH1 and ALDH2 was assessed using RT-qPCR. Enzymes were detected on the protein level by Western blot and im-

munostainings. Metabolite formation by the S9 fraction was used to determine Michaelis-Menten kinetics. Generation of PGE-metabolites in all tested *in vitro* models was measured using LC-MS/MS. CL_{int} derived from the 3D HepaRG model and from S9 was incorporated into a TK model and predicted simulations were compared to available experimental human data.

Our results show that ADH1 and ALDH2 were expressed in the liver, BBB, and brain *in vitro* models (gene and protein expression). Moreover, active metabolite formation was observed in all models tested. 3D HepaRG cells were able to generate metabolite to a similar extent as primary human hepatocytes. Furthermore, metabolite formation in the BBB was approximately 10–30% than that of the liver, whereas metabolism in the brain model appeared to be marginally less. Liver S9 incubations served as a system to estimate enzyme kinetic parameters for the reaction. TK model simulations based on the hepatic clearance derived from both liver systems showed to be close to human experimental data.

In conclusion, the data show expression of ADH1 and ALDH2 and active metabolite formation in all three tested *in vitro* systems. This indicates that metabolite formation can occur, not only in the liver but also in the central nervous system (brain and BBB), potential targets for neurotoxicity. Our results showed that the 3D HepaRG model was able to predict the CL_{int} of PGEs. Furthermore, we propose that the 3D HepaRG model can be applied to study the metabolism of other compounds metabolized via ADH/ALDH. Finally, the generated data helps to further optimize the developedTK model to predict human systemic and brain exposures, thereby supporting the risk assessment of PGEs.

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P19-50
In-chemico reactivity methods based on molecular initiating event of skin sensitization: refinements and possible application to cosmetic formulations

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Skin sensitization is a key endpoint for safety assessment, especially for cosmetics and personal care products. All cosmetics products must be safe under foreseeable conditions of use, and allergenic responses are one of the most frequent adverse reactions noted for cosmetics. The Adverse Outcome Pathway (AOP) for skin sensitization describing the chemical and biological events driving the induction of human skin sensitization are now well understood. Several non-animal test methods have been developed incorporating *in vitro*, *in chemico* and *in silico* approaches to predict sensitizer potential by measuring the impact of chemical ingredients on these key events.

In this work, we have focused on the molecular initiating step (Key event 1) which is based on formation of a covalent adduct between skin sensitizers and endogenous proteins and/or peptides in the skin. A data repository with publicly available data for Direct Peptide Reactivity Assay (DPRA), Amino acid Derivative Reactivity Assay (ADRA) kinetic DPRA (kDPRA) and Peroxidase Peptide Reactivity Assay (PPRA) were assembled for 260 chemicals with animal and human reference data and four relevant physico-chemical properties, highlighting their potential and limitations. Data analyses demonstrated that the test methods' predictivity was consistently reduced for poorly water-soluble chemical and a general tendency for increased false negatives with increasing octanol-water partition coefficient (logKOW) was observed.

The data also showed that DPRA and ADRA can be used interchangeably. Both these assays measure the peptide depletion as the endpoint. However overestimated depletion of the cysteine-based peptide/amino acid derivatives is known in such assays because of the dimerization of the thiol group. To eliminate the false positive prediction due to this, we report here the synthesis and structural confirmation of the dimer of N-(2-(1-naphthyl)acetyl)-L-cysteine (NAC) from the ADRA assay to allow simultaneous determination of (a) peptide depletion using NAC monomer and (b) peptide dimerization using N-(2-(1-naphthyl)acetyl)-L-cysteine dimer (NAC dimer) thus eliminating the overestimation. We present a case study with few test chemicals to demonstrate the importance of this approach. Thus, this simultaneous assay gave a more informed view of the peptide reactivity of chemicals to better identify skin sensitizers.

To expand the scope of refined ADRA methodology beyond the defined molecules and complex mixtures, we have evaluated its potential application to cosmetic formulations as well. To determine the sensitivity of this assay, experiments were conducted with few formulations spiked with known sensitizers and non-sensitizers. The results were promising and could become part of a framework for the skin sensitization safety assessment of cosmetic products.

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P19-51
Unravelling the chemical composition of alternative smoking products

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Electronic cigarettes (E-cigs) and heated tobacco products (HTPs) have become increasingly popular as alternatives to traditional tobacco products (TTPs) due to claims of reduced harm. However, there is ongoing debate about the long-term health effects and overall safety of using these alternative products. Therefore, research is needed to better understand the potential risks associated with E-cigs and HTPs. This study aimed to analyse and compare the chemical composition of three brands of E-cigs, HTPs and TTPs (n=9). After conducting market research, we selected the three best-selling brands of E-cigs, HTPs and TTPs in Portugal to serve as representative samples for our study. The volatile and semi-volatile compounds found in these samples were extracted in triplicate by headspace solid-phase microextraction (HS-SPME) and liquid extraction with dichloromethane, respectively. The headspace and dichloromethane extracts were then analysed by gas chromatography-mass spectrometry (GC-MS). Compounds were identified by comparing the mass spectrum of chromatographic peaks in the sample with a library of mass spectra and standards, where possible. The HS-SPME method allowed the detection of a total of 41 compounds in TTPs, 44 in HTPs, and 53 in E-cigs. Dichloromethane extraction revealed 22 compounds in TTPs, 35 in HTPs, and 43 in E-cigs. Only 7 compounds were common to E-cigs, HTPs, and TPPs. The compounds covered a wide range of chemical classes, including alcohols, aldehydes, esters, ketones, pyridines, and others. HTPs and TTPs have a similar volatile composition (20 compounds in common), especially in the classes of alcohols, ketones, terpenoids, and pyridines. On the other hand, E-cigs contain a higher number of compounds compared to HTPs and TTPs, including several alcohols, esters, lactones, and pyranones. In addition, the volatile composition of HTPs and TPPs showed a high degree of uniformity between different brands, whereas the composition of E-cig brands was more variable. In conclusion, HTPs have a similar volatile chemical composition to TTPs and their impact on human health will then depend on the effects of the different combustion modes. E-cigs show a distinct chemical profile, including several chemical classes of potential relevance for toxicological research.

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P19-52
In silico and *in vitro* genotoxicity evaluation of vildagliptin impurities: vildagliptin cyclic amidine, vildagliptin diketopiperazine and vildagliptine amide

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Establishing the safety of impurities in drug substances or products is crucial. Recent guidelines recognize the assessment of genotoxicity for impurities and determining acceptable limits as challenging issues. While the genotoxicity profile of vildagliptin, an oral hypoglycemic drug, is well investigated, limited knowledge exists regarding the genotoxic potential of its impurities. This study evaluated vildagliptin cyclic amidine, vildagliptin diketopiperazine, and vildagliptin amide for mutagenic and clastogenic potential using Ames and micronucleus tests, both *in silico* and *in vitro*. None of the investigated impurities demonstrated mutagenic or clastogenic potential, indicating they are non-mutagenic and non-clastogenic/aneugenic *in vitro*. These results align with negative in silico predictions, suggesting a strong correlation between *in silico* and *in vitro* data. In conclusion, this study provides valuable insights into vildagliptin's safety assessment by confirming the non-genotoxic nature of its impurities.

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P19-53
Examining specificity in thyroid disruption through read-across potential

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Read-across is regarded as a strategy to reduce animal testing for chemical safety assessment. The OECD Conceptual framework for screening and testing of endocrine disruptors (EDs) includes read-across as relevant non-test information that may be available at Level 1. It is widely recognised that thyroid homeostasis can be affected indirectly through increased hepatic clearance of circulating thyroid hormones, in response to upregulation of phase I and II metabolising enzymes. Induction of these enzymes is often mediated by so-called xeno-sensor receptors e.g. CAR/PXR, but there is significant variation among vertebrate taxa in the presence, role and specificity of these receptors. Moreover, in the context of EU chemicals regulation, which forbids (re) registration of active substances with ED properties, the status of this mode of action for thyroid interference is ambiguous. As set out in the criteria for identifying EDs, "adverse effects that are non-specific secondary consequences of other toxic effects shall not be considered for the identification of the substance as endocrine disruptor." We have interrogated the database of 51 substances subject to Tier 1 determinations under the US EPA Endocrine Disruptor Screening Programme (EDSP), for evidence that liver enzyme induced effects on thyroid parameters in human health models correlate with thyroid activity in the amphibian metamorphosis assay (AMA). We categorised AMA endpoints and effect patterns indicative of thyroid activity were established based on responses to known thyroid disrupters in the OECD validation of the AMA test guideline. These responses were then compared to thyroid-related responses in the male and female pubertal assays also conducted as part of the EDSP. Among the 51 substances in the Tier 1 list 1 programme, the weight of evidence evaluation concluded that 13 affected the thyroid axis. Among those, 2 were concluded to have affected amphibians only, while 9 only affected mammals. Another 3 elicited responses in amphibians and mammals, though it should be noted that 2 of those did not exhibit a clearly thyroidal response pattern in the AMA. Among the 9 affecting the thyroid in mammals only, it was concluded that 5 of them did so indirectly, secondary to induction of liver enzymes. All 5 of those substances are known agonists of CAR/PXR. None of those 5 substances elicited effects in the AMA resembling responses to known thyroid axis disrupters. These data indicate that the AMA model is rather insensitive to perturbations of the thyroid axis consequent to CAR/PXR mediated liver enzyme induction. This suggests that, in relation to thyroid disruption, read-across from amphibians to mammals will be less protective than the

converse, if liver mediated thyroid insufficiency should be regarded as a specific endocrine mediated effect. This assumes greater importance given current interest in development of 'non-animal methods' to support ED testing while reducing animal use.

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P19-54

Data from extended observation period to support evaluation of availability of the skin depot for absorption within *in vitro* dermal absorption studies

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When deriving dermal absorption values for non-dietary risk assessment, a key question is which compartments in an *in vitro* dermal penetration experiment contribute to the systemic dose. The current EFSA Guidance on Dermal Absorption uses the fraction of the terminal receptor fluid (RF) value that penetrates in half of the maximum observation period to decide whether all of the skin depot (minus the first two in a series of tape strips removing the stratum corneum (SC)) will be added to the RF as absorbed or whether the remaining tape strips/SC are considered unavailable for further absorption and then not added. While the latter provides a useful and conservative assumption for the maximum possible absorption over time, there is a risk of considerably overpredicting actual systemic availability for compounds with high skin association and very low/slow penetration. Additional information from *in vitro* experiments with longer observation periods may therefore allow to refine the conclusion about the availability of amounts in skin compartments for absorption.

In total 13 different plant protection products containing different active ingredients were tested in OECD TG 428 compliant *in vitro* human skin dermal absorption studies (6–8 h exposure/24 h observation periods) while adding experiments with an extended observation period (72 h) to obtain further experimental data to inform on possible future absorption.

For the majority of the evaluated products and tested concentrations, absorption of the active ingredient into the RF did not relevantly increase between 24 and 72 h and the amounts remaining in the SC did not indicate relevant penetration into viable skin parts as would be required for future absorption.

It was also shown that the terminal flux from the 24 h experiment (during the last period of RF sampling) is a valid predictor of future absorption by comparison of predicted vs experimental absorption after 72 h.

Considering that the target is to estimate dermal absorption for humans *in vivo*, residues in the SC (or even the whole epidermis) that are sufficiently immobile/do not penetrate into the dermis may be lost by epidermal turnover/desquamation of the SC over time. Therefore an 'outward' flux was calculated by dividing the amount remaining in the SC by the expected time for SC desquamation (14 days for humans) as a measure of how fast the amount in the SC is lost by desquamation and compared to the terminal flux that describes how fast the compound may penetrate through the skin over longer periods. In case the outward flux exceeds the inward flux supports that the amount in the SC will not be available for absorption *in vivo*.

It is therefore proposed to extend the current decision criteria when to consider the amount in the SC as available for absorption or not by an inward/outward flux comparison, which can be further supported or refined by experimental data with an extended 72h observation period.

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P19-55

Working towards the implementation of NAMs for regulatory use in risk assessment – considerations regarding quantitative *in vitro* to *in vivo* extrapolation

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Traditionally, the risk assessment of exposure to chemical substances is based on the extrapolation of animal *in vivo* toxicity data to the human situation, resulting in a Health-Based Guidance Value (HBGV). To extrapolate an animal point of departure (PoD) to a human HBGV, assessment factors are applied to extrapolate between the experimental and real-life exposure scenarios and between populations. Assessment factors also cover uncertainties regarding these extrapolations, and may be used to cover additional uncertainties related to *e.g.* data quality. New Approach Methodologies (NAMs), like *in vitro* and *in silico* methods, can be promising when it comes to reducing animal experiments. When using *in vitro* data, these have to be extrapolated to the *in vivo* situation (using quantitative *in vitro* to *in vivo* extrapolation; qIVIVE), to obtain a human *in vitro*-based PoD and subsequently an *in vitro*-based HBGV to compare to the human exposure.

While in theory straightforward, the use of NAMs introduces various known and unknown uncertainties and extrapolation steps that (ideally) have to be quantified when used for risk assessment purposes. Known uncertainties introduced are related to the choice of *in vitro* systems, the qualitative and quantitative link between *in vitro* effect and *in vivo* adverse outcome, the (response) measurements itself, and the use and quantification of the dose metric *in vitro* (nominal vs. intracellular concentrations). In addition, uncertainties are introduced with the choice of (type of) physiologically-based kinetic (PBK) model, quantification of the PBK model parameters, the allocation of *in vitro* concentration to a certain PBK compartment and the selection of *in vivo* dose metric (relating the *in vitro* exposure duration to the *in vivo* exposure pattern). Currently, qIVIVE is often performed without addressing these known uncertainties. Uncertainties and extrapolation steps introduced when using NAMs for risk assessment need to be addressed, and preferably quantified, to work towards regulatory implementation of NAMs for risk assessment purposes.

Two case studies performed highlight the extrapolation steps needed in NAMs-based dietary risk assessment and provide a first insight in how to deal with the uncertainties. The first case study describes the use of readily available data in the exemplary risk assessment of imazalil focusing on liver steatosis using the Monte Carlo Risk Assessment (MCRA) Platform, whilst the second case study describes the exemplary risk assessment of chlorpyrifos and its metabolites for the inhibition of neuronal proliferation as a model for the onset of neuronal neurodevelopmental toxicity using a newly developed PBK model and an in house *in vitro* assay.

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P19-56

Safety and risk assessment of peptides in cosmetic products

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Recently, there has been an increased interest in launching cosmetic products containing peptides. Peptides are used to prevent or reduce

skin aging in skincare cosmetics. They can be extracted from several sources including plants, animals, or microorganisms, or they can be synthesised. Peptides may be biologically active and have the potential to interact with skin. Thus, in line with the requirements stipulated under the EU Cosmetics Product Regulation ((EC) No 1223/2009), the safety of peptides must be established by selecting an appropriate Point of Departure (PoD) for systemic toxicity and a sufficiently high Margin of Safety (MoS) under cosmetic product use conditions.

This poster presents a weight of evidence (WoE) approach to assess the safety of cosmetic products containing peptides, which is primarily based on the use of read across and BLAST (Basic Local Alignment Search tool). In the context of read across, we have established a stepwise process to identify analogues which are identified using European Chemicals Agency (ECHA)-recommended tools, such as the OECD QSAR Toolbox v.4.6 and the US EPA AIM model. Analogues with relevant toxicological data are further evaluated for their suitability in accordance with OECD guidelines and the ECHA read across assessment framework (RAAF).

To increase the confidence of the safety assessment, an additional analysis is conducted to determine whether the peptide of interest presents sequence similarity to known human proteins. Specifically, a BLAST analysis is performed to compare the peptide sequence to the National Center for Biotechnology Information (NCBI) database and identify the best similarity matches. A high degree of similarity (greater than 80%) with a known human protein can help to support the toxicological profile of peptides. Furthermore, supported by the results of an *in vitro* study, demonstrating the hydrolysis of the peptides, the safety assessment can also be complemented with toxicological data on the individual amino acids. In light of the animal testing ban in the EU, this poster presents the use of a WoE approach, in order to perform a safety assessment of the peptides for use in cosmetics.A case study will be presented to demonstrate the safety assessment approach for data-poor peptides in cosmetics products.

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P19-57

Improved Confidence of Quantitative Sensitizing Potency Assessment for Point of Departure Using GARDskin Dose-Response

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Identification of skin sensitization hazard and potency characterization are central aspects of risk assessment of chemicals. Current legislation advocates a transition from hazard assessment using *in vivo* methods to UN GHS potency subclassification and quantitative risk assessment by use of New Approach Methodologies (NAM:s) as well as Defined Approaches (DA). However, the ability of NAM assays to quantitatively estimate sensitizing potency and thereby establish a point of departure (POD) for next-generation risk assessment (NGRA) strategies is currently lacking.

To this end, the GARDskin Dose-Response (DR) method, adapted from the OECD TG 442E method GARDskin, was recently introduced. The GARDskin DR method evaluates test chemicals in a titrated range of concentrations, in order to investigate the dose-response relationship between the output from the GARDskin prediction algorithm (Decision Values; DV:s) and test chemical concentration. The combined information can be used to derive a quantitative estimation of sensitizing potency, defined as the cDV_0-value, i.e, the least required dose required to elicit a positive response by the prediction model.

The current work focuses on optimizing the ability of GARDskin DR to derive a quantitative POD based on conversion to a composite Potency Value (PV; $\mu g/cm^2$), taking into account both human and *in vivo* reference data sources. A total of 25 chemicals were used to construct predictive regression models fitted to reference PV:s. Results show that the updated models fitted to reference PV:s produced more accurate potency predictions compared with models fitted with, and aiming to predict, only LLNA EC3 and NOEL, respectively. Mean fold-change errors ranged between 2.8 and 3.2, with predicted POD:s being within or close to the range of the variation of the historical *in vivo* data. In addition, uncertainty in predictions was reduced, as estimated by a minimum 2-fold reduction of 95%-confidence intervals, when comparing models fitted to reference PV:s with models fitter with only LLNA EC3 and human NOEL, respectively.

In conclusion, these improvements constitute a major step forward for the ability of NAM:s to assess quantitative sensitizing potency. It demonstrates how GARDskin Dose-Response can accurately estimate a POD and be incorporated into downstream strategies for quantitative risk assessment (QRA), to ultimately contribute to the assessment of safe use levels of chemicals.

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P19-58

Use of transcriptomic signatures of pesticide active substances in human kidney cells to support definition of cumulative assessment groups (CAGs) for risk assessment

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Safety and risk assessments of chemical substances are generally performed on single substances only, while in reality exposure always occurs in the context of the exposome. In recent years, more attention is directed to the possible adverse effects of combined exposure to multiple substances. To allow assessment of health risks upon combined exposure to pesticide active substances in a cumulative risk assessment, the European Food Safety Authority (EFSA) currently works on their grouping in cumulative assessment groups (CAGs). To that end, pesticide active substances are identified that exhibit similar toxicological properties in a specific organ or system. The draft report of EFSA's Panel on Plant Protection Products and their Residues (PPR) for the kidney recently underwent public consultation. The present study aims to use an *in vitro* approach to support definition of CAGs related to kidney toxicity. To that end, human renal proximal tubule epithelial cells (i.e. RPTEC/TERT1 and HK-2) were exposed to different pesticide active substances reported to cause adverse effects to the kidney (i.e. fludioxonil, pyrimethanil, pyraclostrobin, thiabendazole, pyriproxyfen, carbendazim, 2,4-dichlorophenoxyacetic acid) and their effects on the transcriptome were assessed using RNA sequencing. These data were used to assess similarities and differences in mechanisms of kidney toxicity *in vitro*, which may be of use for deciding on inclusion or exclusion of substances in a certain CAG. In addition, HK-2 cells were exposed to concentration-response curves of three substances showing different transcriptomics profiles in RPTEC, i.e. pyrimethanil, pyraclostrobin, and pyriproxyfen, and analyzed using RNA sequencing to identify most sensitive pathways, and differences therein. In future studies, the effects of mixtures of these substances will be studied to assess effects of combined exposure. Additionally, other nephrotoxic substances will be studied and tentatively aligned with the CAG for the kidney currently being established. Altogether, *in vitro* mechanistic data on effects of pesticide active substances in human cell-based test systems are promising to support definition of CAGs, contributing to cumulative risk assessment.

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P19-59
Survey and risk assessment of chemical substances in non-biocidal antifouling paints for private pleasure boats

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Non-biocidal antifouling coatings for pleasure boats are promoted as environmentally better alternatives to biocidal antifouling products. Even though these products do not contain biocides, they may contain other hazardous substances. This study aims to clarify if there are functional, non-biocidal alternatives to biocidal antifouling paints on the Danish market and to investigate the potential environmental and health risks associated with them. The study comprises a survey of non-biocidal antifouling coatings, chemical analyses as well as risk assessments of selected products.

65 coatings marketed as non-biocidal antifouling coatings were found on the Danish market. A total of 28 individual substances was identified as hazardous based on information from the products' material safety data sheets, equalling roughly 50% of all identified substances. Most commonly, the hazardous substances are solvents, which are also used in biocidal coatings.

13 of the 65 products were prioritised for targeted chemical analyses and risk assessment. The basis for the selection was the coatings' availability on the Danish market and the hazardous profile of their containing substances. Seven substances were selected for the consumer risk assessment: solvent naphtha, ethylbenzene, naphthalene, rosin, octamethylcyclotetrasiloxane (D4), 4-methylpentan-2-one (MIBK) and 4-methylpentan-2-one oxime. In the environmental risk assessment, cyclosiloxanes (D4, D5 and D6), medium-chain chlorinated paraffins (MCCP) and zinc oxide were in focus.

The risk assessment methodology used for biocidal antifouling products was adopted for the non-biocidal antifouling coatings in order to allow for a comparison of the risks related to biocidal and non-biocidal coatings.

The human health risk assessment focused on consumer uses of the antifouling coatings. Exposure estimates were compared to health reference values for the general population. Five of the 13 non-biocidal antifouling coatings contain hazardous substances for which the health risks cannot be regarded as controlled. The two substances causing the potential health risks are MIBK and 4-methylpentan-2-oxime. MIBK is a common solvent in coatings and thus not specific to non-biocidal coatings, while the presence of 4-methylpentan-2-oxime is linked to the foul release mechanisms of certain non-biocidal antifouling coatings.

The environmental risk can be anticipated to be controlled for the harbour water and the sediment compartment based on calculated risk quota below 1. Nonetheless, D4, D5, D6 and MCCP are recognized as PBT/vPvB substances and the use of products potentially leading to environmental releases of such substances should be discouraged.

In conclusion, non-biocidal antifouling products, which can be regarded as safe for both human health and environment, are available. However, health risks related to the use of some non-biocidal antifouling products cannot be excluded.

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P19-60
Calculation and comparative hygienic assessment of potential risks when processing agricultural crops using 3RIVE3D technology

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The use of chemical plant protection agents plays an important role in the agricultural sector of the country for the establishment of stable economic growth and the fulfillment of the state's development goals. However, incorrectly selected application methods, inappropriate spraying equipment, poor maintenance of pesticide application equipment, low quality knowledge of workers regarding the safety of the pesticides' application can lead to acute and chronic occupational health disorders.

The aim was calculation and comparative hygienic assessment of potential risks when treating agricultural crops using 3RIVE3D technology.

Materials and methods: 3Rive 3D Application System is a revolutionary crop protection platform that empowers growers to farm more extensively. The system integrates formulation technology, application technology and active ingredients to efficiently cover a larger treatment area in a shorter period of time with fewer refills, saving water, fuel, labor and time.

Field experiments on the study of working conditions when using the Brigade 3Rive 3D, SC formulation was conducted in various soil

and climatic regions of South-East Europe under acceptable meteorological conditions.

Results and discussion: Inhalation risk for workers involved in treatment using the 3Rive3D technology mostly did not differ from this risk for workers using other methods of treatment.

The real dermal risk was significantly lower compared to the risk of workers involved in aerial processing and UAV processing (p=0.009–0.048 according to Wilcoxon's W-test); compared to others, it was insignificantly lower (p=0.262–1.000). There was a similar picture in comparison with the dermal aggravated risk – significantly lower in comparison with the risk for workers involved in air processing and processing with the unmanned aerial vehicles (p=0.009 according to Wilcoxon's W-criterion); compared to others, it was insignificantly lower (p=0.262–1.000).

The significant difference in the complex real and aggravated risks was significantly higher for the tractor driver compared to the corresponding risk of the mixing unit operators during rod processing (p<0.001 according to the Wilcoxon W-test), since the inhalation risk had the main weight in the formation of the complex risk.

Conclusion: It was established that in real conditions, applying pesticides using 3Rive 3D innovative technology in compliance with the recommended agrotechnical and hygienic regulations for safe application does not exceed hygienic standards in the air of the working and drift zones, and it is proven that the potential risk of harmful impact on the agricultural workers body in the case of complex intake through the skin and respiratory tract did not exceed 1 c.u., and therefore is permissible, which allowed to recognize their working conditions as satisfactory.

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P19-61
Combining chemical, biological similarity and metabolite data in read-across approach

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Identifying relevant source chemicals (SCs) is a major challenge in read-across (RAX) assessment. The RAX assessment workflow developed as part of an EFSA funded project (OC/EFSA/SCER/2021/04), integrates information on chemical and biological properties, as well as information on metabolites, to better define relevant SCs. The read-across assessment is performed using the rodent *in vivo* endpoint data from the SC, related to repeated-dose toxicity and prenatal developmental toxicity with oral route of exposure. For this purpose, a project database has been built to store different toxicity studies. Data rich compounds are pesticide active substances and metabolites from triazoles, triazine and carbamates.

One compound from triazole compound class – Propiconazole– was selected as target compound (TC) to explore the performance of the developed read-across workflow. The developed workflow explored three different approaches to generate SC: i) chemical similarity, ii) biological similarity and iii) metabolites. Twenty-seven pesticides from triazole compound class were selected as a reference group with a clear toxicological profile. Structural information was used to identify the relevant SC regarding their chemical properties. For the NAM data, ToxCast summary data consisting of information regarding assay (in) activities were used to calculate the biological similarity. In the first approach, the combination of chemical and biological similarity calculated from ToxCast data identified the majority of reference group compounds and was particularly successful with integrated background knowledge of TC mode of action. The second approach used biological similarity as starting point, which resulted in an overwhelming number of candidate SC originating from diverse compound classes, which was subsequently refined by integrating chemical similarity. The third approach applied shared or similar metabolites from observed *in vivo* data, which efficiently generated SCs mostly only from triazole reference group compounds.

Both integration or modular combination of ii) biological similarity and iii) metabolites information achieved an improvement in the SC selection compared to only considering i) chemical similarity. Moreover, all these approaches can serve as a starting point for the identification of SCs. The developed workflow can support the identification of SCs and could be integrated in future guidance for the RAX of endpoints identified from repeated-dose and prenatal developmental toxicity studies.

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P19-62
Risk characteristics of alimentary cumulative effects of pesticide residues in food products (based on monitoring results)

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Modern agricultural technologies involves the use of a whole range of pesticides. Therefore, the control and toxicological assessment of multiple pesticide residues in food is one of the urgent tasks of food safety.

The aim of the study is to characterize the exposure and assess the risk from the acute alimentary cumulative effects of pesticide residues in some widespread food products.

Methods: Determination of residues was carried out in two model crops – apples and potatoes using GC-MS and/or LC-MS/MS determination method (EN 15662-2008) [1]. Samples of apples and potatoes were obtained from farms in the Kyiv region of Ukraine. In the risk assessment, data on the factors of processing (FP) were additionally used [2]

The active ingredients of pesticides identified in the product samples were grouped according to the effects on the nervous system, thyroid gland and liver [3–5]. The total cumulative exposure (E_{total}) for children 2 to 6 years of age and adults was assessed, as well as the risks of potential cumulative effects for the thyroid gland by the total hazard index and the total exposure margin (MOET) [6].

Results: It was found that the examined samples of potatoes and apples contained residues of two (58%), three (25%) and four pesticides (11%). All potato samples contained imidacloprid, 50% thiamethoxam, 38% fluopicolide, and 13% metribuzin. Deltamethrin was found in 100% of apple samples, thiophanate-methyl in 44%, chloranthraniliprole in 11%. The content of pesticide residues did not exceed the maximum residues levels (MRLs) established in Ukraine.

It has been found that the largest contribution to E_{total} is made by metribuzin, thiamethoxam and fluopicolide. It has been noted that the potential cumulative effect on children is higher, as evidenced by a smaller exposure margin than in adults.

Conclusion: The intake of several pesticide substances with the same mechanism of toxic action can increase their negative impact on human health.

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P19-63
Use of New Approach Methodologies (NAMs) to assess the safety of prostaglandins for use in cosmetics

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In 2018, the German Federal Institute for Risk Assessment (BfR) informed the European Commission that they were concerned that the use of prostaglandins and their analogues as ingredients in cosmetic products might pose health risks for consumers.

Following a call for data conducted in 2020, the European Commission requested the Scientific Committee on Consumer Safety (SCCS) to carry out a safety assessment of the uses of prostaglandins or their analogues (PGA) in cosmetic products. In February 2022, the SCCS released its opinion on the use of prostaglandins and prostaglandin-analogues ('PGAs') in cosmetic products. The SCCS stated that it could not determine safe use concentrations for PGAs in cosmetic products due to the scarcity of toxicological data on the individual PGAs. However, the SCCS also mentioned that it would be ready to assess any new evidence provided to support the safe use of PGAs in cosmetic products.

This poster presents the application of 'New Approach Methodologies' (NAMs) to evaluate the safety of ethyl tafluprostamide, also known as dechloro dihydroxy difluoro ethylcloprostenolamide (DDDE), at use levels in a cosmetic eyelash product formulation. Given the ban on animal testing, *in vitro* methodologies compliant with OECD test guidelines were used to generate data on dermal penetration, irritation, sensitisation and genotoxicity. The assessment of systemic endpoints such as acute toxicity, repeated dose toxicity, carcinogenicity, and developmental and reproductive toxicity was addressed through a read-across approach. A potential analogue for DDDE was identified and assessed using a stepwise process in line with the OECD guidelines and the ECHA's Read Across Assessment Framework (RAAF). To strengthen the confidence in the safety assessment, NAM's based acute toxicity and biological activity assays are planned to provide mechanistic insights and support the read-across hypothesis with additional bridging data.

In summary, this poster presents a comprehensive safety evaluation of DDDE in a cosmetic eyelash formula, demonstrating the use of NAMs to confirm its safe use and to respond to regulatory inquiries regarding the safety of prostaglandin analogues.

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P19-64
Relevance of dog studies for the derivation of health-based guidance values for plant protection products approval

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The current trend of extending the toxicity testing by enhanced mechanistic understanding using human-relevant systems increases the global effort to identify the mechanisms of toxicity and provides an opportunity to re-evaluate the importance of dog studies for the risk assessment of plant protection products (PPPs).

Under Regulation (EU) 283/2013, setting out the data requirements for pesticides active substances, short-term oral toxicity testing in rodents (90-day rat study) and non-rodents (90-day dog study) species are required to address hazard identification and human safety of PPPs, and to support the active substance approval in the European Union (EU). The scientific rationale of using the dog as 'second' species in the regulatory process has been debated since long time and culminated with the elimination of the one-year dog study (OECD TG 452) from the data requirements for the approval of PPPs in the EU and other countries.

The debate is still ongoing, and the scientific challenge remains for unresolved questions: What is the value of a by-default 'second' species when all are a surrogate of humans? What is unique of the dog to be of any benefit in the chemical risk assessment and provide a protective ground for the human population? Are four dogs/sex/group of treatment, as per OECD TG 409, adequately covering the intra- – species variability aspects?

To actively contribute to this debate, EFSA and PPR Panel members reviewed the data from the available dog studies conducted with the PPP active substances previously in the European market and/or currently approved by the European legislation and performed a retrospective analysis of the results on setting Acceptable daily intake (ADI) for pesticides. An overview of the above retrospective analysis, including analyses of the data and future directions, is presented.

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P19-65
Human health risk assessment of metals in PM2.5: evaluating the concentration of metals within residential houses in Porto metropolitan area

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Background: Assessing metal concentrations in particulate matter (PM) is crucial since it can help identify possible pollution sources, understand potential health risks associated with air pollution, and support the development of mitigation strategies in order to safeguard human health and the environment [1,2].

Objective: This study aims to analyze the concentration of metals present in PM with a diameter of 2.5 μm or smaller (PM2.5) collected from indoor and outdoor air samples across 23 residential houses in Porto Metropolitan Area and discern potential contamination sources.

Methods: PM2.5 samples were collected from one bedroom and outdoor area on nitrocellulose membrane filters (0.37 μm pore size; 37 mm diameter, Merck S.A.) using DustTrakTM DRX Aerosol Samplers (Model 8533, TSI Inc. MN, USA) with a flow rate of 3 L/min along seven days. The concentration of metals present in PM2.5 was determined after digestion of the collected samples, using 1.0 mL nitric acid (65%) and 1.0 mL hydrochloric acid (35%) in a microwave system, and through injection into an iCAP™ Q ICP-MS equipment. Overall, 22 metals were evaluated, of which the mean concentrations, standard deviation, and quartiles were computed using SPSS v29.

Results: Higher PM2.5 concentration was detected outdoors than indoors (92.8 vs 66.1 μg/m^3); with an I/O ratio of 0.71. The highest metal concentrations (outdoor vs indoor) were attributed to Calcium (690.4 vs 929.9 μg/m^3), Vanadium (819.9 vs 790.1 μg/m^3), and Aluminium (173.9 vs 190.0 μg/m^3) in both settings. Among others, greater Copper concentration was found indoors (14.95 ng/m^3 vs 10.05 ng/m^3; I/O ratio: 1.49), consistent with prior research [3,4]. This may stem from indoor plumbing pipes and utensils, which can emit particles into the air through corrosion, wear and tear, or off-gassing. Lead concentration indoors also surpassed outdoor levels (7.75 ng/m^3 vs 5.82 ng/m^3; I/O ratio: 1.33). This can be due to exposure to Lead-based paint, indoor smoking, and some household items. Nickel concentration was 2.2 times greater outdoors (50.89 ng/m^3) than indoors (23.12 ng/m^3; I/O ratio: 0.45), possibly linked to industrial activity, traffic emissions, and ongoing construction work. Moreover, seasonal fluctuations, with heightened concentrations during the cold season, were previously reported [5], a trend consistent with our data collection timeframe. While Cadmium, Cobalt, Lead, Titanium, and Thallium exhibited low and comparable concentrations indoors and outdoors, their cumulative impact may be significant.

Conclusions and Future Research: Although higher PM2.5 concentrations were found outdoors, discrepancies in individual metal concentrations were noted. Tailored interventions can detect potential sources, prompt behavior change, and support air quality policies aimed at preserving public health. Subsequent research on carcinogenic and non-carcinogenic risks will be undertaken to better understand the associated health risks.

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P19-66
Industrial case studies in product design and chemical risk assessment

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RISK-HUNT3R aims to develop tools supporting the solution of industrial problems in Next Generation Risk Assessment (NGRA) based on New Approach Methods (NAMS). The project involves research activities developing and refining integrated approaches to testing and assessment based on an integration of experimental and in silico modelling methods. Solutions to problem formulations are pursued using an iterative approach to assessment formulated within an ASPA [1] risk assessment workflow. RISK-HUNT3R has a dedicated work package aimed at the sustainable transfer of NAMS to situations involving industrial and regulatory applications. The competency and tools required to execute solutions are organised within the SaferWorldbyDesign innovation platform [2].

We established a process for formulating, engaging and executing industrial case studies based on NAMS-based NGRA organised as an

ASPA workflow. We describe here results for initial case study work [3] including:

1. In Silico first-based risk assessment of individual compounds as cosmetic ingredients;
2. Hazard evaluation of pesticide compounds across multiple *in vitro* assays;
3. Toxicogenomics- and Metabolomics- based assessment of points of departure for specific mechanism-based concerns;
4. Prediction and evaluation of drug side effects based on iterative workflows combining machine learning, artificial intellgience, knowledge mining, structure-based modelling, bioinformatics, and dose- and time- dependent RNASeq and High Content Screening experiments.
5. Safe-by-Design comparison of alternative ingredients with regards to environmental impact.

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[2] SaferWorldbyDesign, https://saferworldbydesign.com/

[3] The poster will present summary case study results with QR codes/web links provided to further details on each case study

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P19-67
Using Next Generation Risk Assessment (NGRA) to make safety decisions for cosmetic ingredients under regulatory scrutiny

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Purpose: As part of Cosmetic Europe's Long Range Science Strategy (LRSS) systemic toxicity case studies were developed to test and refine the implementation of non-animal-based assessments using an exposure-led NGRA framework. Ingredients were chosen from a chemical list considered by the European commission in 2019 to have potential endocrine activity, and where further safety assessment by the Scientific Committee on Consumer Safety (SCCS) was required. The aim of the NGRA case studies was to understand the likelihood of systemic bioactivity occurring at consumer-relevant concentrations and the ability of the framework to differentiate high-risk from low-risk exposures scenarios.

Methods: A toolbox of high throughput assays was used to generate 1) *in vitro* ADME experiments for parameterising physiologically-based kinetic (PBK) models and 2) bioactivity characterisation using *in vitro* pharmacological profiling of protein interactions, cellular stress assays, and high throughput transcriptomics in 3 cell lines. Points of departure (PoDs) were determined from dose response data using Bayesian statistical models or IC50s for the pharmacological profiling data. These PoDs were then compared with plasma C_{max} estimates from the PBK models to calculate Bioactivity/Exposure Ratios (BERs) for each chemical. Consumer use case scenarios were identified for each case study chemical (UV filters and preservatives) based on regulatory limits (Octocrylene, Butylated hydroxytoluene, Climbazole and Ocytlmethoxycinnamate (OMC)). In addition, four benchmark chemicals (Prochloraz, 4-MBC, Aminoglutethimide and Diethylstilbestrol) with known estrogen activity were included.

Results: Low risk use scenarios were defined for the case study chemicals based on SCCS opinions and regulatory limits, and plasma C_{max} estimates were calculated for each ranging from 0.0034 μM (0.2% climbazole in a face cream) to 0.08 μM (10% OMC in a sunscreen). For each case study the leading PoD from the *in vitro* assays came from either the transcriptomics data or the pharmacological profiling IC50s. Following incorporation of uncertainty analysis in the exposure estimates, BER values were calculated for each chemical-use scenario ranging from 0.4 (10% OMC in a sunscreen) to 21 (0.2% Climbazole in a face cream). Data was also generated for the comparators where high risk exposure scenarios were identified, resulting in BERs ranging from 0.00002 (2.5 mg Prochloraz) to 0.08 (4% 4-MBC in a sunscreen).

Conclusion: Using NAM-based bioactivity data in a risk assessment workflow resulted in full separation of low and high-risk benchmark chemical use scenarios in accordance with safety opinions published by authorities. Higher tier testing could be useful to determine the significance of the *in vitro* results to humans *in vivo* and the likelihood of adverse effects from scenarios resulting in a BER <1. These results build confidence that a low-tier NGRA can distinguish high risk and low risk exposures.

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P19-68
Identification of physico-chemical properties of 79 nanomaterials that are predictive of inflammation and genotoxicity following pulmonary exposure in mice

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Background: Nanomaterial-induced inflammation is a central and early key event in adverse outcome pathways (AOPs) linking pulmonary exposure of nanomaterials to fibrosis (AO173), cardiovascular disease (AOP237) and cancer (AOP303 & AOP451). Genotoxicity, in terms of increased DNA damage, is a key event for cancer development (AOP303 & AOP451). This study aims to identify physico-chemical properties predicting nanomaterial-induced inflammation (neutrophil influx) and genotoxicity (DNA strand breaks) in bronchoalveolar lavage (BAL) cells, lung, and liver tissue.

Methods: Animal studies, where nanomaterials were deposited in lungs of female C57BL/6J mice by intratracheal instillation, were compiled for seventy-nine nanomaterials including multi-walled and single-walled carbon nanotubes, halloysite fibers, nanoclays, graphene plates, silica with or without CuO doping, carbon black nanoparticles, diesel and biodisel exhaust particles, quartz, crocidolite, and various metal oxides. Mice were exposed to 2–3 dose levels alongside vehicle controls and followed for 1, 3, 28 and 90 days. All post-exposure days were not included for all materials. The included physico-chemical properties were: length, diameter, BET specific surface area, reactive oxygen species (ROS)-production and the number of nanodimensions (3 for particles, 2 for tubes/fibers and 1 for nanoplates). We conducted a series of multiple linear regression analyses with neutrophil influx and DNA strand break levels as separate outcome variables. To obtain more symmetric distributions, all variables were log10-transformed. The data set were compiled from 21 animal studies and all analyses were controlled for study variation. Nanodimensions was highly correlated to length for all post-exposure time points. Consequently, the regression analyses were performed without either 'length' or 'nanodimensions'.

Results: We focused on identifying the most robust physico-chemical predictors showing similar associations across the different approaches. For inflammation, dose were strong predictors at all time points. Diameter, length, nanodimensions and ROS were generally predictive for increased neutrophil influx at all the time points except at day 90 post-exposure. The pattern are most clear for analyses using surface area as dose metric. Length and diameter were the most consistent predictors of genotoxicity in BAL cells. Increased diameter was the

most consistent predictors of lower levels of genotoxicity in lung tissue. Length was the most consistent predictor of liver genotoxicity.

Conclusion: Physico-chemical predictors of inflammation and genotoxicity were identified in a dataset of *in vivo* studies in mice pulmonary exposed to 79 different nanomaterials at three dose levels and up to four post-exposure time points.

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P19-69
Analysis of the relationship between cytochrome P450 inhibition and hepatotoxicity of pesticides

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Purpose: Hepatotoxicity is a major cause of drug development discontinuation, and its reliable evaluation method is highly needed. Since it is reported that being a substrate or inhibitor of cytochrome P450s (P450s) is a risk factor for drug-induced liver injury (DILI), we systematically investigated the association between P450 inhibition and DILI. The results demonstrated that most of the drugs that strongly inhibited CYP1A1 or CYP1B1 were with DILI risk [1]. Moreover, mechanistic analyses have suggested that CYP1A1 inhibition may cause liver injury via activating the aryl hydrocarbon receptor [2]. In this study, we analyzed the relationship between hepatotoxicity and P450 inhibition using pesticides to clarify whether the relationship was observed with a different class of compounds and whether P450 inhibition data could be used for hepatotoxicity prediction.

Methods: The results of rat 2-year repeated-dose toxicity studies of 126 pesticides were obtained from the evaluation reports published by the Food Safety Commission of Japan, and the data of males and females were combined for analyses. If each hepatotoxicity endpoint (EP) was observed at any dose for a pesticide, the EP was considered positive for the pesticide. In addition, 5 group EPs (gEPs), including necrosis/inflammation, liver dysfunction, bile duct-related injury, hypertrophy, and dyslipidemia, were defined by grouping similar EPs. If any of the EPs within a gEP was positive, the gEP was considered positive. Test compounds were obtained from commercial sources. Their inhibitory activity against rat CYP1A1, CYP1A2, CYP2B1, CYP2C6, CYP2D1, and CYP3A2 was measured using commercially available recombinant enzymes and luminescent substrates, and a pesticide was considered positive if its inhibitory activity was ≥15%.

Results and Discussion: The number of inhibition-positive pesticides was highest for CYP1A1 (66%) and lowest for CYP2D1 (15%). The association analyses using Fisher's exact test showed the associations of CYP1A1 inhibition with gEPs on hypertrophy and dyslipidemia, as well as increased liver absolute weight, centrilobular hepatocyte hypertrophy, and increased blood cholesterol, consistent with our previous findings [2]. Similar results were obtained with CYP2C6 inhibition as well. In addition, significant associations were observed between CYP3A2 inhibition and gEPs on bile duct-related injury and increased γ-glutamyltransferase levels. These results corroborate our previous findings that CYP1A1 inhibition is associated with hepatotoxicity and suggest that P450 inhibition assays help evaluate the hepatotoxicity of various types of chemical compounds.

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P19-70
Gaps and needs analysis, barriers, opportunities and drivers for implementation of Artificial Intelligence and Machine Learning (AI/ML) tools in regulatory risk assessment

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Within the context of the Horizon Europe Partnership for the Assessment of Risks from Chemicals, the present study aimed at identifying gaps and needs, barriers, opportunities, and drivers for the implementation of AI/ML technologies in chemical risk assessment (CRA) to facilitate further development of scientific criteria for the regulatory acceptance of AI/ML approaches.

Building on our prior review of regulatory and technological landscapes in toxicology and CRA for AI/ML application, this study focuses on three essential domains necessary for successful AI/ML integration into CRA: technology's interpretability and validity, organizational readiness and personnel competence, and the legal framework for technology use. Identified gaps and barriers in the technological domain include the scarcity of high-quality, standardized datasets vital for AI model training and validation, and the prevalent "black box" nature of deep learning algorithms, which obstructs interpretability and regulatory acceptance. Additionally, technical integration issues with existing CRA frameworks necessitate substantial adaptations. Organizationally, there exists a critical gap in requisite expertise among personnel, compounded by resistance to change and limited resources, underscoring the need for dedicated training programs and enhanced stakeholder collaboration. Legally, the existing regulatory landscape fails to fully encompass the nuances of AI, with gaps in guidance and standards, alongside concerns over intellectual property, data privacy, and the assignment of liability. The disparity in legal and regulatory stances on AI across jurisdictions further complicates global harmonization efforts. Main opportunities and drivers for integration of AI/ML tools from a regulatory science perspective lie in the ongoing transition to NAMs and NGRA, leveraging advancements in AI algorithms and computational power to improve toxicology predictions. Organizational drivers such as digital transformation and interdisciplinary training enhance CRA processes and decision-making. Legally, updating frameworks and fostering international harmonization facilitate AI adoption. Public engagement and a focus on sustainability further drive AI's role in CRA, promising more effective risk management and health protection.

In conclusion, the development of scientific criteria for regulatory acceptance of AI/ML approaches should take into account identified gaps and needs. Furthermore, it should be tailored to identified domains of possible AI/ML tools application, namely: (i) Data/evidence management tools; (ii) Data/evidence generation tools – mainly modelling tools helping to create new types of data within hazard and exposure assessment; (iii) Decision support tools that help integrate all the data and make conclusions. Criteria may also benefit from already existing frameworks for the assessment of computational tools in toxicology, e.g., the OECD (Q)SAR Assessment Framework.

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P19-71

In vitro screening for immunotoxicity assessment of nanomaterials, a case study using synthetic amorphous silica

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Manufactured Synthetic Amorphous Silica nanomaterials (SAS-NMs) are the second largest nanomaterials produced worldwide. They are widely used in various applications including cosmetics and food market. Nanomaterials have been recently identified as immune adjuvant possibly involved in the outcome of allergic reactions to environmental allergens. Consequently, a better evaluation of their immunotoxicological potential is needed. However, to date, OECD guidelines do not contain any validated tests dedicated to immune system risk assessment. In line with the 3Rs principle, development of new *in vitro* approach is becoming increasingly relevant.

In vitro evaluation of the adaptive immune response can be based on simple tests involving key cells such as Dendritic Cells (DCs), which capture and present antigens in an immunogenic or tolerogenic manner. In the presence of 'immune danger signals', they undergo maturation, resulting in the expression of co-stimulation and activation molecules, and migrate to draining lymph nodes where they activate naïve T lymphocytes.

We have already demonstrated using a human *in vitro* co-culture model, that SAS-NMs behave as an immunological danger signal by increasing DCs maturation and T-cell response. We addressed the hypothesis that SAS-NAMs surface chemistry could be at the origin of the propagation of these immune danger signals. Indeed, the surface of SAS-NMs contains silanol groups whose reactivity depends on their propensity to establish hydrogen bonds with the polar head of zwitterionic phospholipids in the cell membrane. Surface silanol groups, already identified as critical for cytotoxicity, could also play a determining role in the activation of DCs. To explore this hypothesis, we compared the effects of SAS-NMs by assessing different manufacturing routes and tailoring surface chemistry on the expression of co-stimulatory molecules. As a surrogate model of human DCs, the monocytic THP-1 cell line was used and treated for 16 hours with the materials. Cytotoxicity and expression of CD54 and CD86, markers of THP-1 activation, were measured for each condition. Our results showed that both SAS-NMs increased CD54 surface marker expression with a greater extent for pyrogenic SAS-NMs compared to precipitated SAS-NMs and reducing silanol density led to a downregulation of CD54 expression. We hypothesize that surface chemistry is at the core of the interactions with SAS-NMs leading to DCs maturation. To further investigate membrane interactions, SAS-NMs were also tested using phospholipid bilayer models mimicking cell membrane.

Our work will allow us to develop a relevant strategy to assess immunotoxicity of NMs, considering precise characterizations of surface chemistry, and combining *in vitro* tests using cells andsynthetic biomimetic membrane models. This could lead to a new standard tool for immunotoxicity assessment adapted for nanomaterials.

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P19-72

Comparative risk profile of Dichloromethane (DCM) and non-halogenated solvent alternatives in pharmaceutical manufacturing processes

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Historically, chemical evaluation for pharmaceutical applications focused on hazard information and potency. This approach, known as Green Chemistry, guided selection criteria. These two parameters are also the basis for patient safety considerations regulated by the ICH Q3C Guideline for residual solvents and chemical regulatory restrictions. Recently, Sustainability by Design (SbD) introduced environmental impact as an additional factor when selecting chemicals during pharmaceutical development. Balancing these parameters can be challenging when attempting to avoid regrettable substitutions.

This analysis aims to describe a comparative risk profile of Dichloromethane (DCM) with five non-halogenated solvent alternatives (Ethyl Acetate, Methyltetrahydrofuran, Dimethyl Carbonate, Acetonitrile and Ethanol), within the domain of pharmaceutical manufacturing processes.

A systematic evaluation was conducted, including chemical safety, environmental sustainability, and patient safety parameters. The assessment adhered to the criteria established in the Takeda solvent selection guide. Data from public literature and databases provided hazard classifications, regulatory restrictions, and ICH Q3C classifications. Environmental metrics were assessed using the ecoinvent 3 database, focusing on global warming potential and ozone formation using the midpoint impact assessment method EF3.0 adapted for SimaPro.

Risk quantification employed a scoring system across five domains – hazard potential, regulatory constraints, occupational exposure limits, environmental impact, and patient safety. Scores were assigned on a scale from 1 (indicative of low risk) to 10 (denoting highest risk), facilitating a comparative analysis of the solvent candidates.

Dichloromethane has the highest cumulative risk score. Out of the examined solvents, it is the only substance of concern with environmental regulations and restrictions, hazard potential of chronic effects and a direct greenhouse gas. Out of five alternatives, Ethanol and Methyltetrahydrofuran exhibit low cumulative risk scores due to high chemical safety and comparatively low environmental impact. Results show that non-halogenated solvents are preferable for applicable pharmaceutical processes.

The scoring system enables granular risk assessment, supporting identification of solvents that align with safety and sustainability objectives. By avoiding regrettable substitutions, we enhance safety and sustainability objectives aligned with the principles of Green Chemistry and Sustainable by Design framework.

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P19-73

Commercial mushroom products as a source of biogenic amines

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Central and Eastern Europe have a long tradition of mushroom consumption. Slovak consumers widely use both wild and cultivated species and their mixtures. These food items can contain biogenic amines, attributed to the presence of precursor compounds and their susceptibility to microbial spoilage. Biogenic amines significantly influence food quality and can pose health risks to consumers. In this study, mushroom products available in common retails in Slovakia were analyzed for biogenic amines: spermidine, putrescine, tyramine, cadaverine, histamine, spermine, and 2-phenylethylamine. A total of 54 analyzed mushroom products consisted of products from cultivated species (81%), wild species (15%), and a mixture of both groups (4%). The findings revealed considerable variability in biogenic amine content across individual products, influenced by the mushroom species. The abundance of biogenic amines in the products was found to be in the following order: spermidine >spermine >putrescine >tyramine >tryptamine=2-phenylethylamine >histamine. The dominant biogenic amine was spermidine, present in concentrations ranging from 69.7 to 7,413 mg/kg DW. Putrescine, found in 93% of total products, ranged from 13.5 to 871 mg/kg DW. Spermine was determined to be present in the range of 23.4 to 267.6 mg/kg DW. The presence of tyramine was observed between 24.2 and 949.3 mg/kg DW, tryptamine between 99.0 and 1,261 mg/kg DW, 2-phenylethylamine between 35.7 and 2,611 mg/kg DW, and cadaverine between 19.5 and 272 mg/kg DW. Histamine was found only in two samples of *Agaricus* sp., ranging from 128.3 to 223.5 mg/kg DW. Biogenic amines showed a wider spectrum of occurrence in products containing wild species, especially those belonging to the *Boletaceae* family. Comparison of the mushroom products showed significant differences attributed to the species of mushrooms from which the product was prepared. Principal component analysis revealed three main groups of mushroom products based on biogenic amines: the first group of products containing *Boletaceae* species, characterized by a high content of tryptamine, 2-phenylethylamine, tyramine, and putrescine; the second group of Agaricus species characterized by a high content of cadaverine, spermidine, and spermine; and the third group formed from products containing other species (*Pleurotus* sp., *L. edodes*, *C. cibarius*, *H. tessulatus*, *Ph. nameko*, etc.), characterized by low content of all mentioned biogenic amines. Further research focusing on biogenic amine formation in different mushroom species and their impact on food safety and quality is warranted to understand better and mitigate potential risks associated with mushroom consumption.

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P19-74

Platform for transfer models for hazardous compounds in animal feed

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Feed contaminants are chemical substances that are not intentionally added to feed, but may be introduced in the feed via environmental contamination or during the manufacturing process. Humans can be exposed to these contaminants via consumption of products from animals (e.g. eggs, milk or meat) that have ingested contaminated feed. To assess the possible human health risk, it is essential to determine the amount of contaminants present in animal feed that end up in animal food products. This can be estimated using transfer models.

The website feedfoodtransfer.nl is a publicly accessible platform of chemical-specific transfer models for several farm animals. The models are available with a user-friendly interface and can be used by various stakeholders in the feed and food sector, such as risk assessors, risk managers, feed industry, livestock farmers and other interested parties. The platform is developed by the National Institute for Public Health and the Environment (RIVM) and Wageningen Food Safety Research (WFSR) and contains kinetic models based on previously performed animal studies.

The transfer models can be used to estimate the concentration of a contaminant in edible animal-derived products during and/or after exposure of the animals to the contaminant via feed. These estimates can subsequently be used to determine whether the concentrations in animal products exceed regulatory levels such as maximum levels (MLs). Furthermore, the models can also be used to estimate when the concentration of a chemical compound in an edible product will be below the applicable regulatory level.

Currently, models are available to calculate the transfer of cadmium and dioxins (polychlorinated dibenzo-p-dioxins and dibenzofurans [PCDD/Fs] and dioxin-like polychlorinated biphenyls [dl-PCBs]) in pigs, aflatoxin, dioxins and perfluorooctanoic acid (PFOA)/perfluorooctane sulfonate (PFOS) in dairy cows, and dioxins and dl-PCBs, non-dioxin-like (ndl-)PCBs and organochlorine pesticides in laying hens. More models will be made available on the website soon, including models for ndl-PCBs in broilers and for lidocaine in dairy cows. Model documentation including the underlying R-code will be provided on the website in the coming months.

New partners can join this platform and we are looking forward to expand collaboration on the development and publication of models. With the feed-food transfer models website, RIVM and WFSR want to contribute to a more international standardized approach to assess and manage the risks of substances in animal feed and animal food products.

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P19-75

Integrating NAMs into early-stage screening of novel materials intended for use in Medical Devices: Case studies on the use of GARD for *in vitro* skin sensitization assessment

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The recent advancements in New Approach Methodologies enables the use of *in vitro* method for skin sensitization assessment as part of the biocompatibility testing for medical devices, which is conventionally tested *in vivo*. GARDskin OECD TG 442E is included in ISO 10993-10:2021 as the only OECD validated *in vitro* assay that is compatible with both polar and non-polar extraction vehicles, in line with ISO 10993-12:2021. GARDskin Medical Device is an adaptation of the GARDskin assay, including a pre-sample treatment procedure where solid devices are extracted using both polar and non-polar vehicles.

The aim of this study is to demonstrate the benefits of using GARD for early-stage screening of materials intended for use in medical devices for assessing their skin sensitization potential. Results from two case studies were summarized in which GARDskin Medical Device was used for skin sensitization assessment. The first case study describes the testing of an acrylic-based device with a coating consisting of a UV-cured lacquer, where chemical analysis indicated the potential for skin sensitization. The second case study describes the testing of a polymeric material consisting of Cellulose-Acetate Propionate (CAP) with a plasticizer (Triethylene glycol bis (2-ethylhexanoate), CAS# 94-28-0), with contradictive existing *in vivo* (negative) and *in vitro* (positive) data.

In the first case study, the acrylic-based device induced a positive response in both polar and non-polar vehicles in GARDskin Medical Device and was thus classified as a skin sensitizer. It was hypothesized that the positive results may be due to inadequate curing of the lacquer within cavitary structures of the devices, where UV light exposure was insufficient. To confirm the hypothesis, follow-up testing was performed on an identical device, but without cavities, which was classified as non-sensitizer. *In vivo* data confirmed the outcome of the *in vitro* assay. Consequently, a modification was made in the manufacturing process to prevent the presence of lacquer in cavitary structures of the device.

In the second case study, the CAP material was positive in the non-polar vehicle and was thus labelled as a skin sensitizer. The plasticizer was identified as a potential culprit, considering a borderline negative result in LLNA (SI=2.97) and reported positive clinical data. To support the hypothesis a follow-up study was conducted using another adaptation of the GARDskin protocol capable of providing continuous potency predictions. The results classified the plasticizer as a weak contact allergen. As a result, a different material was selected for the device.

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P19-76
Generic PBK model comparisons: case study methyl eugenol and d-limonene

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Essential oils can be extracted from various aromatic plants and used in multiple products for applications such as bactericidal, virucidal, fungicidal, anti-parasitic, insecticidal, pharmaceutical, and cosmetic. They are liquid, volatile, lipid-soluble, and soluble in organic solvents with a lower density than water. Here, we focus on methyleugenol (CAS 93-15-2) and d-limonene (CAS 8028-48-6), two flavouring agents from a herbal source. D-limonene is under approval as a biocide and is classified as a skin irritant, toxic to aquatic life and could be fatal if swallowed and enters airways. Methyl eugenol is classified as harmful if swallowed and is toxic to aquatic life. Modelling and simulation approaches are becoming critical tools to predict chemical toxicity and rationalise strategies for risk assessment. Physiologically based kinetic (PBK) models are dosimetry models simulating chemical kinetics in the body and are becoming critical tools within the context of Next Generation Risk Assessment. A limited number of physiologically based kinetic (PBK) models are available in the literature covering the flavouring chemical class. Furthermore, the kinetic data of essential oils is often not fully characterised, and multiple knowledge gaps exist. With the progress in science and risk assessment, there is growing interest in developing, reporting, evaluating and applying PBK models, with a focus towards open-source next-generation PBK models. Here, we compare the model outcomes for rats and humans of PKSim, TK-Plate, and an adapted 8-compartment PBK model based on Jones and Rowland (2013). Model performance was compared to plasma data from the literature. The results show that most model predictions were within a 2-fold change of the measured data. Model differences influencing the results are highlighted in the discussion.

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P19-77
Comparing non-clinical safety studies to pharmacovigilance evaluation for assessment of safety: the case of anti-COVID-19 vaccines in France

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Adverse Drug Reaction (ADR) of vaccines are well monitored. However, their predictability and the underlying mechanisms still need to be fully understood and are still considered a significant public health challenge. This study focuses on the COVID-19 vaccination campaign, beginning December 20th 2020 in France, to better understand and anticipate the possible ADR of vaccine components and to build a «Weight of Evidence» (WoE) approach. The project brings together non-clinical safety studies and pharmacovigilance observations in the frame of COVID-19 vaccination. Biomarkers and/or factors related to ADR will be identified and compared to the non-clinical studies carried out during development of COVID-19 vaccines. The integration of these parameters will then be evaluated to see whether it could increase the predictability of non-clinical studies. To do so, the study focused on ADR of adenoviral vector-based, mRNA, and adjuvanted vaccines, of which, five were approved in France: Vaxzevria®, Comirnaty®, Spikevax®, Jcovden®, and Nuvaxovid®. Analysis of PV data allowed us to collect various demographic, clinical and biological data for population stratification and to determine biomarkers, or factors of interest. In line with current knowledge in 2023, different ADR were chosen, depending on their seriousness, their unexpected nature, their effect on compliance or even affecting quality of life. Thus, the following ADR were analyzed: myocarditis/pericarditis, thrombosis (VITT), reactogenicity and menstrual disorders. The number of cases analysed for the ADR mentioned above represented 1884 cases, 122 cases, 35603 cases and 1886 cases, respectively. Preliminary PV results identified potential biomarkers of interest relevant for non-clinical studies, such as troponin and C Reactive Protein for myocarditis/pericarditis and coagulation biomarkers for VITT. Preliminary non-clinical studies results seem to confirm that unexpected ADR could not have been predicted in the current state of study recommendations. Moreover, these results seem to show a difference in the intensity of the immune reaction between vaccines which does not seem to be correlated with the clinic. This could be explained by the different parameters monitored such as acute phase proteins. In conclusion, harmonization of the parameters to be monitored and addition of biomarkers in non-clinical studies have to be considered to build a WoE approach. These results need to be confirmed, in particular with further analysis of data on reactogenicity and menstrual disorders. Analysis of vaccine components, type of pathogen targeted, target population will be added to complete the WoE approach.

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P19-78
Simulating chronic exposure to DEHP with an open-source PBK model: first steps towards aggregate exposure with PK-Sim

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Aggregate exposure (AE) is an individual's combined exposure to a single chemical by different routes of exposure (topical – skin/eye absorption, ingestion, and inhalation) from various pathways (air, water, food, soil, dust, and contact with surfaces) that occur at other times and locations, as the result of multiple sources of exposure [1].

AE varies across individuals, and over time, assessments of populations are described in terms of distributions of short-term doses (acute exposures) and long-term average doses (chronic exposures). Di-2-ethylhexyl phthalate (DEHP), an industrial chemical extensively used in polyvinyl chloride (PVC) plastics, presents a significant concern for AE assessment due to its widespread presence in consumer products such as shoes, gloves, packaging materials, building materials, phar-

maceuticals, personal care products, food contact materials, paints, and adhesives [2,3].

In this study, we apply an open-source physiologically based kinetic (PBK) model, specifically PK-Sim [4,5], to predict the overall internal dietary and consumer AE of DEHP. PBK models are particularly relevant for AE assessments as they can account for exposure from various sources, routes, and timeframes, providing accurate estimations of exposure levels. Furthermore, PBK models can support the prediction of plasma and tissue levels across different populations and life stages, enabling quantitative estimates for individuals with higher exposure risks.

Our PBK model incorporates key parameters related to DEHP metabolism, including the synthesis of its hydrolysis product mono-2-Ethylhexyl phthalate (MEHP) and its elimination through renal excretion [6,7]. By integrating available estimates of consumer and dietary exposure over prolonged periods, we aim to simulate chronic exposure scenarios more accurately.

To validate our model predictions, we propose a comparison with human biomonitoring data collected through the European Information Platform for Chemical Monitoring (IPCHEM) [8]. This comparison will assess the predictive capability of our PBK model in estimating internal doses of both DEHP and its metabolite MEHP.

By providing insights into DEHP exposure levels and dynamics, our study contributes to a better understanding of the potential health risks associated with this ubiquitous industrial chemical. Ultimately, these findings can inform risk assessment strategies and regulatory decisions aimed at mitigating DEHP exposure and safeguarding public health.

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P19-79
A NGRA approach for systemic toxicity: a case study with Erythritol

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Chemical risk assessment is currently undergoing a paradigm shift driven by ethical considerations, regulatory action and the need to ensure the safety of chemicals using efficient, cost-effective and robust methods. Non-animal approaches represent a powerful alternative to improve safety assessments by using more human-relevant tools providing good coverage of key biological targets. Next-generation risk assessment (NGRA) provides a framework integrating data coming from New Approach Methodologies (NAMs) into the decision-making process, allowing for safety assessments to be conducted without the use of animal data. The hypothesis behind the use of NGRA is, that if the internal exposure level of a chemical in humans is below the concentration needed for exhibiting any biological effect, it is unlikely it would trigger any toxicity. Whitin this context, estimates of internal exposures are obtained using physiologically-based kinetic (PBK) models while potential biological effects are assessed by means of points of departure (PODs) from a comprehensive panel of *in vitro* assays. Then, the PODs and exposure estimates can be combined into a single metric often referred to as Bioactivity-Exposure Ratio (BER). Many case studies have primarily focused on chemicals with known modes of action, where the bioactivity and potential toxicity have been assessed based on specific targets affected by the chemical. However, a significant portion of chemicals currently available on the market have limited characterization, requiring an approach that can identify potential toxicity on a broader, systemic level. Erythritol is a sugar alcohol commonly used as a low-calorie sweetener in various food and beverage products. It is naturally occurring in certain fruits and fermented foods. In terms of safety, erythritol is generally considered safe for consumption. It has been approved as a food additive by regulatory and scientific agencies, such as the US FDA and EFSA. These agencies have established an acceptable daily intake (ADI) for erythritol, that was re-evaluated by EFSA in 2023 at 0.5 g/kg body weight/day to be protective for the immediate laxative effect. Here we present a case study applying a NGRA approach for systemic toxicity to Erythritol. We set up a framework to be used as a lower tier screening to flag potential safety concerns. The framework combines *in silico* predictions of toxicity and toxicokinetic parameters, *in vitro* assays including targeted (*in vitro* pharmacological profiling and cell stress profiling) and untargeted (high-throughput transcriptomics) approaches and PBK modelling. By deriving the chemical BER and benchmarking it against established *in vivo* safety levels, we demonstrate the ability of the NGRA framework to drive safety decisions without animal testing.

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P19-80
Application of New Approach Methodologies for the risk assessment of emerging mycotoxins in food

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Mycotoxins, the toxic secondary metabolites of moulds, pose a significant problem for international food systems as well as human and animal health. The situation is expected to worsen as due to climate change, crop-contaminating fungi that until now were predominantly found in (sub)tropical regions are expanding into Europe and North America. While some mycotoxins (*e.g.*, aflatoxins) were studied well enough that food authorities established regulation, a large proportion of fungal metabolites is yet not fully characterized in terms of toxicity and human relevance and exposure. These are referred to as "emerging mycotoxins" and are a recent focus of European food safety assessment.

The goal of this work is to establish a reliable and expandable strategy for evaluation of potentially adverse effects of single exposure and to combinations of emerging mycotoxins (namely beauvericin and moniliformin). It will focus on characterizing the potential of selected emerging mycotoxins in terms of genotoxicity and systemic toxicity.

For this, a set of *in vitro* (toxicity testing in mammalian cell cultures) and computational (PBPK modelling) methods will be used, aiming at the application of animal-free methods that can directly fuel hazard characterization. Furthermore, analytical data from internal surveys and recently published reports and peer-reviewed articles demonstrated the co-occurrence of these emerging mycotoxins in plants. Therefore, we will develop tools to cope with co-occurrence scenarios and inter-individual variability, that can be used as blueprint strategies for the integration of new compounds in toxicity testing. Ultimately, these newly generated *in vitro* and *in silico* data combined with an exposure assessment considering the co-occurrence levels of these emerging mycotoxins in plants would feed a risk assessment fully based on New Approach Methodologies.

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P19-81

Arsenic, lead and essential elements content in tea samples from Turkey: a risk assessment study

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Despite its popularity, tea products may contain elevated levels of both toxic and essential metals, posing potential health risks due to their toxic properties. This study investigates the levels of essential and toxic elements in eight brands of black tea leaves and infusions available in Istanbul, Turkey. A risk assessment was conducted to evaluate the health hazards associated with toxic element intake from tea infusions. For essential elements, the risk assessment compares daily intakes of Zinc, Iron, Copper, and Magnesium from tea infusions against recommended toxicological guideline levels. Results reveal aluminum as the predominant toxic metal, contrasting with magnesium as the prevalent essential element in both dry leaves and infusions. A significant reduction in concentrations of toxic and essential elements in infusions was observed compared to the initial dry tea leaves. Risk assessment of infusion samples indicated negligible non-carcinogenic risks. However, arsenic exhibited a moderate carcinogenic risk. Daily intakes of essential elements from tea infusions fall well below toxicological reference values, indicating minimal risk for consumers. This study enhances our understanding of tea composition, supporting informed decision-making and considerations for product safety in the tea industry.

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P19-82

Assessment of plant protection products and biocides: recent developments and future perspectives on using non-animal methods

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In 2023, the European Commission committed to phasing out animal tests for all regulated chemicals, including plant protection products (PPPs) and biocides. A number of newly developed and validated non-animal approaches are available for regulatory use, but many are not yet being used due to a lack of awareness of or confidence in these approaches. This review aims to inform assessors/regulators, contract research organisations, and the regulated community about 1) the availability of validated non-animal tests that are accepted by EU regulators, 2) approaches to assessing PPPs and biocides that are ready to be implemented and those that will be in the near future, and 3) endpoints that require further resources before non-animal methods will be available for regulatory acceptance.

Examples of validated new approaches that are accepted for regulatory use but not yet being used include OECD test guidelines 467 [1] and 492B [2] – both of which are robustly validated approaches to assessing eye irritation potential. The *in vivo* test for eye irritation has significant limitations, including a lack of reproducibility, and data obtained exclusively from *in vitro* methods can now be used to determine eye irritation potential (guidance document no 263), discriminating among the Globally Harmonised System for Classification and Labelling of Chemicals (GHS) categories for eye damage and irritation. [3] An example of an approach that is ready to be implemented is the OECD Defined Approach on Skin Sensitisation (guideline no 497), which combines data from *in silico*, *in chemico*,and *in vitro* methods and produces results that correlate with human data as well as, if not better than, the *in vivo* local lymph node assay. [4] This testing strategy can discriminate among three GHS sensitiser categories for chemicals. OECD guideline 497 is accepted under the biocidal products regulation, and its use could and should be accepted for PPP active ingredients and products. [5]

In addition, retrospective analyses continue to demonstrate that some *in vivo* tests do not inform risk assessment, and thus waiving of certain tests should be immediately considered. For example, dermal acute toxicity tests rarely inform regulatory decisions, and they should be waived unless the requirement is robustly scientifically justified. [6] Finally, certain endpoints require further resources to develop non-animal approaches. For example, the currently required rodent cancer bioassay lacks reproducibility and relevance, but carcinogenicity assessment is being modernised through mechanistic approaches, including adverse outcome pathway–informed *in silico* models and cell transformation assays. [7]

Many validated non-animal approaches are already available for regulatory use for the assessment of PPP and biocidal active ingredients and products. With further resources and investment, the phasing out of animal tests for all regulated chemicals can be realised.

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P19-83

Employing QSAR modeling for regulatory assessment of TiO_2 nanoparticle safety

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The common use of titanium dioxide nanoparticles ($nTiO_2$) in cosmetics, food, pigments, and photocatalytic remediation raises safety questions. The International Agency for Research on Cancer (IARC) designated powdered $nTiO_2$ a potential inhalation carcinogen (2006), while the EFSA (2021) highlighted genotoxic risks associated with food additive E171 containing TiO_2 nanoforms.

Regulatory bodies like the EFSA (2011) and SCCS (2023) mandate *in vitro* nanoparticle testing, emphasizing detection of mammalian cell gene mutations and chromosomal damage. The Comet assay, while lacking formal regulatory status, is valued as a screening test for DNA damage. However, these *in vitro* assays necessitate specialized laboratories, technical expertise, and substantial resource expenditure.

In response, in silico approaches are gaining traction as initial screening tools for mutagenicity and genotoxicity evaluation. Computational methods, notably Quantitative Structure-Activity Relationship (QSAR) modeling, provide an alternative to experimental research and enable hazard identification at reduced resources.

The present study introduces QSAR models tailored to predict $nTiO_2$ mutagenicity and genotoxicity, utilizing micronucleus test and Comet assay data, respectively. Model construction entailed a comprehensive literature review (2007–2022) with an evaluation of data quality, aligned with EFSA recommendations. Priority was placed on the relevance and robustness of genotoxicity assays and exhaustive reporting of nanoform physicochemical properties. Experimental data, encompassing nanoform and cell line characteristics, were extracted to enable the development of models to forecast mutagenic and genotoxic outcomes across diverse TiO_2 nanoforms and cell lines. Machine learning techniques (Logistic-Principal Component Analysis, L-PCA and supervised algorithms) facilitated the identification of relationships between nanoform/cell line descriptors and observed toxicological effects.

The resulting QSAR models stand among the rare instances capable of predicting regulatory-relevant endpoints for nanoforms, highlighting their value as in silico New Approach Methodologies (NAMs).

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P19-84
Insights into the new information requirements triggered by the revised SCCS Guidance on the safety assessment of Nanomaterials in Cosmetics: Ensuring nano-safety and compliance with the EU Cosmetic Product Regulation (CPR)

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Recent advances in nanotechnology have created new opportunities for innovation in cosmetics. In response, the European Commission (EC) Scientific Committee on Consumer Safety (SCCS) updated its guidance in June 2023 to ensure the safety of nanomaterials (NM) intended for use in cosmetics under the EU Cosmetic Product Regulation (CPR).

The latest revision of the SCCS Guidance on the safety assessment of NM in cosmetics provides a comprehensive overview of the key aspects triggering potential safety concerns. The SCCS emphasised the importance of the appropriate characterisation of the NM and the submission of a relevant dataset considering the specific properties of NM.

Additional physico-chemical criteria have been introduced to strengthen the identification and characterisation of the NM. This includes, amongst others, the full characterisation of chemical composition, including the aspect ratio, surface modification types, stability and homogeneity; analyses of the particle size distribution (PSD) on at least 5 batches; and robust information on solubility and dissolution rate, which are crucial for understanding the likelihood of consumer exposure.

Regarding the toxicological assessment, consideration must be given to the specific properties of the NM to establish an appropriate experimental design. When planning a percutaneous absorption study, appropriate analytical techniques must be included to consider possible adsorption of the NM to surfaces in the absorption rate determination. In the context of genotoxicity testing, cellular uptake of the nanoparticles is a key concern. Unlike for their bulk counterparts, the Ames assay is not considered appropriate for NM due to the size of bacteria and their limited uptake of nanoparticles. The mammalian cell gene mutation assay and the micronucleus test are considered more appropriate for the genotoxicity assessment of NM. It is recommended to perform the studies along with characterisation of the test material in culture media and of its uptake by the cells. The stability of the dispersion of NM in the cell culture medium prior and after the experiment should be determined. It is further recommended to test for NM-induced intracellular reactive oxygen species (ROS), potential formation of free radicals and oxidative damage to cells and tissues.

Based on practical experience, this poster provides insights into the analytical, physico-chemical and toxicological data required to assess the safety of NM used in cosmetic products and to develop SCCS guideline-compliant dossiers.

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P19-85
Alternative approaches to chemical risk assessment: challenging the current animal-based threshold approach

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Background: Humans are exposed to a variety of hazardous chemicals. The identification and management of their potential health impact is mostly based on adversity thresholds, following a 1950s risk assessment paradigm implementing Paracelsus' principle "Sola dosis facit venenum". In fact, if we assume that "only the dose makes the poison", the focus should be to identify the dose that does not produce adverse effects, named toxicological threshold. Science has evolved, and both empirical results and the increased knowledge of the mechanisms governing the interaction of chemicals with biological systems confirms that the toxicological threshold principle is an oversimplification.

Assessment: Certainly, the dose or exposure level is a key factor, but there are many other factors involved in determining the magnitude and likelihood of adverse effects. Even for the homogeneous animal groups used in toxicity tests, it is usual to see differences of several orders of magnitude between the dose that produce effects on some individuals and that producing the effect on all exposed animals.

The emerging mechanistic New Approach Methodologies (NAMs), have triggered the concept of Adverse Outcome Pathways (AOPs) highlighting that the chemical-biological interactions are a succession of connected events. A molecular initiating event (MIE), triggers chains of key events (KE) linked by key event relationships (KER), that finally result in a pathological effect, the adverse outcome (AO), equivalent to the apical effect of toxicity studies. The same KE is connected to several AOPs, and even more important, indirectly related pathways, genetic disorders or specific pathologies may modify the KERs, resulting in individual variability.

We are proposing an innovative non-threshold conceptualization, with some parallelism with that used for genotoxic carcinogens. Even low exposure levels will trigger the MIE, that will progress or not towards KEs and AOs depending on individual conditions and characteristics. It is possible that for some chemicals there is a threshold, but our toxicity tests do not have sufficient power to identify levels relevant for Health Assessments (e.g. in the order of 10^{-5} or 10^{-6}). Instead of connecting chemical exposure to toxicological thresholds, we should assume that individual variability is better explained by probability distributions linking each event with the likelihood for health impacts. The focus is on the observed effects and their mechanistic understanding, linking the event with the likelihood for progression towards measurable health indicators, vs. the possibility for recovery/compensation. The AOP concept is amplified as probabilistic Health Impact Pathways (HIP), addressing vulnerable population groups. Several examples on regulated chemicals, including pesticides and cosmetics, and environmental contaminants, including atmospheric pollutants, are presented.

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P19-86
The value of gene expression data as a surrogate for Human Protein Atlas immunohistochemistry for assessing on-target off-tumour toxicity in target safety assessment

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Immunohistochemistry (IHC) data can be instrumental in Target Safety Assessments (TSAs) to anticipate whether targeting a protein with cytotoxic modalities such as Antibody Drug Conjugates (ADCs) can cause on-target, off-tumour (OTOT) toxicity in normal tissues. The Human Protein Atlas (HPA) is a comprehensive resource consisting of single-cell (sc) and whole tissue (wt) RNA-Seq transcriptomics data for the entire human genome and reliable (HPA antibody reliability score of 'Enhanced') IHC data for 49% of the human proteome across a large panel of normal adult tissues and cell types. HPA IHC data can be used alongside protein membrane expression data to define OTOT risks. This study aimed to assess the utility of RNA-Seq data to identify potential OTOT sites where reliable IHC data is unavailable. We established the concordance of gene-to-protein expression for 38 approved and clinical phase ADC targets with an HPA antibody 'Enhanced' reliability score at both a whole tissue level and at a single cell level in a set of 14 matched cell types. A threshold-based expression level (0 – not detected, 1 – low, 2 – medium, 3 – high) using the median expression of each gene in each sample was used to compare the four categorical 'tissue scores' that HPA applies to its IHC dataset. The data was analysed using different gene expression threshold systems to investigate data concordance using a variable median expression value for each gene and fixed threshold values for the whole dataset. To calculate dataset concordance, all RNA-Seq/IHC combinations were categorised as either 'correct' (both IHC and RNA-Seq tissue score of ≥ 0) points or 'incorrect' (either IHC or RNAseq tissue score=0) points. At the whole tissue level, the results show a 67.1% similarity between IHC and wt-RNA-Seq and a 69.1% concordance with the sc-RNA-Seq dataset. At the matched single cell level, the variable threshold system revealed a 64.5% similarity between IHC and sc-RNA-Seq, and with the fixed threshold scoring system, there was 65.6% similarity. A literature review of IHC data for selected targets without HPA IHC data supported this conclusion. Furthermore, in over 50% of the 'correct' data points, the tissue scores of IHC and sc/wt-RNA-Seq were equal, and there was consistently a large proportion of data points (59% of points with an IHC tissue score of 0 in all comparisons) where the IHC and sc/wt-RNA-Seq were both 0. This indicates that if there is no protein expression in a cell type or tissue by IHC, there is a high probability that the equivalent gene/tissue combination in the RNAseq datasets will show no expression. These results show relatively high concordance between the HPA IHC data and sc/wt-RNA-Seq gene expression in a set of ADC targets. In a TSA context, this study indicates that mRNA transcriptomics is a viable resource during the initial assessment of potential OTOT risks for ADC targets when HPA IHC data is unavailable.

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P19-87
Comparison of growth curves in beef cattle for PBK modelling

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Introduction: Long-term exposure to chemicals can lead to accumulation in various tissues of farm animals. Especially in young animals, growth can lead to an increase in the volumes of blood and other tissues, which in turn alters the distribution and time to reach steady state concentrations in different tissues. Consequently, chemical concentrations in food products may be different depending on the time and duration of exposure relative to age. A physiologically based kinetic (PBK) model for long-term exposure should therefore account for the growth of an animal. The objectives of this study were 1) to identify the best mathematical function to describe the growth curve of beef cattle and 2) the implementation of the growth function into a PBK model to describe the effect of growth on the tissue concentration of PFOS.

Material and methods: Growth data (body weight over time) for multiple breeds of beef cattle were extracted from the literature. Three different sigmoidal growth curves (West, Von Bertalanffy, Richards) were fitted to the data and their Akaike information criterions (AIC) were compared to each other. The fitted curves were implemented in a PBK model of PFOS in beef cattle. The resulting concentration curves were compared to the model without growth curve.

Result: All three growth curves fitted the growth data well, with AICs of 2901, 2903 and 2903, respectively. Growth appeared to be the main driver of the reduction in concentration of PFOS in tissues over the course of simulations; in the non-growth model, PFOS concentration remained constant.

Conclusion/Future direction:

- Based on the comparison, all three growth models are recommended for predicting the growth rate of beef cattle from birth to sexual maturity
- In the PFOS study, growth leads the dilution of tissue concentrations as the time to reach equilibrium is prolonged.

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P19-88
Slovenian surveillance of buckwheat foodstuffs contamination with tropane alkaloids

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Introduction and aim: In recent years, poisoning incidents due to contamination of crops intended for human consumption with *Datura* sp., a toxic plant containing tropane alkaloids, were reported worldwide [1]. In Slovenia monitoring of food products made of buckwheat (*Fagopyrum* sp.) for tropane alkaloids was introduced in late 2003 following a mass poisoning incident [2]. To review the safety of buckwheat food products we analysed the monitoring results from 2004–2023.

Methods: The hyoscyamine (atropine) and scopolamine were determined using liquid chromatography tandem mass spectrometry (API 4000 QTrap LC-MS/MS); level of detection-LOD 0,0005 mg/kg; level of quantification-LOQ 0,001 mg/kg food. Based on an ad-hoc risk assessment following the above mentioned poisoning incident national maximum residue levels (MRL) for atropine/scopolamine mixture in buckwheat food products: 4.0 µg/kg (atropine) and 2.0 µg/kg (scopolamine) were set and used in safety assessments from 2004–2011 [2]. In 2012/13 the point of departure (PoD) for RA was based on decreased heart rate from a human volunteer study, i.e. the Acute Reference Dose (ARfD) of 0.01 µg/kg body mass (bm) for each alkaloid assuming scopolamine to be twice as potent as hyoscyamine [3]. From 2014 onwards the revised ARfD of 0.016 µg/kg bm expressed as the sum of hyoscyamine and scopolamine, assuming equivalent potency served as a PoD [4].

Results: From 2004–2011, the then national MRLs were exceeded in five (4.3%) of the 116 analysed foods samples. From 2012–2023, RA was performed if the residues exceeded the LOQ, which was the case in 31 of 372 analysed buckwheat foodstuff samples. In nine cases (2.4%) the risk was judged as unacceptable. Those foodstuffs were either not put on the market or were recalled.

Conclusion: Our survey indicates that the introduction of buckwheat foodstuffs monitoring for tropane alkaloids has contributed to safety of these products thus preventing food poisoning incidents. It is expected that the compliance with the recent Commission Regulation (EU) 2021/1408 having set the MRLs at 1 µg/kg for each alkaloid [5] will further reduce the likelihood that tropane alkaloid contaminated buckwheat foodstuffs would appear on the market.

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P19-89
Toxicokinetic and alternative methods to support dog study waiver decision making in pesticide risk assessment: triclopyr as a case study

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Under plant protection product Regulation (EU) No 283/2013, the 90-day dog study (non-rodent species) is a data requirement for approval of active substances. However, data shall be generated in accordance with the 2010/63/EU legislation ensuring, whenever possible, the replacement, the reduction and the refinement of animal testing. In this context, the requirement of a 90-day dog study could be questionable when the dog is not a relevant species to address human health risk safety assessment.

It has been observed that the dog is a uniquely sensitive species to some classes of agrochemicals, such as phenoxy- and pyridyloxy-carboxylate herbicides, compared with other species. The aim of this work is to provide a) a retrospective analysis of species sensitivity of Auxin Mimics mode of action herbicides based on the analysis of the available 90-day dog and rat studies, b) a case study of a non-human relevant toxic mode of action for dogs due to quantitative toxicokinetic differences and c) use the case study to provide perspectives on how toxicokinetics and New Approach Methodologies (NAMs) can support decision-making to waive a 90-day dog study.

In the retrospective analyses, the available LOAEL and NOAEL were used to compare potential species sensitivity. Potential differences in target organs were also identified. When available, systemic exposure was used to further refine species differences. Triclopyr was identified as one of the substances with higher sensitivity in dogs compared with rats. Based on the extensive toxicokinetic data package available on triclopyr, it was chosen as a case study of a non-human relevant mechanism of kidney toxicity. Indeed, dogs have substantial quantitative kinetic differences in transporter-mediated (Organic Acid Transporter OAT 1/3) urinary excretion of triclopyr compared to rats, and humans/primates. Therefore, in this case, the use of the dog data to derive toxicological reference values for human risk assessment is not adequate and a waiver for dog studies could have been considered, if this was a new active substance. Interestingly, *in vitro* comparative absorption, metabolism, protein-binding, hepatic and renal clearance studies and PBK modelling on triclopyr further illustrate how NAMs could support decision-making on dog study waivers.

In conclusion, this retrospective analysis and the provided case study allow us to discuss the current scientific and regulatory challenges to waive dog studies. This work illustrates how toxicokinetic and NAMs can be leveraged to support decision-making to eliminate a non-relevant 90-day dog study in the pesticide safety evaluation testing program.

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P19-90
Development of an integrated approach to testing and assessment (IATA) for metabolic disruptors: the DEHP case study

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The increasing prevalence of metabolic disorders underscores the importance of accurately identifying and assessing the impact of metabolic disruptors that interfere with endogenous metabolic pathways and subsequently on human health. This study, conducted within the OBERON project framework proposes an Integrated Approach to Testing and Assessment (IATA) focused on di(2-ethylhexyl) phthalate (DEHP) by leveraging an Adverse Outcome Pathway (AOP) constructed from the integration of multidimensional data sets spanning *in vitro*, *in vivo*, and systems biology domains.

The IATA framework operationalizes a tiered strategy for evaluating DEHP's potential as a metabolic disruptor and is anchored in an AOP that maps the mechanistic pathways from molecular initiating events (MIEs) to adverse outcomes (AOs). Tier 0 initiates the process with *in silico* prioritization and Physiologically-Based Pharmacokinetic (PBPK) modelling based on Human Biomonitoring (HBM) data to establish exposure levels.

Subsequent tiers and subtiers (Tier 1a through Tier 2c) incorporate a comprehensive suite of experimental designs – from targeted *in vitro* assays using hepatic HepaRG and pancreatic EndoC-βH1 cell lines for examining lipid accumulation, mitochondrial dysfunction, and insulin signalling disruptions – to *in vivo* zebrafish models assessing systemic effects. Omics technologies (transcriptomics, metabolomics, and multi-omics) enrich the data landscape, enabling the delineation of early KEs critical to metabolic disruption. The decision-making process for advancing through these tiers is predicated on the evidence threshold thus ensuring methodological rigor.

The *in vivo* tier (Tier 2) further elucidates DEHP's impact on metabolic health, employing zebrafish models for organism-level analysis. This tier's integration of multi-omics data facilitates the construction of systems biology models in Tier 2c, which are pivotal for understanding the complex interactions underlying metabolic disruption.

The developed AOP synthesizes the acquired data to illustrate the mechanistic relationships between DEHP exposure and the etiology of metabolic diseases. This AOP not only serves as a scientific basis for understanding DEHP's metabolic effects but also informs regulatory risk assessment frameworks.

This detailed IATA exemplifies a sophisticated, evidence-based methodology for dissecting the metabolic disruption potential of chemical agents. The strategic integration of computational models with empirical findings across various biological levels demonstrates a holistic approach, crucial for advancing the field of toxicology and environmental health research. Furthermore, it underscores the imperativeness of adopting multidisciplinary strategies in the risk assessment of potential metabolic disruptors.

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P19-91
The Safe and Sustainable by Design toolbox: guiding the user across the innovation stages through the SSbD Wizard

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The objective of the Safe and Sustainable by Design (SSbD) concept is to integrate safety, health, environmental, economic and social considerations with functionality requirements in the development of chemicals and materials across the innovation process. The EC framework for SSbD defines a five step process where Steps 1–3 focus on safety while steps 4–5 focus on sustainability. Within the European Partnership for the Assessment of Risks from Chemicals (PARC), there is a specific task dedicated to the development of the PARC SSbD toolbox. By developing a comprehensive toolbox including all the relevant tools, methods and data, the operationalization of the SSbD framework, as defined by the EC, will be enabled. The PARC toolbox introduces an SSbD Wizard that serves as a user interface that will guide the user through the whole assessment process and create the SSbD assessment workflow based on the user input. In more detail, the SSbD Wizard is designed to take into account the five SSbD steps across the five development stages of chemicals and materials into a final product (stage-gate model), as well as the five SSbD steps. The Wizard commences the assessment by posing a set of questions on aspects that will guide the user towards a proper assessment process (e.g., if the substance in question is novel or existing, its chemical classification, and its potential applications). Furthermore, depending on the innovation stage and the SSbD step, the Wizard will suggest pertinent databases where the user can search and find relevant information. Specific recommendations and disclaimers, such as the evaluation of the prediction reliability of Quantitative-Structure-Activity-Relationship (QSAR) models, will also be included. In conclusion, the SSbD wizard enhances the usability of the PARC toolbox by making a complex analysis systematic and easy to understand and enabling the widespread application of the SSbD concept.

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P19-92
In vitro toxicity assays for investigating the hazard assessment of *Bacillus thuringiensis* strains

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Bacillus thuringiensis (Bt) is an increasingly used biopesticide belonging to the *Bacillus cereus* group, the production of Cry and Cyt toxins being involved in its insecticidal activity. In Europe, some concerns have pointed out that Bt could be involved in bacterial foodborne outbreaks (FBOs). Recently, among 250 FBOs in France, Bt strains, suspected to

be of commercial origin, have been detected in 49 cases (Bonis *et al.* 2021). Developed for regulatory purposes to replace *in vivo* experiments, New Approach Methodologies (NAMs) are certainly promising tools to provide data on Bt potential toxicity. *In vitro* cell assays can be useful to screen for toxicity using various endpoints.

In this study, the toxicity of several Bt strains has been investigated on two human cell models, the intestinal Caco-2 and the macrophage THP-1 cells. A panel of 5 Bt biopesticides (2 *kurstaki*, 2 *aizawai* and 1 *israelensis*), as well as a *B. cytotoxicus* strain isolated from a FBO were selected. The bacterial strains were grown in BHI and supernatants were harvested at the beginning of the stationary phase. Dilutions (from 25 to 0.1%) in cell culture medium (DMEM and RPMI respectively for Caco2 and THP-1) were tested. After a 24 h treatment, cytotoxicity was assessed by measuring the mitochondrial activity with the MTT assay, while the pro-inflammatory response was measured by the release of interleukin 8 (IL-8) using ELISA.

In this presentation, the results obtained with the Bt strains using the 2 cell models will be compared and discussed in light of their usefulness to investigate the pathogenicity of Bt strains and how they can contribute to hazard assessment of biopesticides.

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P19-93

Using high throughput screening asapreliminary tool for predicting environmental risk of chemicals

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The presence of thousands of chemicals in consumer products used daily underscores the necessity of screening a broader range of chemicals beyond the traditional well-studied suspects for environmental impact. Ecotoxicological hazard assessments for individual chemicals are regularly performed using laboratory data derived from standardized tests involving organisms representing key trophic levels, including primary producers, primary consumers, and secondary consumers. The data is summarized to derive a Predicted No Effect Concentration (PNEC) specific to the analysed ecosystem. The PNEC is determined by choosing the most sensitive biotest (representing the most vulnerable trophic level) and applying a suitable assessment factor (AF). The more limited the data set, the greater the inherent uncertainty in the assessment, and thus, the higher the applied assessment factor (AF). The quotient of the predicted environmental concentration (PEC) and the PNEC has become widely accepted standard for the ecotoxicological risk characterization. High-throughput screening (HTS) data and associated predictive models have not yet seen widespread application in risk assessment. A significant obstacle is the disparity between the endpoints measured in HTS assays and those considered in risk assessments. HTS and associated Adverse Outcome Pathways can serve as a preliminary tool to assess the predictive impact of chemicals or their mixtures, potentially reducing the requirement for biotesting. The current objective is to present, justify, and explore the HTS approach for predicting the environmental risk associated with chemicals, employing a combination of accessible data and tools to forecast environmental toxicity. This combination can also be applied to predict the environmental risk posed by chemical mixture, a scenario more prevalent in real-world settings.

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P19-94

A case study on the ^{AX}ILD model: efficacy testing of an antifibrotic drug

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Pulmonary fibrosis is a progressive disease, characterized by scarring and thickening of lung tissue. In the early stages, a symptom is shortness of breath, progressing to respiratory failure within three to five years. While current treatments, such as Nintedanib and Pirfenidone, slow down disease progression, there is no cure on the market yet. Species-specific differences in disease mechanisms hinder the translation of the animal models to humans. Therefore, there is an urgent need for human-based *in vitro* strategies to gain insights into potential therapeutical targets and predictive drug efficacy testing.

The $_{AX}$ILD (interstitial lung disease) model was previously established on the ^{AX}Lung-on-chip system, consisting of human primary alveolar epithelial cells and fibroblast co-culture. Profibrotic cues were induced using a central fibrosis mediator, Transforming Growth Factor β1 (TGF-β1). Read-outs on the barrier function, Transbarrier Electrical Resistance (TER), as well as gene and protein expression of key markers were investigated by RT-qPCR and ELISA. To study the robustness and applicability of the model, the efficacy of Nintedanib, an FDA-approved drug, and a reference compound (RefCmp), were tested.

Our results showed stimulation with TGF-ß1 for 48h led to decreased barrier function and increased expression of profibrotic markers at protein and gene level indicative of extracellular matrix deposition and tissue remodeling. Nintedanib treatment led to a dose-response attenuation in the protein secretion, including collagen IV and PAI-1, and in gene expression of fibrosis-associated markers such as CCN2, COL1A1, and ACTA2. Treatment with RefCmp showed promising effects decreasing collagen secretion compared to TGF-ß1 treated cells.

In conclusion, in this case study the ^{AX}ILD model successfully recapitulated fibrosis specific hallmarks and a dose-response attenuation of key markers in response to the treatment with Nintedanib. This highlights the potential of the ^{AX}Lung-on-chip system for testing the efficacy and safety of new clinical candidates. The technology shows great promise in facilitating decision-making and accelerating drug development while reducing reliance on animal testing.

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P20 | Regulatory toxicology (REACH)

P20-01

FDA SENDIG version 4.0 (the New SENDIG Version): consideration on additions and changes on histopathology-related data

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It has been 7 years since the submission of SEND data to the U.S. FDA became mandatory. SEND dataset must comply with the SEND Implementation Guides (SENDIG), and currently SENDIG versions 3.1 or 3.1.1 or DART v1.1 are in effect. SEND has been kept evolving, and the CDISC has advanced the preparation toward release of upcoming version 4.0. Its internal and public reviews are scheduled to commence in 2024, and v4.0 is expected to be released in 2025. At the time of update from v3.0 to v3.1, pathologists' involvement was even more important since v3.1 requires mapping of non-neoplastic findings to controlled terminologies and population of the distribution and chronicity data into new variables. In the next update to v4.0, significant changes are also expected on histopathology-related data, such as the addition of new test codes and variables.

We have energetically supported SEND data creation to date from the position of pathologists through efforts such as collecting findings with SEND in mind and creating in-house glossaries. For SENDIG v4.0, as is the case with the past, it is important to understand the requirement changes at an early stage and prepare to support the creation of appropriate SEND data.

In this presentation, we introduce and illustrate the additions and changes on requirements to histopathology-related data currently expected in SENDIG v4.0 and discuss points to consider when collecting findings as well as the potential impacts of requirement changes on SEND data creation.

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P20-02
Developing the SciRAPepi tool for assessment of reliability and relevance of observational epidemiological studies

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Robust health risk assessment of chemicals relies on structured and transparent collection and evaluation of data. Non-standard toxicological data and epidemiological (epi) data, for example from academic research studies, are not used to their full potential in regulatory assessments. Reasons for this may be insufficient reporting and uncertainties about the relevance and reliability of the data. A possible solution to increase the use of non-standard studies is to provide tools to facilitate structured and transparent evaluation of the studies according to criteria developed for various study designs, and to support researchers in reporting their data. One example of such an initiative is the Science in Risk Assessment and Policy (SciRAP) online platform (http://www.scirap.org), currently consisting of tools designed to evaluate the reliability and relevance of *in vivo*, *in vitro* and ecotoxicity studies. SciRAP intends to bridge the gap between academic research and chemical risk assessment and policy.

We are now developing an additional tool within the SciRAP platform – the SciRAPepi tool. This tool is intended for assessing the reliability and relevance of observational epi studies, including cross-sectional, (nested) case-control, and cohort studies for use in hazard and risk assessment of chemicals. The draft version of the SciRAPepi has been tested by a group of experts and will be further refined based on their feedback provided to us. The SciRAPepi tool comprises three sections: an introduction with instructions on how to use the tool in Excel, an Assessment section containing specific criteria, and Results. Criteria are divided into three categories: reporting quality (33 criteria), methodological quality (21 criteria) – collectively make up the study reliability, and relevance (6 criteria). The tool covers aspects such as study participants, study design, exposure assessment, outcome assessment, statistical analysis and data processing, ethics and competing interests. Each criterion in the SciRAPepi tool can be evaluated on a scale of not fulfilled/not relevant (0 points) up to fulfilled/directly relevant (1 point). The tool includes functions to increase the weight of specific criteria or remove criteria, depending on the suitability of a specific study design. Guidance is integrated into each criterion in each section, offering comprehensive support for evaluating epi studies. The Results section includes numerical scores and graphical representations, displaying partial results for reporting quality, methodological quality and relevance, and the total numerical score. The reporting checklist, as a part of a tool, provides guidance on how to report epi studies so that they can subsequently be incorporated into risk assessment.

The SciRAPepi tool will help increase the systematic use of reliable epi data in hazard and risk assessment and will provide risk assessors with a broader range of options for assessing epi data for their needs.

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P20-03
Importance of the active and complementary role of sponsors in preclinical research

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Preclinical safety evaluations using animals are no longer a prerequisite for drug discovery[1], together with flourishing of new biotechnology companies and advanced pharmaceutical technologies for contract research organizations (CROs) and supervisions, sponsors play a more vital role in preclinical safety evaluation than ever before. Based on the current Good Laboratory Practices (GLP) framework [2], the study director (SD) serves as the single point of control for preclinical studies, and sponsors should ensure the GLP compliance without relying solely on the assurances of CROs, however there are no specific requirements regarding what and how they perform these tasks.

Indeed, for a harmonized CRO cooperation with high quality, sponsors should actively complement the SD and provide scientific inputs with close study oversight from a global or higher-level view[3]. The main sponsored activities could be listed as follows:

1. Ensuring the overall quality of CROs and personnel quality.
2. Forewarning of particular concern/requests from regulatory agencies or unique study features to CRO for preparation of certain advanced training or specific procedures.
3. Detailing contracts and outlines that specify the schedule, cost, study design, or expectations for report metrics, etc.
4. Manufacturing and shipping representative test/control articles to CRO in sufficient quantities.
5. Facilitating the dosing formulation preparation and validation of formulation and biological analysis.
6. Providing sponsored scientific input for protocol/protocol amendments reviewing and approving them.
7. Reminding for pre-study meeting, important study activities such as sample collection, etc.
8. Maintaining close communication with SD regarding study progress.

9. Staying online for study emergencies and provide a prompt, scientific, effective, and compliant sponsor response if needed.
10. Conducting effective audits of CROs, including on-site visits for key activities or unexpected findings.
11. Providing scientific rationale during deviation evaluation/CAPA if needed.
12. Reviewing study reports in detail to recheck that they truly, accurately, and completely reflect the data obtained during the course of the study.
13. Facilitating activities conducted by a third-party and maintaining close oversight.
14. Ensuring study files are properly archived according to GLP requirements.
15. Ensuring all preclinical findings are reported to supervisors and serving as an effective communication bridge between the CRO and supervisors when necessary.Following the above steps, it is not intended to weaken the role of study directors, instead, it aims to guide sponsors on how to play a more complementary and active role [4]. Sponsors should possess their own toxicology expertise to facilitate more comprehensive intervention throughout the entire dure development process.

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P20-04
Considerations for the development of guidance on dose level selection for developmental and reproductive toxicity studies

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In 2022, the European Chemicals Agency (ECHA) issued advice on the selection of high dose levels for Developmental and Reproductive Toxicity (DART) studies, indicating that the highest dose tested should "*demonstrate an aim to induce clear evidence of reproductive toxicity without excessive other toxicity and severe suffering in parental animals (e.g. prostration, severe inappetence (lack of appetite), excessive mortality as signs of severe suffering) that would compromise the interpretation of co-occurring reproductive effects.*" In addition, a recent publication advocated that the 10% decrease in body weight gain currently used as a criterion for dose adequacy should be universally replaced in test guidelines with a 10% decrease in body weight. In response, the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) gathered a group of experts to evaluate this advice on dose selection and its potential impact on study outcomes and interpretation [1]. Recommendations on dose level selection in existing Organization for Economic Cooperation and Development (OECD) test guidelines and guidance documents were evaluated forcompatibility with the new dose selection advice and proposals. Data on representative DART guideline studies were analysed to determine the impact of a 10% decrease in maternal body weight during pregnancy. Other factors (not related to body weight) that should be considered when selecting high dose levels for DART studies were also reviewed.

The dose selection advice from ECHA is not in line with guidance given by OECD test guidelines. Data analysis indicated that a 10% decrease in maternal body weight during gestation equates to an approximately 25% decrease in body weight gain; this exceeds the existing consensus recommendation of DART experts (from a 2010 ILSI/HESI workshop). Further review of DART studies indicates that high dose selection can be based on other factors such as maternal clinical signs of toxicity, food consumption/nutritional intake, clinical chemistry parameters, circulatory/cardiovascular changes, target organ toxicity, maternal stress and toxicokinetics.

Excessive dose levels that cause frank toxicity and overwhelm homeostasis in pregnant animals have the potential to cause secondary effects on reproduction that are not relevant for hazard characterization or human health risk assessment. Dose level selection should use a biological holistic approach considering all available data and the complexity of the maternal-placental-fetal model.

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P20-05
Polymer REACh on the horizon: technical challenges with polymer materials in several OECD test guideline methods addressing effects on human health and on biotic systems

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Background and Purpose: As part of the European Green Deal and Chemical Strategy for Sustainability REACH registration will be extended to polymers likely including data requirements for analytical, ecotoxicological and toxicological data largely based on existing OECD test guidelines (TGs). Typically, these TGs were validated based on small molecules and their technical applicability as well as predictive capacity is generally not comprehensively evaluated for the complete chemical universe or all types of products including polymers. In contrast to small molecules, however, polymers are inherently characterized by distribution of chain lengths, chemical compositions, endgroups, or architectures. Therefore, the technical applicability of the existing TGs on polymers is potentially limited and involves the risk of generating unreliable data impacting risk assessments.

Methods: Using a set of 17 polymer materials we assessed the technical applicability of several OECD TG methods including aqueous (OECD TG 202, daphnia sp. acute immobilization test), cell-culture based (OECD TG 487, *in vitro* mammalian cell micronucleus test) and reconstructed tissue (OECD TG 439, *in vitro* skin irritation: reconstructed human epidermis test method) based test systems. In addition, we evaluate the technical feasibility of testing extracts (e.g., in analogy to

biological evaluation of medical devices DIN EN ISO 10993). Extracts of 7 polymer materials were prepared and applied in a basal cytotoxicity assay, in an estrogen receptor transcriptional activation assay (OECD TG 455), cell micronucleus test (OECD TG 487) and skin irritation test (OECD TG 439).

Results: Some test material such as polymer materials in the physical form of waxes and foams were difficult to be assessed in most test systems following the respective OECD TGs without special sample preparation. As expected, testing on reconstructed tissue generally presented the least challenging while the daphnia test highly relying on aqueous solubility which was problematic for the majority of polymer materials. Adapting the test protocols including an extraction step similar to medical devices testing facilitated testing in all of the selected test systems.

Conclusion: The development of standardized methods for analytical, ecotoxicological and toxicological evaluations is essential not only for complex test materials such as polymers. We present here challenges applying existing (eco-)toxicity test guidelines for polymers and propose alternative approaches potentially more suitable for polymers.

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P20-06
The role of toxicologists in the Safe and Sustainable by Design framework

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In 2019, the European Commission presented the European Green Deal, which includes the Chemicals Strategy for Sustainability (CSS). The CSS has the goals to improve the protection of citizens and the environment and boost innovation for safe and sustainable chemicals. In line with these goals, the European Commission announced the 'safe and sustainable by design (SSbD)' framework in 2022, a voluntary approach to standardize the innovation process for chemicals and materials.

The SSbD framework consists of 1) a (re)design phase and 2) a safety and sustainability assessment. The design phase is based on an iterative process alternating between stages and gates. Specific criteria have to be fulfilled to pass the gate and enter the following stage. Furthermore, the innovation process is divided in so called technology readiness levels (TRLs) covering the early concept stage (TRL 1) up to an operational environment (TRL 9).

The safety and sustainability assessment, which accompanies the innovation process is divided into five steps, which can be addressed in parallel:

1. Hazard assessment of chemical/material
2. Human health and safety aspects in the chemical/material production and processing phase
3. Human health and environmental aspects in the final application phase
4. Environmental sustainability assessment
5. Social and economic sustainability assessment
6. The (eco)toxicologist will be involved in the first three steps, which require a good knowledge of existing CLP and REACH regulations for the evaluation of hazards and exposure. However, the framework also fosters the use of New Approach Methods (NAMs) to fill data gaps.

The Horizon Europe framework programme is the EU's key funding programme for research and innovation. As requested by the respective calls, many recently funded projects include work packages to apply and test the SSbD framework for the identification of safer and more sustainable alternatives of existing chemicals or to identify safe and sustainable new chemicals. In the following two projects, Luxembourg Institute of Science and Technology (LIST) will be involved in the application of the SSbD framework:

Zero F: development of coating alternatives to replace PFAS compounds in food packaging and upholstery textile value chains. (https://www.zerof.eu/index.php)

SuperBark (Safe, sustainable and high-performance adhesives and coatings): Development of safe, sustainable and high-performance >95% bio-based adhesives and coatings from industrial softwood bark (side stream of forest industry). (https://superbark.eu/)

The SSbD framework is currently being tested through case studies, which provides a unique opportunity for toxicologists to actively develop further and to refine the safety aspects of the framework.

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P20-07
New data indicate high concern for reproductive toxicity of silver and the silver ion

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Introduction: Silver and its salts have been used for centuries in a variety of different products e.g. cosmetics, food supplements, food additives, textiles, medicines, medical devices, biocidal products e.g. surface treatments and surface coatings.

Although systemic uptake of silver from medicines, food supplements and medical devices has been reported, this has generally been considered to be without any adverse consequences to health. Until now, the US EPA, FDA and WHO have considered the critical effect to be a blue-greyish discoloration of the skin and eyes (known as argyria) that has been observed after long-term exposure and the following accumulation of insoluble silver selenide and silver sulfide in the skin/eyes.

In 1991, the US EPA set an oral reference dose of 0.005 mg/kg bw/day to protect against argyria. The US EPA noted that this was a cosmetic effect and not an adverse effect itself and the calculated value was considered of low reliability due to the poor quality of the data.

New data on silver: In connection with a recent evaluation of silver-containing biocidal substances in the EU, the Swedish authority has considered a body of new animal experimental data on silver, and in 2020 and 2023 Sweden made EU classification proposals for metallic silver and silver nitrate based on these data.

After discussion in the Risk Assessment Committee (RAC) at ECHA, the following human health classification for metallic silver was concluded:

Repr. 2, H361f
STOT RE 2 H373 (nervous system)

For silver nitrate, Sweden now proposes the following human health classification (not yet discussed by RAC):

Repr. 1B H360FD
STOT RE 2 H373
Acute Tox 2 H300
Skin Corr. 1A H314
Skin sens. 1 H317
Muta. 2 H341

Regulatory impact and proposal for new reference values

The classification as *Repr. 1B H360FD* is especially noteworthy as this may imply very strict downstream regulation of the use of silver and silver salts in the future. This also indicates concern and the need for limiting exposure to silver ions liberated from articles e.g. surface coatings, textiles, medical devices, etc.

Now, oral reproductive studies in rats should be considered as the most critical studies for the assessment of the Ag+-ion. In these studies, developmental neurotoxicity (adverse histopathological effects in the brain of offspring) and adverse effects on fertility occurred at exposure levels down to 25 mg Ag^{+}-ion/kg bw/day.

From these studies we identified relevant NOAELs as PoD for calculating updated tolerable exposure levels (TE) for consumers according to the REACH regulation and the ISO 10993-17:2023 standard for medical devices:

TE (oral sub-chronic exposure): 0.025 mg/kg bw/day

TE (oral chronic exposure): 0.0046 mg/kg bw/day

Based on an oral absorption rate of 5%:

TE (systemic sub-chronic exposure): 0.0013 mg/kg bw/day

TE (systemic chronic exposure): 0.0002 mg/kg bw/day

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P20-08
The assessment of a potential endocrine activity of Geraniol and its challenges due to missing guidance

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Geraniol is a naturally occurring monoterpene alcohol which can be found in numerous plant-derived essential oils. Due to its various applications as rose and floral scent for consumer applications, (e.g. flavour, repellent, fungicide etc.), Geraniol falls under REACH (EC 1907/2006), Cosmetics (EC 1223/2009), Flavour (EC 1334/2008), Biocides (EC 528/2012), and the Pesticides (EC 1107/2009) regulation.

As an active biocidal ingredient, currently Geraniol is subject to an "Endocrine Disruption" (ED) assessment. The expert group for ED within ECHA concluded "*…that there was no evidence that the substance had an EAS MoA. The experts considered that thyroid histopathology related observations could be considered adverse and that a thyroid disrupting MoA (other than secondary to liver toxicity) should be further investigated.*"

The aforementioned thyroid effects have been observed in a recent "Extended One-Generation Reproductive Toxicity Study" (EOGRTS) conducted jointly under REACH and BPR. At very high doses, effects on thyroid stimulating hormone (TSH) levels, thyroid weight, and histopathology were observed – likely secondary due to liver enzyme induction. Available publications and studies have ruled out a direct effect of the parent compound Geraniol, as no inhibition of DIO, TPO, NIS, and TTR was observed.

To further substantiate this, the ECHA/EFSA Guidance (2018, Appendix A) was followed to clarify the potential ED concern. In a specifically designed repeated dose *in vivo* study in rats, assessing the secondary thyroid effects of Geraniol via enzyme induction in the liver, it was demonstrated that increased liver weights at high doses correlated with minimal centrilobular hypertrophy. Importantly, statistically significantly decreased T4 concentration in plasma, correlated with liver enzyme induction and increased T4 glucuronidation activity. Minimal multifocal hypertrophy of the follicular cells of the thyroid were considered to be secondary to hepatic enzyme induction. Finally, acomparative study of enzyme activity in rat versus human liver cell *in vitro* systems demonstrated that Geraniol displayed a higher T4 clearance (via UGT) in rat hepatocytes compared to human hepatocytes. Hence, Geraniol T4-UGT activity changes seen in rats are considered not relevant to humans, especially as also a direct MoA could be excluded in a weight-of-evidence approach.

ED data requirements and assessments are already in place for EU biocide and pesticide regulations. Recently, ED has been implemented within the EU CLP directive but the CLP guidance on ED criteria is still under development and respective REACH data requirements have not yet been defined. Here we report specifically on our assessment of the endocrine effects of Geraniol as non relevant to humans, and generally on the challenges REACH registrants are facing in the absence of a guidance on the interpretation of such secondary-mediated liver effects on the T-modality.

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P20-09
Evaluating the consistency of judgments derived through both *in silico* and expert application of the Cramer Classification Scheme

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Since its formulation in 1978, the Cramer classification scheme has emerged as one of the most extensively-adopted predictive toxicology tools – owing in part to its employment for purposes of chemical categorisation within the performance of threshold of toxicological concern (TTC) safety evaluation. The characteristics of several of its 33 rules have contributed to the appearance of inconsistencies in relation to degree of hazard (TTC class) attributed to common (particularly food-relevant) substances. We have now investigated the manifestation and origins of these misclassifications, in order to raise awareness of such issues amongst users seeking to apply and adapt the rule-set.

A dataset consisting of over 3,000 compounds was assembled, each accompanied by Cramer class assignments issued by up to four groups of experts. These were complemented by corresponding assignments from *in silico* implementations of the scheme present within Toxtree and OECD QSAR Toolbox software. Consistency between each set of assignments was assessed – revealing that although the extent of inter-expert agreement was very high (≥97%), general concordance between these and *in silico* calls was more modest (~70%). A total of 22 chemical groups were identified as responsible for notable sources of disagreement. In several instances, this discordance could be directly attributable to inconsistencies related to the programming of explicit, structural rules. It is anticipated that this problem should be readily addressable within future releases. The handling of questions necessitating subjective answers (i.e., characterisation of common food components), however is a more substantial issue – with the look-up lists present within the software seemingly inadequate for the task.

It seems desirable that future iterations should rely exclusively upon structural criteria. Analysis was therefore extended so that it incorpo-

rated the approach of an existing scheme constructed along these principles – namely, the 2018 "Revised Cramer Decision Tree" (present within Toxtree). Whilst issues with respect to implementation of certain aspects were identified, a lack of existing documentation meant that its general merits were challenging to assess. Numerous insights into its form and operation were, nevertheless, acquired.

Both the original Cramer 1978 publication, and its subsequent revisions, represent pivotal advancements in the risk assessment of data-poor substances. The *in silico* implementation has offered risk assessors significant advancement in the accessibility of such approaches. As with all models or expert-issued judgements, these are not infallible, and will very likely be subject to continuous evolution. It is apparent that a more complete knowledge of their performance and of relevant structure-activity relationships will be essential in underpinning such progress.

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P20-10
Embracing regulatory compliance: Genetox meets generation SEND

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Purpose: The Standard for the Exchange of Nonclinical Data (SEND) is an implementation of the Study Data Tabulation Model for nonclinical studies that enables the U.S. Food and Drug Administration (FDA) to modernize and streamline the review process. SEND specifies a way to present nonclinical data in a standardized format. This specific data format is well-established and, as a result, patients may benefit from speedier approval of new drugs. The amount of SEND-format data required by the FDA has been steadily growing since 2016, with single-dose and repeat-dose general toxicology, carcinogenicity studies, safety pharmacology studies, and embryo-fetal development studies all being mandated by the FDA. In Spring 2025 this expansion will include *in vivo* genetic toxicology data when SENDIG-Genetox v1.0 becomes mandatory.

SEND implementation and compliance can be challenging and requires effective co-operation between pharmaceutical companies, industry partners and contract research organizations. Here, we examine the upcoming changes and describe how the industry can collaboratively approach *in vivo* genetic toxicology studies utilizing best practices and ensuring regulatory compliance.

Methods: SENDIG-Genetox v1.0 focuses primarily on *in vivo* micronucleus and comet assay data, streamlining these findings into a single domain within the broader SEND framework. This version offers a simplified approach, aligning with the established standards of SEND v3.1.1. The scope of this newly added domain is concise, however, analysis to enhance clarity and usability is relevant.

By explaining the standard-defined categories, sub-categories and tests, we will provide best practices and practical guidance on how to implement the standard. We will showcase enhancements to established genetox data collection tools and demonstrate how pharmaceutical laboratories can adapt current practices to enable adherence to this guideline. A case study of data transformation for compliance, featuring publicly available data, will be presented.

Results: These advancements in SEND regulations signal a broader shift towards standardized nonclinical data utilization beyond mere submissions. By embracing these changes, and looking forward to upcoming genetox SEND requirements, such as unscheduled DNA synthesis and chromosome aberrations, the scientific community can unlock additional value from SEND datasets, driving innovation and insight. The forthcoming requirement for SENDIG-Genetox v1.0 underscores the importance of adaptation and collaboration within the industry. As we move forward, these regulatory updates pave the way for a more streamlined and impactful approach to genetic toxicology studies, ultimately advancing our understanding of compound safety and efficacy.

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P20-11
EU project REMADYL – hazard and risk assessment in the removal of legacy substances from polyvinylchloride (PVC)

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REMADYL aims to develop a process that allows to recycle 'old PVC' and rejuvenating it into market competitive high purity PVC. The removal of the legacy substances (LS), namely short-chained phthalates and lead compounds, are therefore the main goals of the REMADYL process.

The use of short-chained phthalates in the EU is restricted according to COMMISSION REGULATION No 552/2009, Annex XVII and for lead recently, in May 2023, the EU issued Regulation 2023/293 to revise the restriction of lead and its compounds. Rejuvenating 'old' PVC comprises the removal of the LS and subsequent blending of the recovered PVC with virgin PVC. It complies with the current legislations and fulfils the customer's growing demand to use safe and sustainable-by-design recycled materials.

The project work comprises assessing REMADYL by-products, either purposefully introduced to aid removal of legacy substances, legacy substances itself and other substances, originating from 'old PVC', which might be removed.

The hazard assessment of the components was followed by substance and process specific exposure assessment in order to perform a process specific risk assessment.

The conducted exposure assessment included the gathering of detailed information on the involved processing steps e.g. open vs closed processes, exposure points, exposure duration, estimated exposure concentration etc.) and physico-chemical properties of the substance in question itself but also peripheral parameters such as e.g. its mass fraction in 'old-PVC'. The exposure assessment was mainly carried out using the CHEmical Safety Assessment and Reporting tool (Chesar). The exposure to given substances for several tasks within the process was compared to known limit values resulting in risk characterization ratios (RCR).

Tier 1 exposure estimates were calculated in a conservative approach, assuming a duration of 8 hours for the task, normal room ventilation (ACH: 3) and standard gloves with 80% protection level. In the case of an RCR below 1, safe use for the worker can be assumed. In cases of RCR values >1 adequate risk mitigation measures need to be applied (tier 2).

The outcome of the risk assessment for different REMADYL processes will be summarized and discussed.

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P20-12
Tiered approach for nanoparticle characterization and assessment in foodstuff

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The risk assessment of nanosized material is crucial in authorising and approving a new food ingredient (e.g., novel foods) intended to be placed in the EU market. The two recently published EFSA guidelines describe an exposure-driven approach aimed at evaluating nanoparticles' presence and toxicity potential for ensuring consumer safety.

Nanoparticle assessment is a mandatory transversal step of each application related to the food area. The guidelines refer to the engineered, intentionally produced nanoparticles (i.e., nanosized food ingredients with improved bioavailability) and food ingredients containing an unintentional fraction of nanoparticles.

The assessment starts with a chemical characterisation of the food product, considering its intended use and the content of nanosized particles. Quantifying and characterising the nanoparticle fraction can help to rule out the possibility that nanoparticles elicit a biological reaction. On the other hand, where the exposure to nanoparticles cannot be waived or is intentional, the conventional *in vitro* and *in vivo* studies must be adapted to investigate toxicokinetic and toxicodynamic behaviour of the nano-fraction and their potential effects on consumers.This poster intends to provide definitions and practical strategies for assessing nanoparticle risk in new food substances. It will also give an overview of the requirements and recommended methods to support stakeholders in future applications of new ingredients.

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P20-13
Dyes under the reach regulation: a strategy for genotoxicity assessment using NAMs

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Background and Objectives: REACH Regulation (EC) 1907/2006 requires to conduct *in vivo* tests to further assess the mutagenicity potential of chemical(s), when at least one of the *in vitro* tests of the standard battery requested at the Annex VII and VIII level displays a positive result. Organic dyes are a special and vast class of chemicals very often showing positivity when tested in in-vitro mutagenicity test in bacteria, which triggers the performance of a large number of in-vivo tests. It is therefore necessary to develop and use New Approach Methodologies (NAMs) that can be accepted at regulatory level providing reliable and relevant results.

Material and Methods: In silico methodologies, such as Quantitative Structure-Activity Relationship (QSAR) modelling, grouping of chemicals and read across approach are considered reliable alternatives to reduce *in vivo* testing. The present work explores the features and functionalities of an in-silico system, developed on a massive proprietary database of *in vitro* and *in vivo* genotoxicity studies conducted on organic dyes.

Results: The aim of this in silico system is to group similar dye substances based on structural alerts and similarities, and to select tailored samples of each group of substances, considering both expert and statistical criteria, with the ultimate scope of applying the read-across approach in a Weight of Evidence (WoE) tiered strategy for the genotoxicity assessment of dyes.

Discussion and Conclusion: The *in vivo* results can then be used to improve the system and gain insight on the relationship between specific structural features and the *in vivo* genotoxicity of dyes.

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P20-14
Considerations for revising a safe intake of propylene glycol

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Propylene glycol (PG) is used as an additive in food, medications, and other products. Some dietary estimates of PG exposure approach or exceed the current Acceptable Daily Intake (ADI) of PG. In 1974, JECFA established an ADI of 25 mg/kg bw-day, which EFSA reaffirmed in 2018. The ADI for PG is based on a 2-year dietary rat study that reported a no observed adverse effect level (NOAEL) of 2,500 mg/kg bw-day. A total uncertainty factor of 100 was applied to this POD to derive the ADI. However, the available toxicology studies and human data indicate a plausible mode of action (MoA) that could support reducing the total uncertainty factor. We conducted a literature search for PG toxicology information, including studies of toxicokinetics and toxicodynamics in humans, animals, and *in vitro* with the objective to assess support for the application of chemical-specific adjustment factors (CSAFs) for inter- and intra-species toxicodynamics and toxicokinetics. Based on our analysis, human studies were not appropriate to serve as a POD due to several limitations (*e.g.*, pre-existing conditions, co-exposures, and short-term, intravenous dosing). Therefore, we used the same NOAEL in rats that was used by JECFA and EFSA. We also found that there was not enough information on toxicokinetics to derive a quantitative CSAF. However, the available toxicology studies and human data indicated a plausible mode of action (MoA), which involved an increase in serum PG concentrations after metabolic saturation, resulting in serum hyperosmolarity and hemolytic changes. Further, the available data indicated that the toxicodynamics of the hemolytic response is expected to be similar in humans compared to relevant test species (*i.e.*, rats and dogs). Thus, the data supported a chemical-specific adjustment factor (CSAF) of 1 for interspecies toxicodynamic differences. When applied to the derivation of an ADI for PG, the CSAF of 1 would reduce the total uncertainty factor from 100 to 40, resulting in a revised ADI of 62.5 mg/kg bw-day. Our revised ADI for PG is supported by human clinical data, which indicate that 62.5 mg/kg bw-day PG is protective for both children and adults. Moreover, the

reduction of the toxicodynamic portion of the interspecies adjustment factor to 1.0 for PG is consistent with agency assessments in which a UF of 1.0 was applied when a plausible MoA exists and the toxicological data indicated similar toxicodynamics across test species.

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P20-15
Risk assessment of Endocrine-Disrupting chemicals: threshold or not and what about mixtures?

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Introduction: There is an ongoing discussion in the EU regarding the risk assessment of Endocrine Disruptor (ED) substances. Two distinct approaches are being considered: the DNEL Approach (Threshold Approach) and the DMEL Approach (Non-Threshold Approach). A Mixed Assessment Factor (MAF factor) has been proposed to revise the REACH regulation. The MAF factor aims to protect against the combined effects of mixed exposure (such as exposure to multiple chemicals simultaneously).

Methodology: In the present project, exposure estimations from various sources (food, FCM, and medicinal products) were made for six substances considered endocrine disruptors (BHA, BHT, propylparaben, butylparaben, BPA, D4). Also, for each substance, DNEL and DMEL values were derived both with and without using a MAF factor, and using these different values, the corresponding RCR values were calculated from the exposure estimates.

Tolerable exposure levels (DNEL and DMEL values) were derived for each substance using the same "point of departure" for the ED effects. For the DMEL determination, an additional "Large assessment factor" approach was applied using a further assessment factor of 10.

For MAF, an additional factor of 10 was used. This choice was made based on an analysis by the Swedish Chemicals Agency (KEMI PM 8/21) that considered a MAF of 10 to be sufficient to protect against most realistic mixture scenarios.

Results: Based on the identified PoD for the various ED- effects of the substances, the following DNEL and DMEL values were derived *without/with* a MAF factor.

TABLE 1. *DNELs and DMELs for endocrine disrupting effects for T modality (DNELthyr and DMELthyr) and EAS modalities (DNELeas and DMELeas). Without MAF/with MAF. (µg/kg bw/day)*

	D4	BHA	BHT	BPA	Butyl-paraben	Propyl-paraben
$DNEL_{thyr}$	Not relevant	1000/100	250/25	Not evaluated	Not relevant	Not relevant
$DNEL_{thyr}$	Not relevant	100/10	25/2.5	Not evaluated	Not relevant	Not relevant
$DNEL_{eas}$	36/3.6	100/10	Not relevant	0.24/0.024	20/2	20/2
$DMEL_{eas}$	3.6/0.36	10/1	Not relevant	0.024/0.0024	2/0.2	2/0.2

Conclusion: Calculation of DNELs and DMELs used in the risk assessment in this project showed a risk of endocrine disrupting effects that will occur in more exposure scenarios when a DMEL compared to a DNEL approach is used. Also, it was considered appropriate to use MAF, as the extent of single-acting substances and the contribution from different sources has yet to be discovered. Balancing these approaches, threshold and the MAF factor, is crucial for safeguarding public health and addressing potential cocktail effects from mixed exposure in the context of endocrine disruption.

Exposure estimations/RCR values will be in the poster.

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P20-16
The unwritten expectations from FDA on biomedical device testing

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The recent introduction of the eSTAR program by FDA has triggered some discussions about the evidence needed in the process of assessing the biocompatibility of the biomedical devices, since it uncovered new requirements on the evaluation process. Since the end of 2023, FDA considers invalid some biocompatibility tests, despite performed with the same protocol on equivalent devices as other tests, previously accepted and approved by FDA. The new FDA expectations are not within the typical laboratory practices and are not indicated in the Guidance for Industry on ISO 10993-1, issued on September 8, 2023.

The aim of this poster is to share and comments some examples which are based on real notifications from FDA to various Biological Evaluation Report.

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P20-17
GARDskin as a drop-in replacement KE3-method in OECD GD 497 defined approaches

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Recent developments in the regulatory field of skin sensitization assessment have led to the formal acceptance of defined approaches (DA) and integrated testing strategies (ITS), described in the OECD guidance document 497 (GD 497). However, the current implementation of the data interpretation procedures (DIP) only applies to specific assays. Efforts have since been made to soften the definitions of the DIPs from the current assay-specific versions to allow for the use of equivalent validated assays included in the relevant test guidelines (TG) 442C-E.

The GARDskin assay is an *in vitro* test method for hazard assessment of skin sensitizers. It is currently described in the OECD TG 442E, making it an assay relevant for potential incorporation into the DIPs as a monitor for Key Event (KE) 3 in the OECD Adverse Outcome Pathway (AOP) for skin sensitization. However, small adaptations have been warranted to fit into existing frameworks of the DA and the ITSs of GD 497, which requires that a borderline range (BR) is defined for the binary classifications and that a procedure for deriving a potency score is described.

The main readout of the GARDskin assay, on which the binary hazard classification is made, is the SVM decision value (DV). It is a numerical value summarizing induced gene expression levels for a straightforward decision of hazard properties, where the sign of the mean DV decides the classification outcome. The size of the BR was determined from the variability of repeated assessments in the GARDskin's ring trial data. The BR was defined to be between DVs −0.450 and +0.450. The application of the BR showed a slight performance increase among conclusive classifications. Incorporation of the GARDskin assay as the KE3 component in the DA showed maintained performance of the DA at large, compared with the standard configuration, and an increased performance in specific chemical domains, such as for hydrophobic compounds.

A procedure for deriving a potency score compatible with the ITSs in GD 497 was also defined for results of the GARDskin assay. The scoring system was based on the binary hazard classification and on the exposure concentration, as follows: A chemical classified as non-sensitizer obtains a score of 0. A chemical classified as skin sensitizer receives a score between 1 and 3 depending on the exposure concentration, employing thresholds of 56.44 µg/ml and 13.03 µg/ml. With the scoring system, an ITS containing the GARDskin assay as the KE3 component achieved classification performances in line with the current implementation of the ITS.

To conclude, the GARDskin assay was adapted for compatibility with the DA and the ITSs described in GD 497. The incorporation of the GARDskin assay as a component in either DIP produced performances comparable with current versions, or with slight improvements in certain chemical domains, suggesting that the GARDskin assay could act as a relevant component in either DIP.

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P20-18
Method development optimization for intravesical installation in the rat

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Bladder cancer is one of the 10th most common cancers globally and ranks 13th in terms of deaths with approximately 550,000 new cases annually. Intravesical instillation therapy is an effective alternative treatment in the treatment of such cancers. Preclinical evaluation of these therapies requires intravesical installation. This is not a straightforward route of administration, and the following describes the optimization of the instillation to enable the successful evaluation of these drugs. By using anesthesia isoflurane, it was possible to extend the formulation dwelling time to 1.5 hours. To refine the method, the following three measures were taken: (1) the 18 Gauge endovenous catheter was replaced with 22 Gauge sterile plastic feeding tube (22 gavage× 25 mm) to allow a more smooth insertion into the urethra; (2) a heparin cap was connected with the needle to avoid the leakage of infused formulation when the syringe was removed from the infusion needle; (3) a custom-made rat stretcher (28 cm×10 cm) was used to avoid the indwelling needle falling from the bladder when the anesthetized rat was transferred to the isoflurane anesthesia chamber. During the 1.5 hours anesthesia period, the animals were closely monitored until removal 1.5 hours post dose. At recovery the needle fell out from bladder along with urine with spontaneous micturition. Procedure-related findings included transient red urine discoloration noted on the first 3 weekly dosing days, increased incidence in urine occult blood, protein and red blood cells(Table 1) and microscopic findings of minimal or mild multifocal mixed infiltration in the lamina propria and urothelium of pelvis of the unilateral or bilateral kidney, minimal or mild multifocal neutrophilic infiltration in the mucosa of the urinary bladder, minimal multifocal mixed/neutrophilic infiltration and focal hemorrhage in the mucosa, and minimal increased mitotic figures in the urothelium of the urethra in the dosing phase. There were no in life changes, or organ weight and macroscopic changes observed throughout the dosing and recovery phases. Indicating that with these method refinements intravesical instillation is considered suitable for the evaluation of drugs using this route of administration.

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P20-19
Technology-enabled approval acceleration: spotlighting the ICH S1B weight of evidence

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The ICH S1B carcinogenicity testing guideline has been recently revised with a novel addendum describing a comprehensive integrated Weight of Evidence (WoE) approach to determine if a 2-year rat carcinogenicity study adds value when assessing human carcinogenic potential.

Here, we investigate how artificial intelligence (AI) approaches are applied to the six WoE factors within the guidance. We showcase best practices for including technology-enabled, big-data fueled and augmented intelligence platforms in this dynamic and novel WoE framework.

The six WoE factors are: 1. Target Biology, 2. Secondary Pharmacology, 3. Histopathology, 4. Hormonal Perturbation, 5. Genetic Toxicology and 6. Immune Modulation.

The six factors are unique and consider substantially different data. Therefore, the ability to incorporate AI practices varies between each factor. For example, in certain cases where an S1B-like assessment is conducted (e.g., peptides), in silico methods may support the genotoxicity assessment (Factor 5). These tools can predict the likelihood that a compound, based on its structure, will be genotoxic. Similarly, regarding Factor 1: Target Biology, AI is employed to mine databases and retrieve critical information to support the carcinogenicity assessment. Conversely, 4 & 6: Hormonal Perturbation and Immune Modulation are areas with minimal AI or ML (machine learning) appropriate technology developed to support their evaluation within the WoE integrated assessment.

The impact of AI and technology-enabled approaches within the pharma industry is a "hot topic" aiming to reduce time to market and increases confidence in decision making. However, their use and application should be carefully considered and implemented within the specific framework of application.

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P20-20

Retrospective analysis of thyroid hormone measurements from 72 extended-one generation reproductive toxicity studies

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Background: The introduction of the new hazard classes in the CLP Regulation (EC) No. 1272/2008allows classification as endocrine disruptor for human health or the environment based on e.g., thyroid toxicity data. Hence, it is important to assess the quality and reliability of the thyroid hormones data generated in standard regulatory studies.

Objective: The aim of the current work was to conduct a retrospective analysis of the performance of thyroid hormones measurements from extended one-generation reproductive toxicity studies (EOGRTS; OECD TG 443).

Methods: Methodology details and the individual values for thyroxin (T4) and thyroid stimulation hormone (TSH) were extracted from 72 full study reports of EOGRTS. Descriptive statistics, calculation of the coefficient of variation (CV), and the number of values below the limit of quantification (LOQ) were performed.

Results: Thyroid hormones were reported for adults in a majority of the studies. For pups, the results for T4 were reported for F1 in 76% (63/72) of the studies. In studies, which included the F2 generation until post-natal day (PND) 22, T4 was only reported only in 8% (2/23) of the studies.

The T4 and TSH levels in control groups were dependent on age and sex. Adult males had on average 40% higher T4 levels compared to adult females. In pups, at PND 22, mean T4 levels were similar between males and females. Similar pattern was observed for TSH.

The enzyme immunoassay was the most used method for T4 and TSH detection (EIA, n=37 for T4 and n=33 for TSH respectively). Other methods included liquid chromatography-tandem mass spectrometry (LC-MS/MS, n=26) for T4 and radioimmunoassay (RIA) for TSH (n=16) and T4 (n=1). Information, on the analytical method used was missing in 17% of the study reports.

Use of EIAs resulted in higher levels of variation (2–98% for T4 and 17–197% for TSH), compared to LC-MS/MS (7–55% for T4) or RIA (7–179% for TSH). The OECD TG 443 does not specify the acceptable level of Coefficient of Variation; however, the OECD TG 407 does specify the acceptable levels, i.e., 25% for T4 and 35% for TSH.

Furthermore, a high number of TSH values below LOQ was reported for EIA (up to 13%); this was independent of animals' age.

Conclusions: Thyroid hormones are not always measured when required by the OECD TG 443. In addition, it is concerning that thyroid hormone measurements are commonly omitted in F2 and therefore does not allow comparable assessment between F1 and F2 generations.

Particularly for TSH, the analytical methods are not sensitive enough to detect changes in TSH similar to those in T4. In addition, the analytical method used often fail to detect low levels of TSH.

These requirements for performance and reporting of thyroid hormones should be clarified in the OECD TG 443 to facilitate full hazard evaluation of a chemical. This is essential for the independent analysis of the results of the hormone measurement by the risk assessor.

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P20-21

Toxicological testing strategy of a series of gas oils substances under REACH

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Gas oils represent a diverse array of refined (predominantly) C10-C25 hydrocarbon UVCB (unknown or variable composition, complex reaction products or biological materials) substances that are mostly used as diesel fuel and residential heating oil in Europe. Importantly, substance samples of these categories exist within a defined hydrocarbon space, but the quantity of constituents within this space varies significantly between samples. Concawe, the scientific branch of the European Fuels Manufacturing Association, has developed a testing strategy for further assessing the hazard profile and REACH information requirements of 14 registered gas oil (GO) substances from three refining process-defined Categories(other gas oils (OGO), vacuum hydrocracked gas oils (VHGO), and straight-run gas oils (SRGO)), representing over 350 million tons of annual production or import across the European Economic Area. In order to Refine and Reduce *in vivo* toxicity testing required as part of REACH (3Rs), and given the inherent complexity of these substances, chemical grouping and read-across based testing strategies are critical toward an efficient and thorough safety assessment.

Using multiple structural-compositional analyses, anticipated worst-case-in-category samples coupled with samples to best cover the diverse GO hydrocarbon space, of each GO category were identified and selected to be individually studied. The hypothesized worst-case samples selected for each category contained at least 3% polycyclic (three or more ring) aromatic compounds (PACs), a level associated with multiple toxic outcomes in previous studies, as determined by PAC-2 analysis. As part of the testing strategy, *in vivo* bridging studies were conducted according to the Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test protocol (OECD TG 422) in rats by the dietary route of exposure, to inform future higher-tier studies.Supporting our hypothesis, results from the OECD TG 422 studies showed a strong association between the observed repeated dose toxicity and/or developmental toxicity with GO substances with higher levels of PACs. Toxicological observations included target organ effects and developmental toxicity. On the other hand, preliminary examination of test sample compositional information (e.g., paraffinic, naphthenic, and degree of alkylation; as determined by 2-dimensional gas chromatography) had no substantial correlation with observed toxicity. Multiple biological samples (*e.g.*, plasma, urine, liver, etc.) were also collected from these studies for use in New Approach Methodologies (NAMs) to build a robust *in vivo* data repository. This will facilitate further evaluation of substance grouping and the application of read-across, with the potential to refine future testing strategies and reduce the number of animals needed for subsequent toxicological studies, including other petroleum substances.

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P20-22
Endocrine disruptor potential assessment: a fit-for-purpose approach to petroleum UVCB substances

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As part of the European Union's (EU) Classification and Labelling regulation (CLP), new hazard classes are being introduced. Notably, these classes include categories for endocrine disruptors (EDs) affecting both human health and the environment. This new hazard class emphasizes the concept of "One Health", based on the idea that the health of ecosystems is inseparable from human and animal health. Moreover, the next update of the EU Registration, Evaluation, Authorisation and Restriction of Chemicals regulation (REACH) is anticipated to include new requirements to identify ED. ED assessments are complex and data intensive, and multiple mechanisms of action, including effects which occur in the presence of general systemic toxicity, can affect the outcome. The draft CLP guidance for ED does not currently cover UVCB (unknown or variable composition, complex reaction products or biological materials) substances, but it is expected that data obtained from both whole substance and representative constituents will be used to conclude on ED classification. Petroleum substances (PS), complex hydrocarbon materials are UVCBs. They contain thousands to millions of constituents and their composition is variable due to their origin and the refining processes. Therefore, a fit-for-purpose approach for the ED assessment for PS is required.

Our project aims to describe an initial ED data gathering exercise for PS. Some whole substance *in vivo* data were available for mammals and screening data based on quantitative structure–activity relationship (QSAR) and *in vitro* ToxCast bioassays were gathered for 25 selected representative constituents covering eight hydrocarbon blocks. These constituents were chosen to cover a range of structures or carbon numbers within a hydrocarbon block, and were based on data availability and potential flags for ED. Many of the constituents were found to be outside the QSAR applicability domains of the methods. Moreover, despite there were *in vitro* data available for 17 constituents, only data for three were acceptable because analytical data could not adequately confirm the presence of the test item for 14 of them, most of which are volatile.

The acceptable *in vitro* data were useful for prioritising constituents for further investigation, with e.g., phenanthrene and fluoranthene both assigned higher priority. The other screening data were however of limited value, with no scope for ruling out ED activity or considering whether ED activity might differ within or between hydrocarbon blocks. Further work may be needed to develop more reliable QSAR models and *in vitro* data for hydrocarbons. However, given the difficulties with testing already observed in the available dataset, it is currently unknown whether the battery of proposed *in vitro* tests for ED that are expected to be introduced under REACH will be suitable for testing certain hydrocarbon constituents (e.g., volatiles) or whole petroleum substances.

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P20-23
Unlocking a new paradigm for pesticide toxicity assessment in India

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Authorities around the world rely on toxicity data to inform chemical evaluations and regulatory decision-making. Toxicity testing for pesticides in India and other countries, has conventionally relied on a checklist of *in vivo* tests to fulfil data requirements. However, there is now an opportunity for India to modernise its toxicity testing paradigm and harness reliable and relevant approaches such as *in vitro* and *in silico* methods that will better protect human health and the environment, and help India harmonise with other nations while reducing animal use.

This presentation will provide insight into the current regulatory landscape for pesticide testing in India and summarise updates made in the 2023 Central Insecticides Board and Registration Committee guidelines for the assessment of chemical pesticides and biopesticides, highlighting existing opportunities to use *in vitro* and *in silico* approaches, read-across, and waivers based on a weight-of-evidence (WoE) approach.

It will outline ways to address the challenges of adopting non-animal approaches in India, including 1) fostering acceptance of existing and new methods to ensure global harmonisation, 2) expanding the required testing infrastructure, 3) increasing scientific dialogue to clarify data requirements and how existing non-animal approaches can fulfil regulatory needs, and 4) providing training in the use and interpretation of data from non-animal approaches for regulatory decision-making. Instead of taking an animal-heavy check-box approach, India can learn from the experiences and best practices of other countries, including European nations and North America, through the application of WoE approaches, integrating information from use and exposure patterns, existing information and data from non-animal approaches to drive risk decisions to ensure tests on animals are conducted only as a last resort. To facilitate effective implementation, India could also 1) strengthen regulatory language to indicate preference for using data from non-animal approaches, 2) address duplicative toxicity testing requirements for pesticides already registered in India, and 3) engage in transparent discussions on the timely incorporation of new approaches in a regulatory context.

To define key next steps and monitor progress in incorporating cutting-edge, scientifically sound approaches for relevant and reliable toxicity assessment of pesticides in India, these solutions should be outlined in a roadmap. A clear roadmap and greater investment in developing and gaining confidence in the use of non-animal approaches would enable India to embrace a regulatory framework that relies on modern, effective science. As more global regions work towards reducing and replacing animal testing for regulatory purposes, including the assessment of pesticides, India should follow suit to ensure that best science that does not rely on animal testing is used to protect human health and the environment.

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P20-24
Challenges encountered during the initial stages of exposure assessment in REACH registrations

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The REACH regulation has been in force in the European Union since 2007, with the aim to ensure a high level of protection for human health and the environment. Registrants are responsible for the hazard, exposure, and risk assessment of their chemical substances, as well as for the communication of safe use conditions to their customers. Almost two decades since implementation, registrants however still face numerous challenges on the estimation of exposure to a given substance using Tier 1 tools such as Chesar. Exposure assessment is a stepwise process which consists of the collection of key input parameters, description of a lifecycle tree, definition of a strategy, and finally exposure estimation.

The initial stage of selecting key input parameters is critical to the outcome of the overall exposure assessment. Key input parameters affecting the human health and environmental exposure assessment include physico-chemical (PC) properties such as water solubility, octanol-water partition coefficient, vapour pressure and molecular weight, and environmental fate (E-fate) properties such as Henry's Law constant, biodegradation, bioaccumulation, and Log Koc. A scientifically sound rationale is required for the selection of these values, and this can be very challenging when faced with complex substances such as UVCBs (unknown or variable composition, complex reaction products or biological materials) composed of many constituents present at variable concentrations, and not all analytically quantifiable. This significantly affects the overall strategy for selection of the key PC and E-fate values for exposure assessment (i.e., maximum value, average value, value of the most representative constituent).

This poster presents several case studies with UVCB substances where an iterative process was adopted to refine the exposure estimates and conclude on safe conditions of use, within the framework of current European Chemical Agency (ECHA) guidance. Prior to starting an assessment, critical review of the substance's properties and intended uses helped the assessors define the scope. Not only the values for key endpoints but also how the data was generated (estimated vs. experimental, weighted average vs. range, read across, etc.) and the logic behind the selection of these values and their consistent application were part of this exercise, and defined the overall strategy for exposure assessment.Overall, the case studies reflect a tiered approach that should be applied to identify where additional resources should be targeted for the refinement of assessment approaches (e.g., use of new tools), further data generation or gathering (e.g., *in vitro* dermal absorption study, DT50 in sediment/soil), or the consideration of risk management activities (e.g., occupational or on-site treatment).

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P20-25
Reference neurotoxicants based on CLP harmonised classifications

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Purpose: With the current interest to decrease experimental animal testing for regulatory purposes, the need for reliable new approach methods (NAMs) has become evident. NAMs may currently be used for different regulatory purposes such as prioritisation, screening, specific adaptations of some standard information requirements, and/or in weight of evidence for hazard classification. To ensure the continued safe use of chemicals, a NAM (battery) should perform ideally at a comparable level of sensitivity and specificity as the admissible *in vivo* method, especially if they are to replace such *in vivo* tests for hazard and risk assessment. A key element of *in vitro* NAM development is validation, which requires the testing of reference chemicals. Furthermore, the use of a relevant list of reference substances, selected as per transparent criteria, also forms a cornerstone of developing *in silico* NAMs. To obtain an objective view on the predictive capacity of a NAM (battery), claims on sensitivity and specificity should be based on experimental results generated with reference chemicals that were previously scrutinised by independent expert panels on whether the substance has or has not the hazardous property. The validation of NAMs for neurotoxicity is currently a point of focus.

Methods: Within the EU regulatory landscape, the harmonised classifications by the Committee for Risk Assessment, based on transparent classification criteria in CLP Regulation (EC) No 1272/2008, creates the opportunity to objectively define lists of positive reference substances. Neurotoxicity that is not specifically addressed under developmental toxicity may be classified under specific target organ toxicity – single exposure (STOT SE) or – repeated exposure (STOT RE). We screened substances with harmonised classifications STOT SE 1: H370 (nervous system), STOT SE 2: H371 (nervous system), STOT SE 3: H336 (narcotic effects), STOT RE 1: H372 (nervous system), and STOT RE 2: H373 (nervous system) to generate a list of neurotoxicants which may be used for e.g. NAM development.

Results: The following number of harmonised classified substances were identified with nervous system as a target organ:

- STOT SE 1: 10
- STOT SE 2: 4
- STOT SE 3: 82
- STOT RE 1: 17
- STOT RE 2: 3

This list of substances includes chemicals that fall within the scope of REACH and active substances in biocidal and/or pesticidal products. It is important to note that this list is not exhaustive as its composition is subject to regulatory and scientific bias. As such, it should not be considered as a consensus list. Ideally, reference lists generated by other authorities or expert groups are combined with this list and reviewed by an (independent) expert panel to form an appropriate consensus list, which respects official hazard conclusions.

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P20-26
Re-evaluation of erythritol (E 968) as a food additive

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Purpose: This presentation aims at providing an overview on the safety assessment of erythritol (E 968) as a food additive [1]. Erythritol is a sugar alcohol used as a sweetener, obtained by fermentation of a carbohydrate source using non-genetically modified microorganisms. It is currently authorised in the EU in 66 different food categories. It is the first polyol that has recently been re-evaluated by EFSA according to Regulation (EC) No 1333/2008 [2] and Commission Regulation (EU) No 257/2010 [3]. The European Commission (EC) also asked EFSA to consider exempting erythritol from the laxative warning label requirement for foods with more than 10% added polyols as established

by Regulation (EU) No 1169/2011. Lately, emerging literature has suggested possible adverse effects from the long-term use of sweeteners such as cardiovascular diseases, type 2 diabetes mellitus, obesity.

Methods: The risk assessment was carried out by EFSA following structured protocols [4,5], one on the hazard identification and characterisation and one on exposure assessment. The scientific evidence to be assessed were gathered from several calls for data [6,7,8] as well as from studies retrieved in the literature, from January 2002 to September 2023. Dietary exposure to erythritol from its use as a food additive was estimated based on consumers-only, combining food consumption data available within the EFSA Comprehensive Database with the maximum levels according to Annex II to Regulation (EC) No 1333/2008 and both reported use levels and analytical data provided to EFSA following calls for data.

Results: Erythritol is readily, dose-dependently absorbed in humans and can be metabolised to erythronate to a small extent. It is then excreted unchanged in the urine. It does not raise concerns regarding genotoxicity. Based on human interventional data, the EFSA Food Additives and Flavouring (FAF) Panel considered appropriate to set a numerical acceptable daily intake (ADI) of 0.5 g/kg bw per day, which is protective for the immediate laxative effect as well as potential chronic effects, secondary to diarrhoea. The exposure assessment methodology was updated to particularly address this immediate laxative effect and an acute exposure assessment was performed. Both acute and chronic dietary exposure to erythritol (E 968) were above the ADI, indicating that individuals with high intake may be at risk of experiencing adverse effects after single and repeated exposure. The warning *'excessive consumption may produce laxative effects'* is still valid. Current evidence does not show a causal relationship between dietary exposure to erythritol (E 968) and cardiovascular disease risk, while fasting erythritol serum levels may be considered a biomarker of metabolic disturbances. Nevertheless, further research might be helpful to clarify the nature of the association found in some recent observational studies.

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P20-27
Zinc suphide – considerations and challenges for acute inhalation toxicity testing and classification under REACH

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Zinc sulphide is a widely used inorganic powder, and its production has reached quantities greater than 1000 t/year. Previous assessments using a read-across approach with other inorganic zinc substances have proven unreliable for predicting toxicity. Therefore, in accordance with OECD guideline 436, an acute inhalation test was implemented to provide more accurate data. This study is crucial for ensuring the safety of workers exposed to zinc sulphide dust and complying with regulatory requirements for occupational health.

Due to particle specific properties the maximum attainable concentration of zinc sulphide for an inhalation study was not certain. Two dry dispersion systems were used to aerosolize the zinc sulphide powder, and the generated aerosol was supplied to a nose-only inhalation exposure system. The chamber concentration was measured using an aerosol photometer and a glass fiber filter. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of the aerosol were also measured using a cascade impactor. The results showed a maximum attainable chamber concentration of 0.82 mg/L at an MMAD of 1.5 µm over a 4-hour exposure period. The test evaluated respiratory tract effects in rats, including various other endpoints. In the acute nose-only inhalation study all 6 rats showed no specific symptoms, a good general health status and survived post-exposure observation period up to 14 days. From the results observed the status Unclassified was derived according to GHS. Based on the experimental results, an LC50 was not determined but is considered higher than 0.82 mg/L (the maximum achievable concentration).

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P20-28
Toxicological effects caused by particles which exhibit no or very low intrinsic toxicity in inhalation toxicity studies: what are the challenges

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Particles, both natural and synthetically manufactured, that exhibit low or no intrinsic toxicity are commonly used in various daily products and may also be generated during industrial processes. Assessing the inhalation hazard of particles, particularly those with low intrinsic toxicity, can be challenging due to difficulties in the testing and interpretation of sub-acute and sub-chronic rat inhalation studies. To protect workers and consumers, manufacturers and leading scientists joined forces to understand the specificities of particles better and find a workable and more focused approach under the umbrella of "The Particles Platform".

Such challenges include the suitability of high-dose exposures and cut-off limit concentrations for distinguishing between particles with no/low toxicity and those that present an inhalation hazard to humans. In the rat model, such exposures can result in a high epithelial dose in the non-ciliated airways that triggers an influx of inflammatory cells tasked with removing the external stimuli. For toxic particles, this response occurs at lower exposures, reflecting their greater intrinsic toxicity, with the response persisting long after exposure has ceased.

This results in alteration of structure or function (ASF) of the lung as part of a disease pathway. In contrast, for low-toxicity particles, such a response can be a physiological and beneficial defence reaction (adaptive response) with no associated ASF and rapid resolution. However, an additional challenge is that at exceptionally high dose exposure to low-toxicity particles, overloading of macrophage-mediated clearance occurs. This is considered a species-specific phenomenon and results in a rapid accumulation of dose in the alveolar lumen, triggering persistent inflammation with effects that reflect the exposure regimen rather than the intrinsic toxicity.

To differentiate between an adaptive and adverse response using shorter-term studies, it is important to consider the nature of the observed effects, including severity, temporal profile, and reversibility in light of the test conditions. Indeed, the Particle Platform believes there is a need for a deeper understanding of particles' specificities and a framework, such as the one developed by Poland *et al.*[1], to interpret findings and assess the presence of chronic, persistent inflammation. This would enable a consistent and appropriate expert assessment and judgement of hazards (e.g. in the context of the CLP framework).

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P20-30
Comparing OECD TG 422 studies on Gas Oil UVCB substances via the oral (dietary) and dermal route

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Industrial and consumer exposure to Petroleum Substances (PS) is predominantly via the dermal and/or inhalation route. Therefore, historic studies on PS conducted for human health risk assessment were mainly performed via the dermal or inhalation route, depending on the physico-chemical properties of the substance. As part of a testing program to assess the safety of 14 Gas Oil substances (GO) which are refined C10-C25 hydrocarbon UVCBs (unknown or variable composition, complex reaction products or biological materials) Concawe, the scientific branch of the European Fuels Manufacturing Association, conducted oral (dietary) *in vivo* repeated dose/reproductive toxicity screening studies according to OECD TG 422 on representative samples. In fact, the oral route was requested per ECHA guidance for substances for which systemic exposure cannot be demonstrated via the dermal route (ECHA Guidance on IR&CSA R.7.5.4.3.2).

To compare the historic dermal and new oral toxicity data and better estimate the risk under in-use conditions, dermal studies according to OECD TG 422 were conducted on six selected GO for a single high dose group and concurrent control group. The results were critically reviewed and provide an opportunity to further compare systemic exposure via the oral and dermal route. The results were also reviewed in the context of the historic dermal data on related substances, with particular emphasis on the presence and total weight percent of polycyclic (three or more ring) aromatic compounds (PAC) which are suspected to be the main driver of toxicity. The effects observed in the oral studies are qualitatively comparable to those observed in historic dermal studies and correlate with PAC level. The same effects could not be observed at the single dose tested in the dermal studies, which indicates that, under the conditions of these studies, the systemically available dose of PAC was below the effect level. Our results indicate that toxicity via the dermal dose is very unlikely at realistic exposure level in humans. This further supports that real-life exposure considerations should inform hazard testing.

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P20-31
The re-evaluation of sweeteners by the European Food Safety Authority (EFSA): the revised protocols for the assessment of hazard identification and hazard characterisation and exposure of sweeteners

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Purpose: All food additives permitted in EU before 20 January 2009 are subject to new risk assessments by the European Food Safety Authority (EFSA) according to the programme for the re-evaluation of approved food additives set up by Commission Regulation (EU) No 257/2010 [1]. The sweeteners that remain to be re-evaluated are: sorbitols (E 420); mannitols (E 421); acesulfame K (E 950); cyclamates (E 952); isomalt (E 953); saccharins (E 954); sucralose (E 955); neotame (E 961); salt of aspartame-acesulfame (E 962); maltitols (E 965); lactitol (E 966) and xylitol (E 967). The risk assessments of thaumatin (E 957) [2], neohesperidine DC (E 959) [3] and erythritol (E 968) [4] have been completed. This presentation aims to provide an update on EFSA's work on the re-evaluation of sweeteners, with a focus on the latest revisions made on the protocols for the assessment of the hazard identification and characterisation [5] and on the exposure assessment of the sweeteners.

Methods: To fill in the data gaps identified during the initial assessments, in addition to the first calls for data, more recently EFSA launched other calls for data [6,7,8] to invite interested business operators to submit additional information not provided during the previous calls. Moreover, the two protocols originally developed and published in 2020 for the assessment of the hazard identification and characterisation of sweeteners [9] and the exposure assessment [10], have been revised and further updated following their implementation in the three finalised opinions listed above (E 957, E 959 and E 968).

Results: The main revisions of the protocol on the exposure assessment and of the protocol on hazard identification and characterisation[5], summarising the different steps to be applied during the risk assessment, are described.

Regarding the protocol on the exposure assessment, this has been updated to address the acute effect noted for erythritol (E 968), the first polyol assessed, and a methodology for an acute exposure assessment was developed.

Regarding the protocol on hazard identification and characterisation of sweeteners, the main updates concerning the general approach in respect to the inclusion or exclusion criteria of studies to be evaluated, the evaluation of the risk of bias, the data extraction, the weight of evidence and a harmonised approach for assessing genotoxicity studies were described. An overview of the process of translating the final rating of confidence in the body of evidence into levels of evidence for adverse effects on health or no adverse effects on health was also introduced. Finally, an overview of the process of integration of human and animal evidence to develop hazard identification conclusions, expressed in terms of likelihood of an association between the intake of the sweetener in question and an adverse effect on health, were reported. The updated protocols are implemented in the assessments of the sweeteners that remain to be re-evaluated.

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P20-32
Evaluating the exposure-response relationship in 1,3-Butadiene and leukaemia studies

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1,3-Butadiene (BD) exposure's link to leukaemia is under regulatory scrutiny. The assessment methods for BD exposure risks have evolved from early animal and limited human studies to advanced exposure-response modelling with comprehensive quantitative epidemiology data.

A literature search was performed in Medline/Pubmed to identify all human studies on leukaemia mortality associated with BD exposure. The electronic search spanned from inception of records until July 23rd, 2023, using the search term: Butadiene AND (leukaemia OR leukemia OR myeloid OR lymphoid) and was restricted to humans. From these results various statistical models and factors influencing exposure-response modelling were evaluated with a focus on the Synthetic Styrene-Butadiene Rubber (SBR) industry cohort study conducted by the University of Alabama at Birmingham (UAB).

From the analysis it emerges that peak exposures (i.e., tasks with exposures ≥100 ppm) to BD may be more influential in the dose-response relationship than cumulative or long-term exposure. We recommend utilizing beta-coefficients derived from the latest SBR study update, employing Cox proportional hazard modelling, non-lagged and non-transformed cumulative BD exposure, and adjusting for age and peak BD exposure. This review reveals that the estimates of the beta-coefficients for dose-response effects are remarkably consistent across time and statistical model selection. The significant variation in estimated cancer mortality values arises from additional assumptions needed for metrics like the Excess Leukaemia Risk or the Occupational BD Effective Concentration.

This review provides insights into exposure-response modelling for BD exposure and leukaemia mortality, highlighting the importance of peak exposures. In conclusion, evidence to endorse the utilization of the latest SBR study update is provided in this review This study boasts a large cohort, longitudinal exposure quantification of 47 years, inclusion of both genders and a 65-year follow-up period covering over 50% of the cohort. Thus, it has the highest statistical power of the available cohort studies to estimate potential effects of BD exposure. By acquiring and utilizing data from such high-quality human study available, there is no need for exposure-response extrapolations from animal studies for cancer endpoints. The recommended statistical approach offers a reliable basis for regulatory risk assessment and public health population metrics.

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P20-33
Creating a chemical, sensory and risk assessment profile of tobacco products, within the context of regulatory toxicology

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Introduction: In Europe, the Tobacco Products Directive (TPD) is responsible for regulating tobacco product to mitigate their toxicity and appeal. According to the TPD, tobacco products that impart a characterising flavour other than that of tobacco are not allowed on the EU market, creating the necessity for the detailed and methodologically solid risk assessment of products that enter the EU market. Very recently, Heated Tobacco products (HTPs) are also subjected to enhanced reporting obligations via TPD 2014/40/EU of the European Parliament and of the Council of 3 April 2014 which mandates that Member States (MS) prohibit the placing on the market of tobacco products with characterising flavours. Notably, on November 23, 2022, a pivotal development occurred within the EU as the prohibition on placing flavoured heated tobacco products on the market came into force. The aim of our work was to develop a methodology for creating a profile (sensory, chemical, toxicological) for tobacco products on the EU market.

Methods: Tobacco products available on the EU market were selected, from three major product categories. Each product was assessed for the sensory properties through descriptive profiling by trained assessors in triplicate for potential odour attributes, and the average odour intensity for each individual attribute was calculated for each of the three test sample replicates. In parallel with the sensory analysis, a qualitative chemical assessment was performed. In general, a full scan analysis of all compounds by headspace solid-phase microextraction analysis (HS-SPME-GCMS) was performed with each sample blinded and analysed in triplicate. From each analysis, the peaks in the chromatogramme were automatically detected and subsequently characterised using spectral

libraries. In addition, the use of chemical standards for the verification of identified peaks of the chemical assessment was applied. The European Union Common Entry Gate (EU-CEG) data was supplementarily assessed with regards to the products chemical composition.

Results: HTPs, cigarettes and roll your own tobacco underwent detailed sensory and chemical evaluation using the standard operating procedures that were developed. The sensory and chemical analyses identified common traits between the three groups of tobacco products with regards to their composition, additives that could indicate characterising flavours and concentrations of substances that can be used in comparative risk assessment, in unburnt form. Commonalitites between reported vs declared compounds was also noted.

Conclusions: Through the combination of detailed sensory and chemical analyses, it is possible to identify characterising flavours in tobacco products. Combined with the toxicological data available within EU-CEG it is possible to create a tobacco product profile for regulatory assessment. Further assessment through inhalation toxicology risk assessment techniques is warranted.

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P20-34
Genotoxicity evaluation of Propyl gallate: dissecting ambiguous *in vitro* findings and validating *in vivo* assays

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Propyl gallate (PG, CAS 121-79-9) is a widely used food additive (E310) known for its antioxidant properties. Despite its widespread use, genotoxicity assessments of PG have yielded inconsistent results, in bacterial and mammalian cells requiring further analysis to ensure consumer safety.

There are several *in vitro* studies that have evaluated the genotoxicity potential of propyl gallate. Most bacterial reverse mutation assays found PG to be non-genotoxic, but one specific study reported weak genotoxicity in an oxidative damage-sensitive Salmonella strain. Mammalian cell studies also varied, with some showing increased chromosomal aberrations and mutagenic responses, particularly in rodent cell lines.

A notable *in vivo* comet assay from 2002 showed no mutagenicity but did not meet the information requirements as it did not comply with key parameters of OECD TG 489, including the number of dose groups and appropriate controls, the number of animals and cells per sample, and the provision of detailed tail DNA data. The European Chemicals Agency (ECHA) rejected this study and requested additional information to clarify the genotoxicity assessment. As some publications suggest that PG may be a cross-linker, an *in vivo* COMET assay may not be meaningful as cross-linking could lead to a false negative result. To address this gap, additional *in vitro* testing was performed using the Toxsys ToxTracker® assay and a modified *in vitro* COMET assay. In the ToxTracker® assay, markers indicative of DNA damage to the genome – Rtkn-GFP and Bscl2-GFP – and the p53 response exhibited activation, regardless of the metabolic system's presence. Furthermore, reporters of oxidative stress – Srxn1-GFP and Blvrb-GFP – were activated in both conditions. These results merit consideration as they implicate the activation of pathways associated with DNA damage, oxidative stress, and p53-response. Following these studies, a modified *in vitro* COMET assay was initiated to clarify the lack of DNA cross-linking capacity of PG. The absence of cross-linking in the modified *in vitro* COMET assay allows the use of a standard *in vivo* COMET assay as a follow-up, which could otherwise be confounded by false negatives in cross-linking scenarios.

In summary, the integrative genotoxicity assessment of PG incorporates a broader range of *in vitro* assays, provides a path through previous ambiguities, and allows a standard *in vivo* COMET assay to be performed without the risk of the result being scrutinised by regulatory authorities as a false negative due to proposed cross-linking activities. This rigorous approach provides a robust understanding of the impact of PG on genetic stability and assures regulators and consumers of its safety profile in its current applications.

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P20-35
Analysis of the available datasets on genotoxicity and reproductive toxicity of the approved biocidal active substances

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Annex II of the Biocidal Products Regulation (BPR) 528/2012 sets the information requirements for the approval of the biocidal active substances. The Annex II was revised in 2022 introducing changes in the information requirements regarding genotoxicity and reproductive toxicity.

The current poster investigates whether the studies submitted for those two endpoints at the time of the initial approval of the active substances will be adequate to meet the new information requirements for the renewal of approval. In addition, the poster presents specific quality characteristics of the studies.

Regarding genotoxicity, it is investigated whether the *in vivo* studies are the appropriate ones and can indicate exposure of the target tissue(s), and whether the *in vitro* testing was performed at sufficient levels of cytotoxicity.

Regarding reproductive toxicity, it is presented whether the two-generation reproduction toxicity study (OECD TG 416) has been conducted with the 2001 protocol and includes investigation of endocrine disruption parameters.

The outcome of the present analysis contributes to the identification of possible data gaps in the genotoxicity and reproductive toxicity datasets to be addressed ahead of the application for renewal of approval of biocidal active substances.

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P21 | Environmental toxicology

P21-03
Risk assessment of PFAS in home-produced chicken eggs in the Netherlands

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Aim: Certain environmental contaminants can transfer to products of animal origin such as meat, milk or eggs via animal feed, soil, water and/or grass. This also applies to Per- and Polyfluorinated Substances (PFAS). At the end of August 2023, the Dutch newspaper NRC published a study showing that home-produced chicken eggs from the Dordrecht area (within a six kilometres radius around the Chemours chemical plant) contained high concentrations of PFAS. According to NRC, consumption of these eggs could lead to health risks. The Netherlands Food and Consumer Product Safety Authority (NVWA) is the competent authority that monitors compliance with laws and regulations for food in the Netherlands. Home-produced chicken eggs are not

subject to the supervision of the NVWA because they are not placed on the market for commercial sale. Nevertheless, the NVWA attaches high priority to consumer health, and consequently the NVWA was concerned by this situation. Therefore, the Office for Risk Assessment and Research (BuRO) of the NVWA assessed the risk to the health of Dutch consumers if exposed to PFAS over a longer period, via the consumption of home-produced chicken eggs.

Methods: The risk assessment of PFAS in home-produced chicken eggs in the Netherlands is based on the method followed by the Codex Alimentarius and the working method of the European Food Safety Authority (EFSA). This method consists of four steps: hazard identification, hazard characterization, exposure assessment and risk characterization.

Results: Home-produced eggs in the Netherlands can contain high concentrations of PFAS. Depending on the way in which the total PFAS concentration is calculated, the mean total PFAS concentration in home-produced eggs varies between 1.4 ng PFAS per gram total egg and 4.6 ng PEQ12 per gram total egg. The 95th percentile of the total PFAS concentration in home-produced eggs is between 5.5 ng PFAS per gram total egg and 19 PEQ per gram total egg. At this time the source of the PFAS contamination is unknown. It is therefore not possible to predict in advance which eggs from private individuals contain these high concentrations and which not. Compared to home produced eggs, the mean total PFAS concentration (0.044 ng PFAS per gram total egg or 0.058 ng PEQ per gram total egg) and the 95th percentile (0.28 ng PFAS per gram total egg or 0.28 ng PEQ per gram total egg) in commercial eggs are lower. The weekly PFAS intake through the consumption of home-produced eggs containing the high PFAS concentrations exceeds the health-based guidance value (i.e. maximum safe intake) considerably. The ratio between the total weekly PFAS intake by children and adults through the consumption of home-produced chicken eggs and the maximum safe intake ranges from 2.7 to 102. This means that the weekly PFAS intake through the consumption of these eggs over a longer period may result in health risks. This does not apply for the consumption of commercial eggs.

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P21-04
Effect and mechanism of transferrin on kidney ferroptosis induced by UV-photoaged polystyrene nano-plastics

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Background: Nanoplastics (NPs, <1000 nm), produced from industrial nanomaterials or degraded from fragmentation of ubiquitous plastic products, are emerging nanopollutants. These NPs that persist in the environment for long periods undergo further aging under various environmental factors. However, there is still a lack of in-depth understanding regarding the changes in toxic effects caused by the aging process on the molecular structure and physicochemical properties of NPs themselves, which may lead to inaccurate assessment of the potential risks of NPs exposure in the human body.

Method: We artificially aged pristine polystyrene (PS) NPs to obtain ultraviolet (UV) -aged PS NPs (aPS NPs). The morphological and physicochemical changes of PS NPs before and after aging were characterized. In a mouse oral exposure model, we compared the nephrotoxicity of PS NPs and aPS NPs. Then examined ferroptosis-related markers in mouse kidney tissue and human tubular epithelial cells (HK2). The effect of aPS NPs on the adsorption and structure of transferrin (TF) was studied by fluorescence spectroscopy, UV spectroscopy and circular dichroism. The binding sites on proteins were simulated by molecular docking. Finally, the effect of aPS NPs-TF complex on ferroptosis was observed.

Results: The result indicated that UV irradiation resulted in a reduction in the particle size of PS NPs and a significant decrease in Zeta potential. Compared to PS NPs, a large number of oxygen-containing functional groups were generated on the surface of aPS NPs. aPS NPs exposure induced more serious destruction of kidney tissue structure and function along with characteristic changes in ferroptosis, and significantly affected the metabolic patterns of various small molecule metabolites in renal tissue. Then vitro experiments revealed that aPS NPs-induced cell death in HK2 cells involved ferroptosis, which was supported by the use of ferrostatin-1. aPS NPs can enhance the binding of serum TF to its receptor on the cell membrane by forming an aPS NPs-TF complex, leading to an increase in intracellular Fe^{2+} and then exacerbating oxidative stress and lipid peroxidation, which renders cells more sensitive to ferroptosis.

Conclusion: UV aging significantly alters the size, morphology, and physicochemical properties of PS NPs. aPS NPs increased the biological toxicity of PS NPs by inducing ferroptosis. Serum TF could form corona protein on aPS NPs surface for an long period, which can promotes the binding of TF to transferrin receptors on the cell membrane, rendering cells more susceptible to ferroptosis.

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P21-05
Exploring response of oxidative stress biomarkers to environmental lead and cadmium in testes of European brown bear (*Ursus arctos*)

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The detection of cadmium (Cd) and lead (Pb) in liver and kidney samples of European brown bear in levels surpassing toxicity benchmarks has raised concerns regarding potential risks to the male reproductive system. The health effects of reproductive toxicants in individual males have population-wide implications. Environmental exposure of wildlife to potentially toxic metals like Cd and Pb was shown to affect the testicular antioxidant system, which is essential for the maintenance of testes and sperm functions. As the reproductive toxicity of Cd and Pb is species specific, our aim was to explore markers of oxidative stress in the testis of free-ranging brown bears (N=28) from Dinara-Pindos population in relation to the presence of the two toxicologically most relevant metals for terrestrial mammals. Two pollutants were positively associated (r_P=0.45, p=0.026), thereby implying a similar source of exposure. Accumulated Pb negatively correlated with testis width (r_P=-0.44, p=0.038). An increase in cadmium was associated with higher activity of catalase (CAT; r_P=0.61, p=0.0012), and a decrease of reactive oxygen species (ROS; r_P=-0.48, p=0.014). As expected, testes of adult bears (≥4 years) had higher mass, length and width than those of subadults. Males sampled in April (breeding season, N=17) showed higher level of ROS in testes than bears in non-breeding season (October, N=11), while total antioxidative capacity (TAC) marker showed opposite trend. Finally, when health indicators (mass and size of testes, biomarkers of oxidative stress) were modelled with pollutants while controlling for breeding season and maturity status (adults *vs*. subadults), a significant effect was observed solely for Cd regarding CAT activity (b=0.03, p=0.0096, R^2=0.33). Based on the findings, it appears that chronic exposure of brown bears to potentially toxic metals in their environment could lead to alterations in the activity of antioxidative enzymes, specifically in relation to Cd-induced enhanced oxidative stress in the testes.

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P21-06
Relative toxicity study of polystyrene, polypropylene, and polyethylene microplastic fragments in the respiratory system of mice

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Background: The presence of microplastics in atmospheric environments has become an issue of global concern, as studies have shown that inhalation is a major route of human exposure. However, the adverse effects of these persistent pollutants on the respiratory system and their underlying molecular mechanisms are sparsely reported.

Method: In this study, the pulmonary toxicities of dominant microplastic fragments such as polystyrene (PS), polypropylene (PP), and polyethylene (PE) were investigated in C57BL/6 mice. Mice were intratracheally instilled with 5 mg/kg of PS, PP, or PE daily for two weeks, and the inflammatory responses in BALF cells and lung tissues were assessed in comparison with the control group using ELISA, western blotting and histopathological studies.

Results: The numbers of inflammatory cells such as macrophages, neutrophils, and eosinophils in the BALF of PS-instilled mice significantly increased as compared to those in the control. In addition, the levels of inflammatory cytokines and chemokines, including IL-1β, IL-6, MCP-1, MIP-1α, MIP-2, and KC increased in BALF of PS-instilled mice. However, no significant inflammatory responses were observed in PP- and PE-stimulated mice. Results also showed that TLR2 and TLR4 increased in lungs of PP- and PS-stimulated mice, respectively, while TLR1, 5 and 6 remained unchanged in all mouse groups. Furthermore, significant elevations in levels of NF-κB proteins, as well as NLRP3 inflammasome components (NLRP3, ASC, and Caspase-1) were observed in lung tissues of PS-instilled mice compared to the control.

Conclusions: Overall, these results suggest that PS microplastic fragment stimulation induces pulmonary inflammation by NLRP3 inflammasome activation via the TLR4/NF-κB pathway. However, PP and PE fragments induced no significant pulmonary alterations based on our experimental conditions. The work provides baseline data helpful for future studies in our understanding of the potential respiratory effects of inhalable microplastics.

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P21-07
The Environmental Health Language Collaborative (EHLC): harmonizing toxicology data use and sharing

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Purpose: The extensive generation of data is rapidly advancing opportunities to answer large-scale, complex questions in toxicology and environmental health sciences. However, a significant challenge remains around the development and use of a harmonized language to allow for streamlined workflows to find, share, and reuse this data. The Environmental Health Language Collaborative (EHLC) was created to facilitate a community-driven effort to advance the development and adoption of harmonized language approaches within toxicology and environmental health science fields. This presentation describes EHLC activities and data harmonization and sharing opportunities for toxicologists. Our aim in this presentation is to inspire EuroTox professionals' collaborative engagement with EHLC and bring awareness of available resources to help advance environmental health sciences.

Methods: EHLC has grown into a robust community that is led by an Executive committee made up of transdisciplinary subject matter experts from multiple institutions. The collaborative currently supports three active use case working groups focusing on Data Discovery, Data Harmonization, and Biomarkers and Biological Processes of Exposure. Additionally, EHLC hosts webinars and workshops to share relevant data harmonization information and methods with the scientific community.

Results: EHLC has planned and hosted two major workshops and numerous webinars collectively attended by ~300 participants. All three use case workgroups are currently developing manuscripts focusing on pain points, potential solutions, and existing best practices in the corresponding fields. To encourage engagement within the community, EHLC shares activity updates, opportunities, and key resources via the EHLC Community Listserv and the comprehensive EHLC website, which also has foundational information describing the mission of the collaborative.

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P21-08
Health based quality criteria for a mixture of environmental pollutants on the beaches and in sea water

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In the period of 1956–1973, Grindstedværket was allowed by the authorities to deposit wastewater in Kærgård Klitplantage, an area on the coast of southwest Denmark. The amount of deposited chemicals in wastewater has been estimated to be more than 1500 tonnes, including sulfonamides, organic nitrogen compounds, barbiturates, fenols, chlorinated solvents, benzene and toluene. This has led to a leak of the chemicals to an area of about 500,000 m^2, going west to the sea over a coastal stretch of 800 m. The leakage of chemicals led to prohibition against bathing off the polluted coastal stretch from 1964. The question is now how to determine quality criteria based on the toxicology of groups of substances and the exposure of beach visitors.

The exposure of humans, especially children, was modeled according to three scenarios:

1. visiting the beach, occasional contact with polluted water by digging into polluted groundwater
2. playing and bathing in puddles on the beach
3. bathing and svimming in the sea

16 different barbiturates and 13 sulfonamides were grouped and assessed with regard to toxicity and tolerable daily intake (TDI). In addition, certain single substances were also assessed to produce a TDI for them. Group assessments were based on the few wellknown members of the group, whereas most of the intermediates and derivatives had only scant available data. Since sulfonamides are used as antibiotics, the lowest contration likely to induce antimicrobial resistance was also estimated for the sum of sulfonamides because of the risk of cross-resistance.

When assessing the toxicological risk, it was taken into account that the substances can be both absorbed through the skin upon contact with water and sand, and that a certain amount of ingestion can also take place during play at the beach and during swimming/bathing. Prerequisites in the exposure models were eg. children's body weight at 13 kg; oral daily ingestion of sand 0.1 g/day; sand skin contact daily average 1 g/day; sand skin contact maximum 10 g/day; bathing water oral 50 mg/hour; skin surface child feet and hans 1500 cm^2; skin surface whole body 5300 cm^2; frequency of stay 7 days/year; frequency swimming at sea 7 days/year; duration of stay 4 hours/day; duration of bathing/swimming 2 hours/day, dermal absorption velocity in cm/hour was estimated for each substance from log Kow and molecular weight.

After allocation of fractions of TDI to other known exposures the models resulted in the following proposals for health based quality criteria: Proposed quality criteria, amount per liter of water

Substance/group	Scenario 1	Scenario 2	Scenario 3	Resistance criterion
Barbiturates (sum)	0.4 mg/L	0.2 mg/L	0.05 mg/L	
Sulfonamides (sum)	466 mg/L	259 mg/L	7.6 mg/L	16 µg/L
Meprobamate	588 mg/L	310 mg/L	8.8 mg/l	
Sulfanilic acid + acetylsulfanilic acid (sum)	90 mg/L	90 mg/L	32.5 mg/L	
Anilin	0.1 mg/L	0.03 mg/L	0.005 mg/L	
Ethyl urethane	0.0004 mg/L	0.0001 mg/L	0.0045 µg/L	

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P21-10
Libraries of micro- and nanoplastics: variations in size, shape, surface oxidation, and fluorescent labeling

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Microplastics (MPs), plastic particles less than 5 mm in size, are ubiquitous in the environment, while nanoplastics (NPs) are defined as plastic particles less than 1 µm in size. Recent detection of MPs in human tissues such as the lung, placenta, and blood highlights inevitable human exposure through inhalation or ingestion. Moreover, MPs are pervasive in various environmental including air, rivers, and even drinking water. However, the full extent of their effects on human health remains unclear. Despite their diverse physicochemical properties, such as size, shape, and surface oxidation induced by environmental factors like ultraviolet radiation and waves, research on MPs often neglects these complexities when assessing their biological effects. As a result, there is a dearth of particles reflecting environmental MPs and NPs, with most research employing plastic particles that represent only one physicochemical property due to their availability. To address this gap, we endeavored to establish libraries of MPs and NPs that consider size, shape, surface oxidation, and fluorescence labeling to facilitate kinetics analysis.

Polyethylene (PE), Polypropylene (PP), Polystyrene (PS), and Polyvinyl chloride (PVC) were chosen as polymer types due to their global production volumes. We obtained fragmented particles ranging from 20 to 250 µm in diameter for each polymer and established a protocol for surface oxidation using these particles. Surface-oxidized MPs (oxiMPs) were generated through irradiation with vacuum UV light at 172 nm under air to simulate environmental conditions. Environmental MPs were collected from a sandy beach facing Osaka Bay for comparison. Analysis using ATR-IR and XPS revealed the presence of hydroxyl and carbonyl groups in both environmental MPs and generated oxiMPs. To produce smaller particles (NPs, less than 1 µm in diameter), we selected PE and PS and employed a previously published precipitation-based method. Scanning electron microscopy confirmed the successful generation of nano-sized particles. For fluorescence labeling of MPs, we attempted to label each particle with Nile Red, observing successful labeling in MPs. Additionally, we modified the protocol to incorporate Qdot, a fluorescent label with high brightness and photostability, for NP labeling, which was validated through microscopic analysis.

The establishment of libraries of MPs and NPs reflecting their complex physicochemical properties enhances the relevance of MPs and NPs research to environmental contexts. These libraries are available for distribution upon request through future collaboration.

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P21-11
Analysis of zebrafish AHR interacting protein (AIP) mutants

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Animals are frequently exposed to environmental pollutants including aromatic hydrocarbons that elicit their toxicity through interaction with the aryl hydrocarbon receptor (AHR). There is interindividual variation in sensitivity to AHR agonists, but the mechanisms are not fully understood. Several populations of Atlantic killifish evolved resistance to high levels of pollution [1]. GWA studies identified the AHR interacting protein (AIP) as a possible resistance gene [2–4]. AIP's roles include facilitating the stability and nuclear translocation of the AHR, but its exact function remains poorly understood. To further investigate AIP's role in mediating toxicity two AIP mutant lines were developed.

The AIP mutant lines were generated using CRISPR-Cas9 technology, inducing a deletion in exon 2 (*aip*^{wh86} line) or exon 5 (*aip*^{wh239} line) of the gene. Both resulted in an early stop codon and truncated a protein. Homozygous mutants from both lines die at 8–9 days post-fertilization (dpf). No such effect is observed in AHR mutant lines. Due to larval lethality, spawning had to be performed using adults heterozygous for the mutation resulting in offspring of unknown genotype. 5dpf larvae were prepared for imaging and RNA-seq analysis using genotyping methods designed to preserve the sample for later analysis. These methods implemented a TaqMan genotyping assay allowing rapid and accurate analysis. RNA-seq was performed on pooled larvae using Lexogen sequencing and differentially expressed gene (DEG) analysis was performed. Microscopic imaging was performed until 9dpf using a Keyence fluorescence microscope.

Brightfield images of zebrafish demonstrated morphological changes in the craniofacial structure of AIP mutant larvae. DEGs were identified by comparing the wildtype (WT) and mutant larvae using a Log2(FC) cutoff of 1 and an adjusted p-value cutoff of 0.05. In the *aip*^{wh86} line, 626 transcripts were in higher abundance and 603 were lower when compared to the WT. In the *aip*^{wh239} line, 564 transcripts were in higher abundance and 430 were lower when compared to the WT. 48% of DEGs were shared between the lines while the remainder were unique to either line. In both lines biological process gene ontology terms were significantly enriched when imputing the DEGs. Some terms were shared between lines including extracellular structure and immune response. Terms unique to the *aip*^{wh86} line included cell cycle and chromosomal organization terms. Terms unique to the *aip*^{wh239} line included defense response and biotic stimulus terms.In conclusion, the viability and morphology of zebrafish are altered in two unique AIP mutant lines. RNA-seq analysis of the lines yielded a large dataset including thousands of DEGs. This dataset can be further analyzed using transcriptomic analysis and offer leads for mechanistic studies. Further, the novel methods used to genotype larvae will be implemented in experiments that assess the toxicity of AHR agonists in the mutants.

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P21-12
Gas6-Axl signal promotes indoor VOCs exposure-1 induced pulmonary fibrosis viapulmonary microvascular endothelial cells–fibroblasts cross-talk

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Volatile organic compounds (VOCs) as an environmental pollutant were associated with respiratory diseases. Pulmonary fibrosis (PF) was characterized by an increase of extracellular matrix, leading to deterioration of lung function. The adverse effects on lung and the potential mechanism underlying VOCs induced PF has not been elucidated clearly. In this study, the indoor VOCs exposure mouse model along with an ex vivo biosensor assay was established. Based on scRNA-seq analysis, the adverse effects on lung and potential molecular mechanism were studied. Herein, the results showed that VOCs exposure from indoor decoration contributed to decreased lung function and facilitated pulmonary fibrosis in mice. Then, the whole lung cell atlas after VOCs exposure and the heterogeneity of fibroblasts were revealed. We explored the molecular interactions among various pulmonary cells, suggesting that endothelial cells contributed to fibroblasts activation in response to VOCs exposure. Mechanistically, pulmonary microvascular endothelial cells (MPVECs) secreted Gas6 after VOCsinduced PANoptosis phenotype, bound to the Axl of fibroblasts, and then activated fibroblasts. Moreover, Atf3 as the key gene negatively regulated PANoptosis phenotype to ameliorate fibrosis induced by VOCs exposure. These novel findings provided a new perspective about MPVECs could serve as the initiating factor of PF induced by VOCs exposure.

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P21-13
The protective effects and molecular mechanisms of Baicalin against the cytotoxicity of Phenylarsine oxide

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Objective: Phenylarsine oxide, a derivative of organo-arsenic compounds previously deployed as chemical weapons, is characterized by high toxicity, chemical stability, and resistance to degradation. Despite its global prohibition, environmental residues pose ongoing risks of exposure. Few agents are currently known to mitigate its toxicity. Baicalin, a flavonoid derivative found in the roots of the medicinal plant goldenrod, has demonstrated antioxidative and anti-inflammatory properties. However, its protective effects against Phenylarsine oxide toxicity remain unclear. This study aims to investigate the protective effects of baicalin against Phenylarsine oxide-induced cytotoxicity in the human epidermal keratinocyte HaCaT cell line and elucidate the underlying molecular mechanisms.

Methods: HaCaT cells were exposed to 500 nM of Phenylarsine oxide in the presence of varying concentrations (0–100 μM) of baicalin for 24 hours. Flow cytometry and fluorescence microscopy were used to assess reactive oxygen species (ROS) generation, mitochondrial mem-

brane potential (MMP) reduction, and cell death. Changes in apoptosis signaling protein expression were analyzed via Western blotting.

Results: Treatment with 500 nM of Phenylarsine oxide significantly reduced cell viability and induced cell death in HaCaT cells. This treatment suppressed Sirt-3 expression, increased ROS production, and decreased MMP. Moreover, it enhanced the activation of JNK, p38, and caspase-3 while inhibiting AKT activation. Co-treatment with 50 μM of baicalin reversed the Phenylarsine oxide-induced suppression of Sirt-3 and AKT expression, reduced ROS generation, and notably inhibited caspase-3 activation and apoptosis induction. Conversely, pretreatment with nicotinamide and LY294002, inhibitors of Sirt-3 and AKT, respectively, one hour before co-treatment significantly attenuated the protective effects of baicalin.

Conclusion: Baicalin effectively protected HaCaT cells from Phenylarsine oxide-induced cytotoxicity by restoring Sirt-3 activity, thus inhibiting excessive ROS generation. This led to reactivation of AKT, attenuation of JNK, p38, and caspase-3 activation, and protection against Phenylarsine oxide-induced cell death. These findings suggest the potential therapeutic use of baicalin in countering Phenylarsine oxide toxicity, warranting further investigation into its clinical applications.

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P21-15
Effects of autogenous vaccine on 20 antimicrobial drug residues in Nile Tilapia (*Oreochromis Niloticus*) farm, Thailand

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The application of the autogenous vaccine in poultry, cattle, swine and sheep served as a curative and preventive measure, and it has proven to be a very efficient method of antimicrobial therapy. However, there are limited study on the effect of autogenous vaccine against *Streptococcus agalactiae* in Nile tilapia (*Oreochromis niloticus*). Therefore, this study determined the occurrence of antimicrobial drug residues after using autogenous vaccine against certain disease in the Nile tilapia farm in Thailand. An autogenous vaccine was developed using the most common *Streptococcus agalactiae* (Killed virus vaccine) and treat via the feed coated during March 2023 – March 2024. The samples collected every two months annually were 18 waters, 18 soil, 70 blood and 70 meat samples. The 20 antimicrobial drugs of Sulfadiazine, Lincomycin, Trimethoprim, Oxytetracycline, Amoxicillin, Norfloxacin, Ofloxacin, Levofloxacin, Ciprofloxacin, Enrofloxacin, Ampicillin, Chlortetracycline, Sulfamethoxazole, Doxycycline, Tetracycline, Erythromycin, Tylosin, Nalidixic acid, Tiamulin and Clarithromycin were analyzed by using Liquid Chromatography-Mass Spectrometry Triple Quadrupole (LC-MS/MS). The Mann-Whitney U test was performed for comparisons of the concentrations of antibiotics between non-autogenous vaccine and autogenous vaccine group using GraphPad Prism version 8.0.1(2018). The result revealed that none of 20 antimicrobial drugs residue detected in the meat, water and soil, whereas only amoxicillin was detected in the blood sample. However, there was no significantly difference of amoxicillin levels between autogenous (11.39±21.04 ng/ml) and non-autogenous group (12.90±24.80 ng/ml) at $P>0.05$. These results concluded that intervention of the autogenous vaccine did not alter fish growing efficacy compared with those did not use the vaccine. The fish are well growing and healthy while long term drug residue in its environment must be carefully admitted.

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P21-18
Development of a highly sensitive reporter gene cell line for detecting estrogenic activity in drinking water

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Fresh water sources are under extreme pressure due to increased urbanization and chemical use which results in an extensive release of synthetic chemicals into the environment. With an average daily drinking water consumption of 2 liters, very low levels of contaminants can become a health risk due to life-long exposure [1]. To date, most drinking water treatment facilities are not designed to remove micropollutants and hormone-like chemicals. Consequently, there is a risk that

chemicals present in the raw water, will also be present in the drinking water [2]. In order to improve water quality monitoring, effect-based methods, which are bioanalytical tools based on mammalian cells, detect and quantify the induced activity of chemicals on different toxicological endpoints [3]. The aim of this study is to develop a highly sensitive reporter gene cell line for continuous monitoring and rapid detection of estrogenic activity in drinking water, where the reporter activity can be measured directly in the cell culture medium without need for cell lysis.

The reporter gene cell line is based on human breast cells (MCF-7) transiently transfected with the reporter gene for estrogenic detection ER-NanoLuc (pNL2.3-ER[secNluc/Hygro] plasmid) and exposed for 24 hr to estradiol (E2) in concentrations ranging from 0.0007 pM to 10 nM. The process for developing this reporter cell line involved the optimization of culturing and exposure conditions (exposure time, cell viability and effects on plate coating), optimization of the reporter gene assay limit of detection (plasmid concentration, transfection efficiency and relative potency factors) and assessment of the sensitivity level by testing real water samples without solid phase extraction.Our results have shown that this reporter gene cell line is highly sensitive for estrogenic activity detection at very low concentrations in cells transfected with 5 or 10 ng of the plasmid. Given that we observed a slightly more sensitive response in cells transfected with 5 ng of the plasmid, we decided that this would be the optimal concentration for all subsequent experimental procedures. The developed reporter gene cell line was able to detect E2 in concentrations as low as around 50 pg/L E2 equivalents, which is around 10 times more sensitive compared to other commonly used mammalian reporter gene assays for estrogenic activity [4]. Next, we wanted to assess the effect that exposure to E2 had in cell viability. Results indicated that none of the E2 tested concentrations decreased cell viability. Next, we tested transfection efficiency of the plasmid. Our results displayed that transfection was uniform trough all treatments. Given that this an ongoing project, data generation regarding exposure time, relative potency factors and sensitivity level in water are still in progress.

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P21-19
The secondary organ translocation of inhaled ultrafine particulate matter in mice

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As global air quality worsens, concerns about the impact of air pollution on human health are increasing. However, there is a lack of information about the biokinetics of ultrafine particles, which are a major component of ambient particles and pose significant hazards and risks to human health. In this study, simulated ultrafine particulate matter (sUPM) was synthesized using a spark discharge soot generator, and carbon black nanoparticles (CB) and diesel exhaust particles (SRM 2975) were used as reference particles. sUPM was treated in mice at 25 μg/mouse using the intratracheal instillation method, and the organ burden was analyzed at 0 h, 7 days, 1, 2, and 3 months after instillation using proteinase K and a UV-Vis spectrometer. The results showed that sUPM was significantly translocated to lung-associated lymph node (LALN) and spleen from 1 to 3 months, while other organs showed no detectable levels. The translocation pattern of the reference materials was similar. The study compared the extrapulmonary translocation pattern of the three particle types with intravenous injection as a systemic circulation route or intrapleural injection as a lymphatic route. Both exposure routes showed typical translocation organs such as the liver, lung, spleen, and LALN. Therefore, the unique translocation pattern of sUPM from the lung to the spleen may be due to the finest particles, like singlet and least agglomerated particles, that escape from the lung. These findings are significant as the particles can avoid surveillance in the liver sinusoids but are trapped physically in the open circulation system of the spleen. Further research is necessary to study the effect of these particles on secondary organs.

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P21-20
Lung accumulation of polyethylene microplastics may cause chronic inflammatory response

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Starting with the technology of adding sulfur to harden natural rubber, plastic manufacturing technology has been developed innovatively during this period, and the application of plastics has been accelerated in various industries including food packaging, textiles, toys, tire manufacturing, construction, and furniture, due to its sundry advantages such as low cost and strong durability. Polyethylene (PE) plastics accounted for about a third of the total plastics market in 2017, and low-density PE and high-density PE occupy 15.7% and 12.8% of global plastic production, respectively. Considering that environmental exposure to airborne microplastics has attracted public concern due to the increased plastic product usage, we tried to identify the toxicity of PE microplastics (PE-MPs) instilled repeatedly for 14 days and 90 days. When instilled intratracheally PE-MPs for 14 days (0, 250, 500, or 1000 μg/lung), there is no dead animal. Meanwhile, the total cell number and the chemokine level increased dose-dependently in the lungs of the treated mice. In addition, we found that instilled PE-MPs accumulated in the lung tissue of mice exposed during 90 days, maintaining their intact form. PE-MPs elevated the total number of pulmonary immune cells, and the pulmonary level of cytokines and chemokines which contribute to pro- and anti-inflammatory responses increased with dose. Furthermore, infiltration of inflammatory cells, accumulation of macrophages, and multinucleated giant cells were observed in mice exposed to PE-MPs. Herein, we suggest that pulmonary accumulation of PE-MPs causes chronic inflammation, suggesting that it is an inducible lung disease.

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P21-21

Dimethyldodecylamine oxide induces cytotoxicity via damage of mitochondria and cell membrane

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The importance of personal and public health hygiene to prevent infectious diseases has been further heightened during the COVID-19 pandemic, and dimethyl dodecyl amine oxide (DDAO) is the most frequently used surfactant in producing household chemicals, including detergent, cleaning agents, and disinfectants. Like the skin, the respiratory tract is a representative exposure route to household chemical products, but data on the potential adverse health effects of these household chemicals on the respiratory system is very limited. In this study, we sought to identify the toxicity mechanism of DDAO using the BEAS-2B cell line derived from human bronchial epithelial. When exposed for 6 hours, cell viability rapidly decreased above 10 ug/ml concentration, and LDH release was detected significantly above 20 ug/ml concentration. Meanwhile, contrary to our expectations, the level of intracellular reactive oxygen species decreased in a concentration-dependent manner, and mitochondrial volume and produced ATP amount also decreased significantly in DDAO-treated cells compared to the control. In addition, TEM images revealed that mitochondrial damage and the formation of lamellar body-like structures and autophagosomes are induced in the DDAO-treated cells. Taken together, we suggested that mitochondrial damage and cell membrane leakage may be closely associated with DDAO-induced cytotoxicity. Furthermore, we propose further study is needed to identify DDAO-inducible pathological changes.

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P21-23

First data on polybrominated diphenyl ethers occurrence and a follow-up on polychlorinated biphenyl levels in European brown bear as a bioindicator of terrestrial environment contamination

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Polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) are two classes of persistent organic pollutants (POPs). Due to their persistence and lipophilicity, these chemicals are spread in aquatic and terrestrial ecosystems. Long lived, high level trophic feeders, tend to accumulate POPs in lipid tissues, which is attributed to biomagnification process, which can lead to adverse health effects. While there is ample data on POP contamination of aquatic ecosystems, comparable research on terrestrial wildlife is scarce. The main objective of this study was to determine the mass fractions and profiles of seven PCB and seven PBDE congeners in the adipose tissue of European brown bears (*Ursus arctos*). Additional goal was to compare the current PCB levels with the results of a previous study on the same population in the same area to examine the temporal pattern of PCB levels over a ten-year period. Subcutaneous adipose tissue was obtained from brown bear human-induced mortalities (hunted or traffic-killed; N=27) captured in April and October 2021 and 2022, in the Gorski Kotar and Lika regions of Croatia. The sum of the mass fractions of PBDEs detected in the adipose tissue of the bears ranged from 0.011 to 0.463 ng/g of lipid weight (lw), and from 0.911 to 30.39 ng/g lw for PCBs. The difference in median values between these two POP groups was two orders of magnitude, and they were 0.022 ng/g lw and 2.897 ng/g lw, for PBDEs and PCBs, respectively. PCBs thus accounted for over 94% of the total measured mass fractions. Compared to the results from 2010/2011, similar PCB congener profiles and median values of the sum of the PCB mass fractions were obtained, suggesting that PCBs have reached a steady-state in the Croatian terrestrial environment. In general, the POPs profile consisted predominantly of the BDE-47, PCB-153 and PCB-180. PBDE and PCB mass fractions measured in the samples from April hunting season were higher than those of samples from the October hunting season, indicating possible impact of winter hibernation on increase in lipophilic compounds concentration due to body fat loss. On the other hand, no significant differences in POP levels were noted between the two sampling years. Relatively low POP levels detected in Dinara-Pindos brown bear population align with the data on the generally low contamination of the Croatian terrestrial ecosystem with those two POP classes. The observed steady-state of PCBs in these sparsely populated regions of Croatia underlines the ubiquity, resilience and persistence of PCBs in the atmosphere.

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P21-24

Arsenic in hair and nails of pet dogs as biomarkers of exposure to inorganic pollution from a gold mining area

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Water, soil, and wildlife in a partially remedied gold mining area in Salsigne (Orbiel valley, south France) are predominantly contaminated with arsenic (As), but also with lead (Pb), bismuth (Bi), copper (Cu), cadmium (Cd), mercury (Hg), and zinc (Zn). Since the mine closure in 2004, tonnes of waste deposits are exposed to frequent winds, rain drainage and regular flood events, resulting in dry and wet deposition of fine metal(oid) particles to surrounding area, and leaching of particulate and dissolved metal(loid)s to Orbiel catchment. Nevertheless, there is a severe lack of exposure studies in domestic animals, pets and in humans from that area. Here we applied non-invasive sampling of pet dogs, in order to detect arsenic in their organism, which can also serve as good sentinels of long-term exposure to environmental pollutants in the human population. Hair (N=49) and nails (N=14) from dogs residing in mining (up to 10 km from Salsigne mine) and control areas (central France, 12 km SW from Lyon) were collected with the owner's permission, along with biometric, dietary and outdoor routine data of the canines. Arsenic levels in hair and nail samples were quan-

tified by inductively coupled plasma spectrometry following washing and digestion procedures. Dogs residing in mine-neighbouring communes (N=27) had higher (Welch's t-test, t(46)=2.26, p=0.029) level of As in hair (mean±SEM 232±73, range 17.8–1759 μg/kg dry mass) compared to dogs from the control area (N=22, 70.9±21.2, 21.0–506 μg/kg dry mass). Likewise, nails of dogs from the mining area (N=8, 722±288, 84.5–1952 μg/kg dry mass) had higher levels of As than control dogs (N=6, 137±35, 30.4–285 μg/kg dry mass), but the difference was not significant (Student's t-test, t(12)=1.29, p=0.22). High correlation between hair and nail As levels (r_S=0.71, p=0.041, N=14) was determined. The sex and age of studied dogs had no influence on As levels in their hair and nails. There was no discernible correlation between the duration of residence in a mining region or regular swimming in the nearby tributaries of the Orbiel River and levels of As in hair. The As levels in the nails of dogs from Salsigne mining area were comparable to those detected in wild mammals (snowshoe hare, muskrat and red squirrel) from Giant gold mine in Canada. However, their hair contained higher As levels in comparison to urban dogs from Sydney, Australia, which reported levels similar to those of dogs from central France (control area for this study). Research has demonstrated that As found in the keratinized matrices of pet dogs cohabiting humans can serve as reliable indicators of pollution with As caused by centuries long mining activities.

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P21-25

Difference in metal(loid)s levels in cut and plucked hair of European brown bear (*Ursus arctos*)

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The persistent non-essential metal(loid)s arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) can cause nephrotoxic, haematotoxic, neurotoxic, and negative reproductive effects even at low levels. From the practical point of view, hair sampling is the best method for monitoring metal(loid)s levels in animals. However, the method of sampling (cutting, plucking or snagging) can have an effect on the values obtained. Hair follicles with blood vessels are present in plucked or snagged hair and consequently metal(loid) levels are expected to be higher than in cut hair where only hair shafts are analysed. The difference in metal(loid) (As, Cd, Pb and Hg) levels in cut and plucked hair was investigated in free-ranging brown bears (N=28, 17 adults and 11 subadults) from Dinara-Pindos population, while assisting to human-induced mortalities (hunted or traffic-killed) in 2021 and 2022 in Croatia. Arsen levels were similar to previously published studies from the same population and there was no statistical difference between cut and plucked hair, two age groups or season of sample collection. Mean Cd and Pb levels were slightly lower than in previously published study in cut hair from Dinara-Pindos and Carpathian population. Lead levels were higher in plucked than in cut hair (mean±SD=529±568 μg/kg and 377±267 μg/kg, respectively; t(25)=-2.12, p=0.044), while, contrary to expectation, Hg levels were higher in cut than in plucked hair (mean±SD=173±171 μg/kg and 154±154 μg/kg, respectively; t(26)=3.49, p=0.0017). Subadult (<4 years) bears had higher Hg levels than adult (≥4 years) bears in both plucked and cut hair (t(25)=4.13, p<0.001 and t(26)=4.75, p<0.001, respectively). In addition, bears sampled during breeding season (April, N=17) had higher levels of Pb and Cd in plucked hair than bears sampled during non-breeding season (October, N=11). Based on our findings, age and method of hair sampling could influence metal(loid) levels in the hair of European brown bears. We believe that for the purpose of standardization and comparisons across studies using hair in monitoring of elements, whether follicles were included into extracted samples or not, is methodologically crucial and should be reported.

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P21-26

Impact of airborne microplastic bio-Corona formation on alveolar epithelial cell interactions

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Introduction: In recent years, the inhalational exposure to airborne microplastics has emerged as a significant concern globally, given their presence in both air and human samples. Microplastics have the ability to adsorb various substances onto their surfaces, forming a corona. Within the alveolar region, microplastics interact with alveoli lining fluid containing surfactant lipids, proteins, albumin, and antioxidants, potentially inducing chemical and physical modifications to the corona. This study aimed to investigate whether microplastic corona formation within the alveoli microenvironment affects cellular uptake, localization, cytotoxicity, and morphological alterations in alveolar epithelial cells using three distinct microplastics.

Methods: Polystyrene, polyamide, and polyethylene terephthalate microplastics were exposed to artificial surfactant lipids and albumin. Assessment of lipid and protein attachment to microplastic surfaces was conducted using fluorescent/brightfield z-stack imaging and spectrophotometry. Dynamic light scattering monitored changes in agglomeration and surface charge due to corona formation. For instantaneous membrane-level interactions, optical tweezers coupled to a confocal microscope captured lipid dynamics using raster image correlation spectroscopy. Cellular uptake, cytotoxicity, and morphological changes upon exposure to microplastics were measured over 20 hours using digital holotomography. Additionally, accumulation and cell localization of microplastics were measured using 4-dimensional confocal imaging of the actin cell skeleton over 16 hours.

Results and conclusions: Microplastics underwent surface coating with surfactant lipids and albumin, leading to alterations in their physiochemical properties. The extent of corona formation appeared to be dependent on the type of plastic. This study provides important insights into the effect of corona formation on cellular interactions, from initial seconds of membrane interaction to long-term responses such as uptake, compartmentalization, and toxicity. These findings demonstrate significant modification of microplastics' physiochemical characteristics when exposed to alveolarlipids and proteins, suggesting the formation of a bio-corona. This bio-corona is likely to impact the tox-

icokinetics of microplastics following inhalation exposure, highlighting the importance of considering corona formation in assessing their biological effects and associated health risks.

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P21-27

Inflammatory and genotoxic effects of nanoplastics and benzo[a]pyrene in zebrafish

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Nanoplastics (NP) and benzo[a]pyrene (BaP) are major environmental pollutants that have attracted significant attention due to their potential adverse effects on ecosystems. NP, due to their small size (<100 nm in diameter) have a high surface-to-volume ratio which potentiates adsorption of other compounds present in the environment, including other contaminants. In this work, we aimed to understand if the combined exposure of zebrafish (*Danio rerio*) to NP and to an environmental carcinogen, BaP, led to different toxic effects compared to individual exposure to either contaminant.

Zebrafish were exposed for 28 days (75% water change and recontamination every 48 h) to the following treatments: i) control; ii) NP via dietary intake of *Daphnia magna* previously contaminated with 25 nm diameter polystyrene nanoparticles (PSNP) at a concentration of 500 $\mu g.mL^{-1}$; iii) waterborne exposure to BaP (25 nM); and iv) combined exposure to NP and BaP.

At the end of the experiment, fish were sacrificed, and samples collected to evaluate DNA strand-breaks in blood (Comet assay) and changes in the transcript levels of genes associated with inflammation (IL1ß), detoxification (Cyp1a) and tumour progression (rab1a) and suppression (tp53) in the liver and intestine (qRT-PCR).

All treatments significantly increase DNA strand-breaks, especially in the group exposed to both NP and BaP (49±17% DNA in the tail). In the liver, all genes analysed were upregulated. IL1β mRNA was increased (up to 3-fold) by all treatments in the liver, in line with previous findings on the immunotoxicity of these contaminants. Cyp1a (3-fold) and rab1a (8-fold) were particularly upregulated by BaP exposure, which is a clear indication of active metabolism and tumorigenicity of this compound. For tp53, the highest values were observed in the BAP+NP combination (15-old). In the intestine, only BaP exposure had an effect over the expression of all genes.

Interestingly, although the results of the Comet assay point to a stronger genotoxic effect of the combined exposure of BaP and NP, at the same time, tp53 transcription was enhanced by this same treatment. These results highlight the intricate interactions of nanoplastics with other environmental contaminants.

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P21-28

Health risk contamination of Mercury in blood and meat after using autogenous vaccine against *Streptococcus agalactiae* in Nile Tilapia (*Oreochromis Niloticus*)

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Streptococcus agalactiae (*S. agalactiae*) is a zoonotic human and animal pathogen that causes major economic losses to the aquaculture industry and fatal outcomes in Tilapia farming worldwide in recent years. However, there are no report on the effect of autogenous vaccine against *S. agalactiae* in Nile tilapia (*Oreochromis niloticus*) and other related xenobiotics yet. Since, Mercury (Hg) is a toxic metal in the environment and become the World Health Organization's foremost chemicals of concern. Hg contaminated fish consumption could possess health risk to human. Therefore, the aim of this study was to evaluate and compare Hg contamination among autogenous and non-autogenous vaccine against *S. agalactiae* in the Nile tilapia farm in Thailand. An autogenous vaccine was developed using the most common *S. agalactiae* (Killed virus vaccine) and treated via the feed coated during March 2023 – March 2024. Eighteen of soil and water samples, seventy of blood and meat samples were collected every two months annually. Hg analysis was operated using Mercury analyzer and the data were tested using GraphPad Prism. The Mann-Whitney U test was applied to compare Hg concentration between non-autogenous vaccine and autogenous vaccine group. Results revealed that the Hg concentration in raw water, cultivated reservoir, and last pond were 0.060±0.055, 0.037±0.040 and 0.186±0.360 µl/L respectively. While Hg concentration in soil of raw water, cultivated reservoir and last pond was 0.023±0.0008, 0.022±0.0004 and 0.022±0.0022 mg/kg, respectively. Additionally, the Hg concentration in soil significantly related with the Hg in the meat from autogenous group ($r^2=0.34$) at $P<0.05$. However, Hg level was not significantly difference in the blood sample between non-autogenous vaccine (0.03±0.08 µg/L) and autogenous vaccine group (0.05±0.11 µg/L) at $P>0.05$. Whereas, the level of Hg concentration their meat of non-autogenous vaccine group (0.009±0.004 mg/kg) was significantly higher than autogenous vaccine group (0.006±0.002 mg/kg) at $P<0.05$. The concentrations of Hg were below the limits for fish proposed by World Health Organization (0.5 mg/kg). Thus, the autogenous vaccine could help alleviate health effects from Hg accumulation in their meat. However, in depth immune response mechanism as well as other protective effects should be carried on in further study.

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P21-29
Assessment of lung function changes in mice exposed to polyhexamethylene guanidine phosphate

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In the humidifier disinfectant inhalation incident, which caused numerous deaths due to an unknown lung disease in 2011, as of February 29, 2024, 1,851 out of 7,913 total victims have passed away. Identification of the victims is still ongoing, and 882 individuals among them have yet to receive compensation recognition. Recognition of humidifier disinfectant exposure requires medical verification, including clinical, and tissue pathology. Among these, images of the lung CT scan are the most crucial evidence. However, many victims complain of abnormal lung function despite normal lung CT scan data, necessitating research on lung function evaluation following humidifier disinfectant exposure.

This study exposed polyhexamethylene guanidine pohsphate (PHMG-p), a causative agent of humidifier disinfectant, to C57BL/6 mice via intratracheal instillation at three different doses(n=10). The single exposure group was observed for 2, 4, and 8 weeks after exposure, while the 5-times exposure group(5 times/week) was observed for 10 weeks after exposure. Control groups included a non-treated group and a group treated with 0.9% normal saline(n=10).

Anesthesia was induced with intraperitoneal injection of ketamine(50 mg/kg) and medetomidine(0.5 mg/kg) before lung function test. A breath tube was inserted into the trachea and connected to a DSI Buxco Pulmonary Function TEST device. The lung function values represent the mean of six mice per experimental group.

In the single exposure group, chord compliance(Cchord) decreased in proportion to the exposure doses, while the maximum pressure change over the breath(dPmax) and lung resistance(RI) values increased at 2 weeks. Additionally, vital capacity(VC), tidal volume(TV), and forced expiratory volume(FEV) decreased. However, these changes were not observed at 4 and 8 weeks. In the 5-times exposure group, Cchord decreased, and RI increased at 10 weeks depending on the exposure doses. VC, TV, total lung capacity, and FEV also decreased.

In conclusion, our results suggest that short-term and temporary exposure by PHMG-p can decrease lung compliance and elasticity, but lung function reduction restores without further exposure. In conclusion, our results suggest that short-term, single exposure to PHMG-p can decrease lung compliance and elasticity, but reduced lung function recovers without further exposure. Conversely, persistent exposure sustains lung function reduction over a longer period, and the risk of lung function deterioration is high due to restrictive ventilatory defect, which by pulmonary fibrosis induced by PHMG-p.

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P21-30
Effects of black carbon particles on human monocyte-derived macrophages *in vitro*

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Background: Black carbon constitutes a fundamental element of particulate matter in air pollution and is linked to adverse health outcomes, heightened vulnerability to respiratory infections, chronic obstructive pulmonary disease (COPD), and asthma. In addition to being a vector for particulate matter formation, pure black carbon on its own represents a mixture of very fine carcinogenic particles, small enough to enter the bloodstream and reach other organs. In human airways and lungs, macrophages play a pivotal role in addressing and executing a response to various inhaled particles, including black carbon. When exposed to particulate matter, macrophages initiate an innate immune response characterized by phagocytosis, antigen presentation, activation of transcription factors, and subsequent upregulation of proinflammatory cytokines. Simultaneously, they activate the transcription of anti-oxidative enzymes. In the case of black carbon particles, the activation of macrophages may contribute to the development of respiratory diseases.

Objectives: Our objective was to investigate the impacts of black carbon on human monocyte-derived macrophages *in vitro*. The outcomes of this investigation provide novel perspectives on the involvement of macrophages in lungs exposed to black carbon, with potential implications for the pathogenesis of various diseases. In addition, we explored potential counteractive measures.

Methods: We conducted a comparative analysis of two types of commercially obtained black carbon particles using various physicochemical methods and assessed their biological effects on monocyte-derived macrophages. In parallel, the candidate post-biotic preparation (lysate of beneficial upper airway-residing bacteria) was tested. For quantitative analysis of phagocytosis, we utilized confocal microscopy and *CellProfiler*, an open-source cell imaging tool. We quantified black carbon-induced alterations in cell viability, morphology, and particle uptake/phagocytosis. Simultaneously, inflammation and oxidative stress biomarkers were evaluated through Western blot (Nrf2, NQO1, HO-1, p62, p-p62, LC3A/B), ELISA (IL-6, IL-8, IL-1β), multiplex analysis, and RT-PCR. These comprehensive methodologies provided a thorough examination of the biological effects of the two black carbon particle types.

Results: Both black carbon types induced similar responses in macrophages, including particle uptake, cytokine production, and oxidative stress-related protein expression. The alterations we observed suggest an activation of the Nrf2-mediated antioxidant response, impairment in autophagy, and a potential decrease in cellular defense mechanisms against oxidative stress. The ameliorating role of the candidate post-biotic preparation was detected and quantified. These findings imply

potential pathways contributing to the onset of chronic inflammatory lung diseases and also therapeutic strategies to modulate them.

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P21-31
Predictive metabolomic signatures for safety assessment of three plastic nanoparticles using intestinal organoids

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Background: Nanoplastic particles are ubiquitous environmental contaminants with potential health risks, and mouse intesticular organoids provide an accurate *in vitro* model for studying these interactions. Metabolomics enables precise study of cellular and organoid responses.

Materials and Methods: This study used mouse intestinal organoids to explore differences in metabolites and toxicity mechanisms following exposure to three nanoplastics (PS, PTFE, and PMMA) using a cellular model.

Results: PS, PTFE, and PMMA exposure reduced mitochondrial membrane potential, intracellular ROS accumulation, and oxidative stress, and inhibited the AKT/mTOR signaling pathway. Untargeted metabolomics results confirmed that three types of nanoplastic particles modulate cell state pathways by modulating fatty acid metabolism, nucleotide metabolism, necroptosis, and autophagy.

Conclusion: Nanoparticle exposure induced metabolic toxicity in intestinal organoids. PS-NPs, PTFE-NPs and PMMA-NPs are cytotoxic and inhibit the AKT-mTOR signaling pathway by inducing oxidative stress and reducing mitochondrial membrane potential to induce apoptosis and necrosis.

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P21-32
Genotoxicity and phytotoxicity evaluation of Norfloxacin (NOR) and intermediate of NOR after ZnO treatment

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Extensive increase in the consumption of Norfloxacin (NOR) results in NOR contaminations in worldwide environments. ZnO nanocomposite was fabricated as photocatalyst and used to degrade antibiotics with excellent efficacy [1,2]. However, the effects of degraded NOR on living organisms remain unknown. Two independent experiments were conducted to investigate the effects of degraded NOR on onion roots and mung bean seedlings. In the first experiment, 10 ppm of intact NOR was degraded using ZnO to produce 0.55 ppm and 0.22 ppm of NOR, and degraded metabolites (DNOR). Onion bulbs were grown with deionized water (DW) for 24 hours. Subsequently, bulbs with growing roots were transferred to 4 sets of test tubes filled with DW, 10 ppm NOR, 0.55 ppm and 0.22 ppm NOR with DNOR. There were 10 replicates for each set of test tubes. Root lengths were determined at a 24-hour interval for 72 hours. After 24 and 48 hours, roots grown in 10 ppm NOR were significantly longer ($p<0.05$) than those grown in DW, 0.55 ppm and 0.22 ppm NOR with DNOR. After 24, 48, and 72 hours, roots supplied with 0.22 ppm NOR with DNOR were significantly ($p<0.05$) shorter that those supplied with DW, 10 ppm NOR, 0.55 ppm NOR with DNOR, suggesting adverse effect of 0.22 ppm NOR with DNOR on onion root growth. In cytological studies, onion root cell division growing in all treatments for 24 hours was observed under microscope. The results suggested genotoxicity caused by DNOR. As compared to DW, 0.22 ppm NOR with DNOR caused a reduction inmetaphase, anaphase and telophase indices leading to a decrease in mitotic index by 31.4%. Application of 0.22 ppm NOR with DNOR to roots resulting in a largest increase by 2.15-fold in total chromosome aberrations (TCAs). The contributions by anaphase bridge, lagging anaphase, disturbed metaphase, and sticky metaphase were detected and varied depending on concentrations of NOR and DNOR. In the second experiment, 10 ppm NOR was degraded by ZnO to produce 0.44 ppm, 0.329 ppm and 0.198 ppm NOR with DNOR. Petri dishes were prepared by placing filter papers on the dishes, and soaking filter papers in 5 ml of Murashige and Skoog medium for seed germination and seedling growth. Five seeds of mung bean were placed on the top of filter papers in each dish. Treatments comprising of 5 ml DW, 10 ppm NOR, 0.44 ppm, 0.329 ppm,and 0.198 ppm NOR with DNOR were randomly applied to petri dishes. There were ten replicas for each treatment. All petri dishes were kept in the dark at ambient temperature. On day 7 after seedling growth, root and shoot length, number of lateral roots, fresh weight and dried weight were recorded. There were no significant effects of any treatments on fresh weights, dry weights or shoot lengths, number of lateral roots of mung bean. Only 10 ppm NOR caused a significant reduction ($p<0.05$) in root lengths by day 7 of seedling growth. The results suggested that the degraded products of NOR by ZnO were not harmful to mung bean seedlings.

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P21-33
Damage towards human red blood cells by brominated flame retardants (BFRs) – molecular aspects

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Brominated flame retardants (BFRs) are synthetic compounds whose task is to reduce the flammability of polymeric materials, commonly used in industry as well as to produce everyday objects. The widespread use of these compounds is associated with human exposure to BFRs [1]. The most used BRF is tetrabromobisphenol A (TBBPA), whose annual production exceeds 220,000 t [2]. Tetrabromobisphenol S (TBBPS) was introduced into the market as a TBBPA substitute. There is very limited data on the presence of TBBPS in the environment and its effect on living organisms.

This study aims to identify the molecular mechanism of TBBPA and TBBPS damage in red blood cells leading to hemolysis. Previous research

has shown that TBBPA above a concentration of 10 μg/ml induces intense hemolysis of red blood cells and leads to an increased level of methemoglobin formation [3]. It has also been shown that TBBPA induces oxidative stress in cells and decreases ATP levels [4]. An appropriate level of reducing factors and an ionic balance maintained largely by ATP-dependent ion pumps are needed to maintain cellular homeostasis.

Therefore, total glutathione levels and some ion-dependent ATPases activities were determined in normal human red blood cells. Both parameters are crucial in cell functioning and are directly related to the protection of cells against oxidative stress.

Erythrocytes were isolated by centrifugation (600xg, 10 min, 20°C) from erythrocyte-leukocyte platelet buffy coats purchased from the Regional Centre of Blood Donation and Blood Treatment, Lodz, Poland. Erythrocytes suspensions in PBS with 5% hematocrit were treated with TBBPA (10–50 μg/ml) and TBBPS (10–100 μg/ml) and incubated for 24 h at 37°C. Glutathione content was measured using a commercial Glutathione Assay Kit (Sigma-Aldrich, Cat. no CS0260), and ATPases activities were determined with a commercial ATPase Activity Assay Kit (Sigma-Aldrich, Cat no. MAK113) with ouabain as a Na,K-ATPase inhibitor.

The obtained results indicate that TBBPA from a concentration of 15 μg/ml causes a decrease in the total ATPase activity. However, for TBBPS we do not observe any inhibition of the total ATPase activity up to a concentration of 50 μg/mL, although the Na,K-ATPase activity is slightly reduced. In the case of TBBPA, the total glutathione content remained at the level of non-treated cells up to a concentration of 15 μg/ml. From a concentration of 20 μg/ml, a decrease in glutathione content was observed, proportional to the increasing TBBPA concentration. In the case of TBBPS, no decrease in glutathione levels was observed up to a concentration of 100 ug/ml.

These results correlate with a threshold increase in hemolysis initiated by TBBPA at a concentration of 15 ug/mL.

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P21-34
SARS-CoV-2 omicron infection aggravates lung fibrosis through IL6/ CSF/ RANTES activation and dysfunction of lipid metabolism in mice with PHMG-induced lung injury

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Objective: There are many clinical cases showing that patients with underlying diseases may be more vulnerable to infect SARS-CoV-2. However, there is a lack of experimental evidences on the severity of SARS-CoV-2 symptoms depending on the presence or absence of underlying diseases. This study aimed to assess the effects of infection SARS-CoV-2 omicron in a mouse model of polyhexamethylene guanidine (PHMG)-induced lung injury.

Methods: To construct lung injury model, transgenic mice expression human ACE2 (K18-hACE2) were intratracheally administered with PBS or PHMG (0.6mg/kg). After a 7-day period, these infected with SARS-CoV-2 omicron via intranasal administration. Mice were grouped into the following groups: vehicle control (VC), PHMG, SARS-CoV-2, and PHMG + SARS-CoV-2. To evaluate effects, we executed analysis of histopathology, cytokine/chemokine expression, and RNA-sequencing.

Results: In analysis of BAL fluids, total cell count and SARS-CoV-2 infection related cytokine (IFNγ, IP-10, and MCP-1) significantly increased in the group of SARS-CoV-2 alone. Whereas, IL6, CSF and RANTES known as cytokine storm related factors were significantly increased in the group of PHMG+SARS-CoV-2. Lung histological findings were characterized by increased severity of granulomatous inflammation/fibrosis, cell infiltration, and macrophage activation in the group of PHMG+SARS-CoV-2 compared to the group of PHMG alone or SARS-CoV-2 alone. Chronic inflammation observed in the group of SARS-CoV-2 alone. Using the next-generation sequencing (NGS) analysis, we categorized the genes with the greatest expression changes in infectious mice with lung injury. 38 genes were classified as upregulated genes, and 98 genes as downregulated genes. In Gene ontology (GO) and disease ontology (DO) using these genes, the results showed that up-regulation of immune system process and fibrosis related genes in the infectious mice with lung injury. On the other hand, gene expression related lipid metabolism is down-regulated.

Conclusions: This study demonstrates that infection of SARS-CoV-2 omicron may aggravates lung fibrosis in mice with PHMG-induced lung injury through induction of cytokine storm and dysfunction of lipid metabolism. Although future studies will be needed to determine for biomarker or therapeutic strategy, these results are expected to provide molecular distinction occurring when patients with lung injury infected SARS-CoV-2.

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P21-35
Cardiorespiratory effects of indoor ozone exposure during sleep and the influencing factors: a prospective study among adults in China

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Ambient ozone (O3) is recognized as a signifcant air pollutant with implications for cardiorespiratory health, yet the effects of indoor O3 exposure have received less consideration. Furthermore, while sleep occupies one-third of life, research on the health consequences of O3 exposure during this crucial period is scarce. This study aimed to investigate associations of indoor O3 during sleep with cardiorespiratory function and potential predisposing factors. A prospective study among 81 adults was conducted in Beijing, China. Repeated measurements of cardiorespiratory indices reflecting lung function, airway inflammation, cardiac autonomic function, blood pressure, systemic inflammation, platelet and glucose were performed on each subject. Real-time concentrations of indoor O3 during sleep were monitored. Associations of O3 with cardiorespiratory indices were evaluated using linear mixed-effect model. Effect modifcation by baseline lifestyles (diet, physical activity, sleep-related factors) and psychological status (stress and depression) were investigated through interaction analysis. The average indoor O3 concentration during sleep was 20.3 μg/m3, which was well below current Chinese indoor air.

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P21-36
Effects of air pollution in asthmatic children: personal exposure to pollutants and biomarkers of effect in exhaled breath condensates

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The deleterious effect of air pollution on human health is of great concern in several regions of the world. It was recently estimated that about 9 million of deaths are attributable to air pollution, mostly to particulate matter (i.e. PM), which refers to a complex mixture of airborne chemicals. Although air pollution concerns all regions of the world, its level is rising in low-income countries and children living in developing countries are the ones the most at risk (100% of children are exposed to $PM_{2.5}$ and 83% to indoor air pollution).

The present project *AIRQALI 4 ASMAFRI* (*A4A*) aims to examine the air pollution-asthma relationship in Cotonou, a urban West Africa city. The *A4A* project will be a unique opportunity in sub-Saharan Africa to combine original environmental, medical, and social data based on a cohort follow-up of 720 urban schoolchildren. Thus, this project will contribute to addressing Africa's data gap on indoor and outdoor air quality and personal exposure and on asthma and health care use.

Finally, we will follow up a sub-sample of 300 asthmatic children for 30 months, and we will collect i) environmental data on collective (living area) and individual (home, school, commute) air pollution exposure; ii) monitoring data on health respiratory status; iii) survey data on economic and psychosocial vulnerability, lifestyle, and asthma and air pollution knowledge; risk perception associated with a polluted environment; and pollution avoidance behaviour.

The characterisation of air pollutants in schools and schoolchildren's neighbourhoods of residence will be achieved from outdoor concentrations of CO, NOx, PM_{10}, $PM_{2.5}$, O_3, VOCs hourly measured by multi-pollutants portable analysers. The personal pollutant exposure will be assessed using portable measuring devices (NOx, $PM_{2.5}$, O_3) and Radiello passive badges (BETX).Numerous studies have proposed the synergistic effects of oxidative stress and inflammation as the main biochemical pathways of toxicity and health effects induced by air pollution. To estimate the effects of pollutant exposure on lung function, we will investigate several biomarkers detected in exhaled breath condensates (EBC) related to oxidative stress and inflammation such as Tumor Necrosis Factor alpha, interleukins (IL-2, 4, 5, 6, 8, 13, 14 and 17) and leukotrienes (B4 and E4). Finally nitric oxide (FeNO), carbon monoxide (CO) as well as the measurement of respiratory function by spirometry will be followed.

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P21-38
Heavy metals in the blue crabs (*Callinectes sapidus*) collected in coastal lagoons and shallow marine waters of Northwestern Adriatic Sea

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Purpose: The Atlantic blue crab (*Callinectes sapidus*), endemic to the eastern coast of America, is listed among the worst invasive species introduced into the Mediterranean. It was reported in the Emilia-Romagna region lagoons (Italy) in 2015 [1], and in very recent times it colonized all the Italian coastal areas [2]. This species poses threat to shellfish farming and biodiversity. A potential mitigation strategy is the increase of the fishing pressure, using this species as a food source for humans and animals. However, a risk for human health is the possibility of crabs accumulating pollutants, such as heavy metals (HMs). HMs are an important indicator of pollution, especially in the aquatic environment, due to their high toxicity and persistence, non-biodegradability, tendency to bioaccumulate in the trophic chain (biomagnification). The aim of this study was to evaluated the concentrations of 6 elements (lead, cadmium, chromium, mercury, nickel, arsenic) in the edible tissues of crabs collected along the Emilia Romagna coasts.

Methods: From August 2023 to March 2024, 30 samples were collected. All crabs were separated by sex, measured (carapace width), weighted and tissues pooled. An in-house triple quadrupole inductively coupled plasma mass spectrometry (TQ-ICP-MS) method was used to analyze 30 pool (15 for each sex) (152 specimens) of muscle and 30 pool of hepatopancreas, after a wet mineralization process with concentrated nitric acid. The method has been validated for detecting HMs in accordance with Regulation (EC) 333/2007 [3], with a limit of quantification (LOQ) of 5 µg/kg. The results obtained by males and females were compared for the concentration of HMs.

Results: HMs investigated in muscle tissues and hepatopancreas of male and female *C. sapidus* individuals were Pb, Hg, Cd, Ni, As, and Cr. No sex-related differences were observed for any metal. Preliminary results showed no tissue-related differences for metals such as Hg, Cr and As. For Pb, Cd and Ni, significant differences for the hepatopancreas and muscle tissue ($p<0.001$) were observed. Pb concentration in the hepatopancreas was threefold higher than in the muscle, with an average concentration of 0.061 mg/kg. Cd concentrations were several times higher in the hepatopancreas (1.67 mg/kg) and were lower in muscle tissue ($p<0.001$). Ni concentrations were fivefold higher in the

hepatopancreas (0.506 mg/kg) than in the muscle. The present study is a preliminary evaluation of the concentration of heavy metals in *C. sapidus* sampled along the coasts of Emilia Romagna. The results have shown that no significant differences are observed between male and female individuals. A different accumulation capacity of HMs in the analyzed tissues was observed. In particular, significant accumulation of metals such as Pb, Cd and Ni was demonstrated in the hepatopancreas, while low levels observed in the muscle.

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P21-40
Indoor air pollution from volatile organic compounds in residential buildings in the European Union between 2010 and 2023

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Long-term exposure to volatile organic compounds (VOCs) in indoor environments can lead to diverse adverse effects including damage to the central nervous system and cancer. This study summarises data on levels of 16 VOCs in residential buildings in the member states of the European Union (EU) reported between 2010 and 2023. We obtained data on VOC concentrations by means of systematic literature searches in PubMed, Scopus and Web of Science databases. 1390 articles were identified, of which 112 were reviewed, with 33 yielding relevant data. We used these data to compare indoor levels of VOCs reported in the literature searches with their reference concentrations (RFC). The data extracted from each study were used to determine the concentration distribution for each VOC separately. The resulting concentration distributions were grouped by country, then weighted by the number of buildings studied and combined by VOCs using probabilistic Monte Carlo simulations. We show that the concentration of acetaldehyde in Romania (median: 68.6 μg/m^3, 1st and 99th percentiles: 16.1–362.3 μg/m^3), benzene in Cyprus (median: 3.43 μg/m^3, 1st and 99th percentiles: 0.2–44.5 μg/m^3) and formaldehyde in Romania (median: 89.4 μg/m^3, 1st and 99th percentiles: 8.8–247.1 μg/m^3) reached above their RFC of 100, 30, and 100 μg/m^3, respectively. In addition, we demonstrated that the level of trichloroethylene in France (median: 0.4 μg/m^3, 1st and 99th percentiles: 0.004–24.1 μg/m^3), Germany (median: 0.08 μg/m^3, 1st and 99th percentiles: 0.02–1.9 μg/m^3), and Lithuania (median: 6.9 μg/m^3, 1st and 99th percentiles: 3.8–11.9 μg/m^3), and xylenes in Lithuania (median: 1.1 μg/m^3, 1st and 99th percentiles: 0.5–177.6 μg/m^3) and Portugal (median: 3.8 μg/m^3, 1st and 99th percentiles: 0.6–39.4 μg/m^3) were above their limit value of 2 and 100 μg/m^3, respectively. Our research reveals that residential exposure to VOCs continues to be a public health concern in the EU. Although the EU has implemented limits for the emission of certain VOCs, further measures are necessary to limit the use of these chemicals in household products.

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P21-41
Deciphering sex-specific disruption in the nervous and reproductive systems through interactions of 4-methylbenzylidene camphor and nanoplastics in adult zebrafish

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Environmental pollution from plastic, particularly micro/nanoplastics (MNPs), is currently a topic of significant concern among both the scientific community and the general population [1]. We have observed that exposure to MNPs can induce neurotoxicity [2–4], cardiovascular toxicity [5], and endocrine-disrupting effects [6]. The main contributors to MNP pollution are personal care and cosmetic products (PCCPs), which commonly contain microbeads used for their abrasive properties, prevalent in dental and skincare applications. Microbeads are favored for their durability, cost-effectiveness, and superior performance compared to natural alternatives like inorganic powders or crushed shells [7]. However, due to the widespread use and disposal of these products into drains, MPs end up in wastewater systems, accumulating in various ecosystems and persisting for extended periods without degradation. 4-Methylbenzylidene camphor (4-MBC) is a key component of ultraviolet (UV) filters, crucial for protecting human skin from UV radiation's harmful effects [8]. Its widespread presence in anti-aging creams, body lotions, and other consumer products has led to its extensive distribution in surface water, sediment, and various organisms [9]. MNPs act as carriers for 4-MBC in both PCCPs and the environment. Our previous research showed that 4-MBC induces estrogenic effects through the brain-liver-gonad axis in zebrafish larvae aged 3 to 5 days post-fertilization, at concentrations relevant to environmental conditions (1.39−15.4 μg/mL) [10]. However, knowledge gaps persist regarding sex- and tissue-specific accumulation and potential toxicities from chronic coexposure to 4-MBC and MNPs.

In this study, adult zebrafish were exposed to environmentally realistic concentrations of 4-MBC (0, 0.4832, and 4832 μg/L) with or without polystyrene nanoplastics (PS-NPs, 50 nm, 1.0 mg/L) for 21 days. Sex-specific accumulation was observed, with higher concentrations in female brains, while males exhibited comparable accumulation in the livers, testes, and brains. Coexposure with PS-NPs intensified 4-MBC burden in all tested tissues. Dual-omics analysis (transcriptomics and proteomics) revealed dysfunction in neuron differentiation, death, and reproduction. 4-MBC-co-PS-NP exposure disrupted brain histopathology more severely than 4-MBC alone, inducing sex-specific neurotoxicity and reproductive disruptions. Female zebrafish exhibited autism spectrum disorder-like behavior, disruption of vitellogenesis, and oocyte maturation, while male zebrafish showed Parkinson's-like behavior and spermatogenesis disruption. Our findings underscore that PS-NPs enhance tissue accumulation of 4-MBC, resulting in sex-specific impairments in the nervous and reproductive systems of zebrafish. This discovery enhances understanding of ecological risks posed by 4-MBC and PS-NPs in aquatic environments, emphasizing the need for nuanced assessment.

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P21-42
Impact of Organic Extractable Matter (OEM) from $PM_{2.5}$ collected in Lebanon on human bronchial epithelial cells: insights into autophagy, mitophagy, and cell senescence Activation

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Nowadays, air-pollution-derived fine particulate matter ($PM_{2.5}$) is fully acknowledged to be a significant public health problem. $PM_{2.5}$ generally corresponds to a complex mixture of both inorganic (e.g. metals, ions) and organic (e.g. polycyclic aromatic hydrocarbons, PAHs, dioxins) chemical and biological (e.g. pollen, fungi, bacteria) components.

The critical role of the $PM_{2.5}$ organic extractable matter (OEM) in the adverse health effects is still fragmented. This study aimed to investigate the toxicological effects of OEM of $PM_{2.5–0.3}$ and quasi-ultrafine particles $PM_{0.3}$ collected in southern Lebanon, on normal human bronchial epithelial cells (BEAS-2B). The study also explores their involvement in activating processes associated with autophagy, mitophagy, and/or cell senescence.

The chemical characterization revealed the presence of a wide range of organic chemicals, notably PAHs, nitrated-PAHs (N-PAHs), and oxygenated-PAHs (O-PAHs), dioxins and furans. Genotoxic effects occurred in BEAS-2B cells with cell survival events and cell cycle deregulation, as supported by alterations of the protein expression of pP53, total P21, pH2AX, total MDM-2, pCHK-1 and 2, and the high concentration of 8-OHdG. Additionally, exposure to PM and OEM revealed an activation of the autophagy mechanism, as detected through the expression of LC3B marker.

Furthermore, there was evidence of mitophagy and regulation of mitochondrial quality control through the gene expression of *Fis1*, *MFN1*, *MFN2*, *OPA1*, *DRP1*, and *MEF*. Moreover, cell senescence was observed through the expressions of MMP9 and MMP3 proteins.

Given the original data reported in this study, future complementary works are also needed to better decipher the critical role of $OEM_{0.3}$ and the activation of processes involved in autophagy, mitophagy, and/or cell senescence.

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P21-43
Assessment of acrylamide levels in popcorn samples by liquid chromatography-mass spectrometry

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The European Commission published in 2019 a list of foods in which the presence of acrylamide (AA), a toxic substance formed during food processing that should be monitored [1]. Among these foods are cereal-based snack products such as popcorn. This snack is widely consumed either at home or at the cinemas, and its consumption has increased in recent years [2]. It is common to find microwave popcorn in butter, salted, sweet, and cooking varieties on the shelves of food establishments. Therefore, the aim of this study is to conduct a market study to determine which type of popcorn contains a higher content of AA. For this purpose, more than 70 samples of popcorn were acquired from several food establishments in Spain and cooked in the laboratory following the manufacturer's instructions for subsequent determination. Extraction was carried out through liquid extraction using water, and quantification was performed through liquid chromatography coupled to mass spectrometry (LC-MS/MS). The results obtained showed that there is no significant difference in the AA content among the different flavors of popcorn; however, a significant difference between the levels of AA was found between the microwave-cooked popcorn and those intended to be cooked in a popcorn maker or pan. From the data obtained, AA is frequent in measurable amount in popcorn and the ingredients present in the microwave popcorn bag as well as the cooking method are important factors to control for reducing AA levels in this popular snack.

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P21-45
Development of LC-HRMS untargeted analysis methods for neurological extracellular vesicles into environmental exposure science

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Extracellular vesicles (EVs) are tiny, membrane-enclosed particles that arise from parent cells and are excreted by diverse cell types into their immediate surroundings. The intersection of extracellular vesicles and exposomics presents exciting opportunities for understanding how environmental exposures influence intercellular communication and molecular signaling within biological systems. Emerging evidence suggests that environmental stressors can modulate the release, composition, and function of extracellular vesicles, thereby influencing cellular responses and disease outcomes. Moreover, EVs themselves can serve as carriers of environmental pollutants, toxins, and other bioactive molecules, contributing to their systemic dissemination and potential health effects. High-resolution mass spectrometry (HRMS) enables exceptional sensitivity and heightened selectivity by accurately measuring the mass of specific compounds under investigation.

The aim of this study was to develop and validate a highly sensitive liquid chromatography-high resolution mass spectrometry (LC-HRMS) method for quantitatively analyzing EVs and plasma from pooled samples, then used to analyze xenobiotics. EV concentration from astrocytes was measured via ELISA. In the samples analyzed, a wide range of metabolites and xenobiotics were detected in both plasma and neuronal EVs, with some xenobiotics showing comparable concentrations between EVs and plasma. For certain xenobiotics in EVs, we observed concentrations of up to 90% of those found in plasma. Certain compounds like Caffeine or DEET were found exclusively in plasma. The detection of pesticides such as atrazine, dichlorvos, and chlorfenviphos (with respective Mz of 216.02556, 220.95316, and 358.979886), and Detection of pesticides in EVs at 89%-135% of plasma levels, within EVs suggests that investigating the interaction between EVs and exposome factors could unveil novel mechanisms pertinent to environmental health and neurological disorders such as Parkinson's or Alzheimer's disease. within EVs suggests that investigating the interaction between EVs and exposome factors could unveil novel mechanisms pertinent to environmental health and neurological disorders such as Parkinson's or Alzheimer's disease.Integrating molecular analyses of EVs with comprehensive exposomic assessments offers the potential for gaining deeper insights into how environmental exposures shape cellular communication networks. Moreover, this approach may lead to the identification of biomarkers for exposure assessment and early disease diagnosis.

In summary, the study of extracellular vesicles and exposomics provides a comprehensive and nuanced understanding of the interplay between environmental exposures and biological systems. This approach enables the discovery of novel mechanisms linking environmental factors to disease expression, offering insights into previously unexplored pathways of disease development and progression.

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P21-46
Toxicological effects of particulate matter emitted in a university cafeteria

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People spend more than 90% of their time indoors, including homes, workplaces, or schools. This makes them vulnerable to indoor air pollutants from a short distance (Cincinelli and Martellini 2017). Among these indoor environments, university cafeterias are a common place where students and staff relax, socialize, and have quick meals or snacks. However, the health impacts of the particles emitted in these spaces are not yet known (Alves *et al.* 2020; Gebrehiwot 2022). This study aimed to evaluate the mutagenicity and the potential toxicological effects of PM_{10} emitted in a university cafeteria using human alveolar epithelial cells (A549).

The sampling campaign was conducted for one week at the Léon University cafeteria. Samples were collected simultaneously indoors and outdoors during the opening hours. A background sample was collected on Sunday representing the non-occupancy period. The PM_{10} total organic extracts were tested for cell viability using the MTT assay on A549 cells. Flow cytometry was performed to analyze the effect on cell cycle dynamics and the production of reactive oxygen species (ROS). Cells were exposed to PM_{10} at the IC_{20} concentration both for ROS and cell cycle assays. The mutagenicity of the PM_{10}-bound polycyclic aromatic hydrocarbons (PAH) was assessed using the Ames test, where S. *typhimurium* TA98 strain with and without metabolic activation by the S9 fraction (rat liver microsomal fractions) was used.

The results demonstrated a significant cell viability decrease in all tested samples. Indoor PM_{10} showed the highest decrease of about 30% in cell viability at the maximum concentration tested (150 µg/mL) compared to outdoor and background PM_{10}, which showed a decrease of about 15% ($p<0.05$). Regarding the mutagenicity results, no mutagenic effects (mutagenicity ratio <2) were observed for all tested PAH extracts, both with and without metabolic activation by the S9 fraction.

Tests on ROS production and cell cycle dynamics are currently ongoing. The preliminary results on ROS production indicate an increase in ROS levels in indoor PM_{10} at the IC_{20} concentration when compared with outdoor and background PM_{10}. Additionally, changes in cell cycle

dynamics were observed for all samples. These changes include an increase in the percentage of cells at G0/G1 phase accompanied by a decrease in cells at S and G2 phases. This suggests an arrest at G0/G1 phase where cells remain quiescent without replicating DNA.

According to these findings, PM_{10} collected in a university cafeteria induces toxicity in lung cells. Since the cafeteria is a popular space for socializing and having quick meals and snacks, it is important to conduct further research to fully understand the health impacts of these pollutants and take necessary measures to mitigate any negative outcomes.

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P21-48
Toxicity of subway related nanoparticles in human lung cell models

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Air pollution in cities poses a significant health risk and particulate matter is classified as a Group 1 carcinogen. Among the various pollutants, nanoparticles – often defined as particles smaller than 100 nm – are believed to play a critical role in toxicity due to their higher surface-to-volume ratio and reactivity compared to larger particles. Metal-containing nanoparticles can for instance participate in Fenton-like reactions, leading to oxidative stress and DNA damage. Subways are a major source of airborne metal-containing particles in urban areas. Particles from the subway have previously been suggested to be more toxic than particles from tire wear, road dust, or wood combustion. However, research on the specific role of nanoparticles in subway particle toxicity remains limited.

In our study, which is part pf the European nPETS project that focuses on understanding toxicity and health effects of nanoparticles from different transport modes [1], we compared the toxicity of nanoparticles from different subway materials including rails, wheels and third rail. All particles were generated in the laboratory from electrodes of the same material as in the Stockholm subway system with a spark discharger. The respective particles, along with nanoparticles generated from iron electrodes as a reference, were thoroughly characterized regarding elemental composition, size and form. By using a human lung cell model (A549) and monocyte-derived macrophages (dTHP-1), the subway related nanoparticles were tested for cytotoxicity, genotoxicity, oxidative potential as well as inflammation response by different approaches including the alkaline comet assay, real-time quantitative PCR and MSD multiplexing.

The results showed that the nanoparticles mainly consisted of iron and had a primary size of 7–10 nm but formed agglomerates. Only low cytotoxic effects were observed in the doses tested (10–200 μg/mL), but a clear and concentration-dependent increase in DNA damage was noted in both cell lines investigated. Also, increased expression of the pro-inflammatory cytokine *IL-8* was noted upon exposure of dTHP-1 cells, but not in A549. However, no or less clear changes were noted for the other genes tested (*IL-1β, IL-6, TNF-α, HMOX, GADD45*). No changes in release of cytokines (IL-8, IL-6, TNF-α, IL-1β) were observed and none of the nanoparticles tested led to an increase in acellular ROS formation. Overall, we could only find minor differences in the toxicity between these nanoparticles. Taken together, our results indicate relatively low cytotoxicity and inflammatory potential of agglomerated nanoparticles of subway-related materials but a dose-dependent increase in DNA damage was observed.

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P21-49
Airway impedance in Mexican children exposed to artisanal brick kiln fumes

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Background: During artisanal brick production, a large amount of gases such as carbon monoxide, nitrogen oxides, sulfur dioxide and solid particles less than 10 and 2.5 microns are emitted into the atmosphere. Artisanal brick kilns are particularly common in certain marginalized areas in Central Mexico, posing a real health threat for inhabitants near communities, especially for children.

Purpose: To measure airway resistance and reactance in children exposed to artisanal brick kiln fumes and to compare results in the same parameters in non-exposed children (control group).

Methods: We recruited children from 6 to 12 years old, both genders, exposed and not exposed to artisanal brick kiln fumes. Clinical and sociodemographic history as well as frequency of respiratory symptoms were obtained through a validated questionnaire. Airway impedance was evaluated by pulse oscillometry with the Vmax® Encore system oscillometer following quality criteria of the American Thoracic Society (ATS).

Results: 84 non-asthmatic children were included; 25 (29.8%) of them were exposed to artisanal brick kiln fumes (exposed group), while 59 (70.2%) were non-exposed (control group). The exposed group showed a higher frequency of allergic rhinitis (OR=4.6, 95% CI: 1.02–21.3). Also, we observed that resistance at 5 Hz (R5Hz) and 20 Hz (R20Hz) were significantly higher in exposed children (p=0.04). Results from a generalized linear model showed that, after adjusting for tobacco smoke and wood smoke exposure, variable "place of residence" was the factor most associated with oscillometry alteration, since living near an artisanal brick kiln increased between 4 to 15% R5 Hz parameter.

Conclusions: Artisanal brick kiln fumes exposure in children aged 6 to 12 was associated with airway impedance alterations. Proximal and peripheral airway resistance is significantly higher in exposed children. Children exposed to artisanal brick kiln fumes present 4.3 times greater risk for presenting proximal airway obstructive alterations.

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P21-50

Sex hormone status of male Wistar rat offspring prenatally exposed to pyrethroid insecticide

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Infertility presents worldwide health issue that affect 15% reproductive-age couples, with male factors responsible for 40–50% overall cases. Exposure to endocrine-disruptive chemicals (EDCs) from the environment, including pesticides, is one of the factors associated with impaired male fertility. Pyrethroids are world-widely used insecticides that can act as EDCs and affect male fertility by disrupting sex hormones, among others. Exposure during *in utero* development when gonadal sexual determination and testes formation occur presents a critical sensitive window of vulnerability to EDCs. Alpha-cypermethrin (α-cyp) is a pyrethroid insecticide whose effects as EDC on male sex hormones is still unexplored. Thus, the aim of this study was to evaluate the effects of prenatal exposure to α-cyp on hormone levels of foetal and pubertal male offspring. For this purpose, pregnant Wistar rats were orally exposed to α-cyp at 1, 10 and 19 mg/kg bw/day, diethylstilbestrol (positive control), corn oil (solvent control) and water (negative control) from the 6th to the 21st day of gestation (DG). The gravid uterus was dissected under general anaesthesia at 21st DG and the blood from male foetuses was sampled. After confirmation of the puberty onset male offspring were weighed, testes were isolated under general anaesthesia and the blood for hormone analyses in serum was sampled. The testosterone and progesterone serum levels were measured by the enzyme-linked immunosorbent assay (ELISA) using commercial kits and according to the standard protocol supplied by the kit manufacturer. No effect of prenatal α-cyp exposure was observed in body mass and testes weight of pubertal male offspring. Prenatal exposure to α-cyp significantly affected testosterone levels in foetal male offspring serum and progesterone levels in pubertal male offspring serum. Testosterone levels in pubertal male offspring serum did not different between groups. These findings suggest that prenatal exposure to α-cyp disturbs sex hormone levels in foetal and pubertal male offspring and call for further research of endocrine disruptive properties of this pyrethroid insecticide.

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P21-51

Does 5G exposure activate UCP1 adaptive non-shivering thermogenesis?

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Introduction: The emergence of the fifth generation (5G) of wireless technology has led to major advances in the telecommunication industry. However, the rise of 5G network has raised major concerns about the possible health effects caused by radiofrequency (RF) exposure [1]. Young people are more exposed to 5G RF due to their higher mobile usage. Children have an elevated specific energy absorption (SAR) of wireless radiation compared to adolescents [2]. Previous RF studies have shown that rats adopted some thermoregulatory responses similar to those observed in cold environment [3,4]. Adaptive non-shivering thermogenesis plays a vital role to maintain the body's temperature within its normal range when exposed to cold stimuli [5]. Specific effects of 5G exposure on this mechanism are unknown. Based on our previous research into 2G RF effects, we aim to assess how 5G (3.5 GHz) and 2G (900 MHz) exposure influence UCP1 adaptive thermogenesis in rats, and thereby evaluate the impact of 5G exposure on thermoregulation.

Methods: Wistar rats of different ages were used: young (3-week-old) and adolescents (8-week-old). They were randomised into 3 subgroups: 5G group (3.5 GHz), 2G group (900 MHz), and control group (SHAM). Animals were exposed to their respective RF signals for a period of 1 or 2 weeks at an intensity of 1.5 V/m during 2 sessions of 1 hour per day. SAR values for the 5G and 2G groups were estimated to 0.07 mW/kg and 0.24 mW/kg respectively. At the end of exposure, RT-qPCR was carried out on the collected brown adipose tissues (BAT) to study genes implicated in this thermogenic pathway (UCP1, PPAR-α, PGC1-α, PRDM16). A Shapiro–Wilk normality test and a one-way ANOVA followed by post hoc Tukey's test were used for statistical analysis.

Results: Results showed that exposure to RF signals (5G and 2G) did not significantly modify the transcriptomic activity of UCP1, regardless of exposure duration or age. However, few cofactors were significantly upregulated in both age-groups, only after 2 weeks exposure to 5G. The transcriptomic expression of PRDM16 was four times higher in young 5G exposed rats compared to control group ($p<0.05$). Adolescent 5G exposed rats showed a slight upregulation of PGC1-α compared to SHAM ($p<0.1$) and PPAR-α compared to the 2G group ($p<0.1$). Our results indicate that one-week exposure is insufficient to induce transcriptional changes in UCP1 adaptive thermogenesis. Also, 2G exposure (900 MHz) did not alter this pathway in both age-groups.

Conclusion: Our findings suggest that 5G exposure could influence specific thermoregulatory cofactors similarly to those activated by cold stimuli. The PRDM16 gene is crucial for BAT hyperplasia and the browning of white adipose tissue (WAT), two physiological responses commonly observed upon cold exposure. These processes are currently being studied to better understand the underlying mechanisms between RF exposure and thermoregulation.

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P21-52

Paraquat disrupts KIF5A-mediated axonal mitochondrial transport in midbrain neurons and its antagonism by melatonin

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Paraquat (PQ) is a broad-spectrum herbicide used worldwide and is a hazardous chemical to human health. Cumulative evidence strengthens the association between PQ exposure and the development of Parkinson's disease (PD). However, the underlying mechanism and effective interventions against PQ-induced neurotoxicity remain unclear. In this study, C57BL/6J mice were treated with PQ (i.p., 10 mg/kg, twice a week) and melatonin (i.g., 20 mg/kg, twice a week) for 8 weeks. Results showed that PQ-induced motor deficits and midbrain dopaminergic neuronal damage in C57BL/6J mice were protected by melatonin pretreatment. In isolated primary midbrain neurons and SK-N-SH cells, reduction of cell viability, elevation of total ROS levels, axonal mitochondrial transport defects and mitochondrial dysfunction caused by PQ were attenuated by melatonin. After screening of expression of main motors driving axonal mitochondrial transport, data showed that PQ-decreased KIF5A expression in mice midbrain and in SK-N-SH cell was antagonized by melatonin. Using the *in vitro* KIF5A-overexpression model, it was found that KIF5A overexpression inhibited PQ-caused neurotoxicity and mitochondrial dysfunction in SK-N-SH cells. In addition, application of MTNR1B (MT2) receptor antagonist, 4-P-PDOT, significantly counteracted the protection of melatonin against PQ-induced neurotoxicity. Further, Kif5a-knockdown diminished melatonin-induced alleviation of motor deficits and neuronal damage against PQ in C57BL/6J mice. The present study establishes a causal link between environmental neurotoxicants exposure and PD etiology and provides effective interventive targets in the pathogenesis of PD.

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P21-53
Investigation of the toxicity of 2-mercaptobenzothiazole in the human kidney cell line HK-2

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2-Mercaptobenzothiazole (MBT) is widely used in industry, particularly in rubber production. It is found in many products such as car tires, cables, rubber gloves, gaskets, shoes, toys, and swimsuits made from rubber. MBT is also a degradation product of biocides used in paper and leather products. Sodium and zinc salts of MBT are used as fungicides, microbicides, and bacteriostats. Occupational exposure generally occurs through dermal and inhalation routes. However, MBT has been detected in leakage waters of rubber installations and has been found in drinking water at parts per billion (mg/L) levels due to its mixing with drinking water. Exposure can occur through the consumption of wastewater containing MBT, contamination of food with wastewater, dermal contact with dust in the air, or inhalation of aerosols containing MBT. In addition to industrial use and occupational exposure, MBT poses a risk of oral exposure in humans due to its use as a pesticide. Substances taken into the body orally pass through the liver and kidneys for metabolism and excretion. Therefore, the liver and kidneys are the organs most threatened by toxic substances. Previous studies have demonstrated the hepatotoxicity of MBT, suggesting its potential for nephrotoxicity as well. The International Agency for Research on Cancer (IARC) has classified MBT as "probably carcinogenic to humans" (IARC Group 2A) based on limited evidence of its carcinogenicity in humans causing bladder cancer and sufficient evidence of carcinogenicity in experimental animals. The German Federal Institute for Risk Assessment has determined that emissions of 2-MBT from consumer products should be minimized as much as possible, and the European Chemicals Agency (ECHA) has included 2-MBT in the Community Rolling Action Plan (CoRAP) and called for its inclusion in bio-monitoring programs. In our study, the cytotoxicity, genotoxicity, apoptosis, and oxidative stress induction potential of MBT in the human kidney proximal tubule HK2 cell line, which has a high exposure risk and toxic potential, were investigated. The IC50 values obtained from the MTT and NRU tests for cytotoxicity were found to be 146.82 μg/ml and 86.03 μg/ml, respectively. It was observed that MBT induced ROS in a dose-dependent manner, and a dose-dependent increase in apoptosis was also detected. Results from the Comet assay were normalized to tail lengths of cells, revealing a dose-dependent increase in DNA damage. However, more research is required to validate our findings.

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P21-54
Searching for links in binding patterns among androgen and progesterone receptor ligands

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There is a growing concern that some chemicals of anthropogenic and natural origin occurring in the environment may cause endocrine-disrupting effects in humans and wild animals. Androgen (AR) and progesterone (PR) receptors belong to a superfamily of nuclear receptors. Ligand binding domains of AR and PR are very similar; therefore, multiple compounds can bind to both receptors. Nevertheless, it is still unclear if a compound with an agonistic or antagonistic effect on one of the receptors will exert the same effects (agonistic or antagonistic, respectively) on the other receptor or vice versa. Surprisingly, studies that have reported a link between compounds with (anti-)progestogenic and (anti-)androgenic activities have focused only on the (anti-)androgenic activities of progestins. However, progestins are only one group of pollutants, and many more (anti-)progestogenic and (anti-)androgenic compounds are likely to be in the environment.

This study aims to search for links among compounds that bind AR and PR using *in vitro* bioassays and quantitative structure-activity relationship (QSAR) models. We targeted 64 compounds and 16 environmental water samples to be tested using PR and AR CALUX assay. Compounds found to be active on both receptors will be analyzed using QSARs to predict the activity and physicochemical properties of the compounds based on the analysis of the structures of molecules to their respective measured activity.

Our preliminary results found that flutamide, mifepristone, ulipristal acetate, bisphenol A, bisphenol AF, bisphenol TMC, 4-diethylamino-7-coumarin, benzo(a)pyrene, benzo(k)fluoranthene, bicalutamide, leflunomide, 4-methyl benzylidene camphor and homosalate

were antagonists of both AR and PR. Also, medroxyprogesterone, levonorgestrel, altrenogest, etonogestrel, gestodene, 17-alpha-ethinylestradiol and dexamethasone were agonists of both receptors. Cyproterone acetate, dienogest, nomegestrol acetate and progesterone, were agonists of 1 receptor and antagonists of the other. However, dihydrotestosterone, diclazuril, diclofenac, fipronil, diuron and flufenacet were found to be receptor specific (thus, they were active on 1 of the receptors and inactive on the other).The next step of this study will be to perform QSAR on the active compounds and test environmental water samples to determine the cooccurrence of the (anti-)progestogenic and (anti-)androgenic activities.

Such a study would aid researchers in characterizing both AR and PR ligands and give insight into the probability that a compound could exert multiple activities *in vitro*.

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P21-55

Human biomonitoring of parabens and dialkyl phosphates in urine and amniotic fluid from mother-child pairs cohort from the population of Sevilla (Spain)

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There is an increasing concern about the exposition during prenatal stages to environmental pollutants and their impact on the cognitive, motor, and intellectual development of the child. Among all population groups, pregnant women and newborns are two of the most vulnerable groups since these substances can cross the placental barrier and access the fetus, even from the earliest times of gestation. Parabens are a widespread group of endocrine-disrupting compounds (EDCs), with confirmed transplacental passage. The usage of cosmetics, pharmaceuticals, and consumer products during pregnancy that may contain parabens has led to the need for monitoring surveys. Also, organophosphates (OPs) are a large group of chemical substances that have been widely used as pesticides in agriculture and households. Dialkyl phosphate metabolites (DAPs) are hydrolysis products of OPs that have been widely used as bioindicators to reflect exposure to OPs.

Human biomonitoring (HBM) allows us to identify and eliminate possible sources of exposure, studying possible relationships between these pollutants and health problems.

The aim of this work was biomonitoring the levels of 7 parabens (methyl, ethyl, propyl, isopropyl, butyl, isobutyl and benzyl) and their main hydroxybenzoic acids metabolites (4-hydroxy, 3,4-dihidroxy and 3,4,5-trihydroxy) and 7 dialkyl phosphate metabolites (dimethyl, diethyl and dibutyl phosphates; dimethyl and diethyl thiophosphates and dimethyl and diethyl dithiophosphates) in a cohort of mother-child pairs in Seville (Spain) and to establish potential relationships between these chemicals and newborn's parameters at birth, pregnancy details and the epidemiologic information obtained using an extensive questionnaire data. Samples of maternal urine and amniotic fluid were taken at the time of delivery, previous signing of the informed consent, and the epidemiological questionnaire.

The extraction was achieved by EME (a simple liquid-liquid microextraction technique based on the use of an electric potential to achieve a selective extraction across an organic solvent known as supported liquid membrane (SLM)). The extracts were analyzed by ion pair liquid chromatography coupled to a triple quadrupole mass spectrometer (UPLC-TQ-XS, Waters™).

In most samples of both urine and amniotic fluid, the presence of parabens and/or their acidic metabolites was detected. Specifically, p-hydroxybenzoic acid was the compound that could be quantified in the highest number of samples. Additionally, the percentage of positive samples in amniotic fluid was higher than in urine samples for most of parabens studied. On the contrary, DAPs were detected only in some of the analyzed samples, which is reasonable due to the banning of most OPs in Europe. All values were inside normal ranges for the study population.

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P21-56

Shedding light on the epidermal alterations induced by blue light and heated tobacco products co-exposure: an *in vitro* study

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In recent years, tobacco manufacturers have been introducing alternative products due to the health risks associated with smoking. The most similar to cigarettes are heated or modified-risk tobacco products (HTP), has become particularly popular among young people since they contain lower levels of carcinogenic pyrolysis products formed by burning cigarettes and have different tastes and aromas. Especially after the COVID-19 pandemic, adolescents and young adults began to consume HTPs more frequently and, in addition, they started to use technological devices such as mobile phones and tablets persistently due to rapidly increasing online activities. Depending on the time spent in front of these devices, people are exposed to the increasing intensity of blue light (BL) emitted from electronics every day. It is known that BL might penetrate the skin more than UV. However, the phototoxic potential of BL on the skin is ignored in daily exposure. Interestingly, almost all studies conducted on tobacco focus on cigarettes or e-cigarettes, and the possible effects of HTPs on the skin have not yet been elucidated. The present study investigates how co-exposure to BL and HTP, two ever-present environmental factors in young people's lives, may lead to negative health outcomes by elucidating the potential adverse outcome pathways in human keratinocyte cells. For this purpose, the tar phase of HTP was extracted with a slight modification of our previous method [1], and the cells were exposed to BL for 30 and 120 minutes at 13 J/cm^2 in a closed chamber [2], where the control cells did not receive any BL or HTP. The current study employed the MTT assay to assess the dose-dependent cytotoxicity of HTP and BL exposure on HaCaT cells. The ROS levels were quantified with DCFDA to evaluate oxidative stress via fluorospectrometric analysis. Furthermore, the expression levels of matrix metalloproteinase-1 (MMP-1)and procollagen type-1, the key regulators of skin aging and epidermal integrity, were detected via ELISA. Results indicated that BL exposure led to a time-dependent cytotoxicity in HaCaT cells whereas only HTP did not exert a notable dose-dependent cytotoxicity up to 60 µg/mL. However, co-exposure of HTP+BL led to a remarkable decline in the cell viability of HaCaT cells, particularly in 120-min exposure. In parallel, elevation in the intracellular ROS generation, MMP-1 level, and

a decrease in procollagen-1 levels were observed in all HTP+BL groups ($p<0.05$). To conclude, it is expected that the findings obtained might enable the elucidation of toxicity pathways of both HTP and BL-mediated skin damage and might contribute to the development of specific adjuvant treatment methods. In addition, it is thought that enlightening the effects of HTPs on the skin might help to increase awareness, especially among the young population of high-ranked daily smoker countries.

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P21-58
AhR-activating polycyclic aromatic hydrocarbons disrupt metabolic pathways in human hepatocyte-like cells

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Polycyclic aromatic hydrocarbons (PAHs) represent a widely distributed group of environmental pollutants with numerous impacts on human health. They form a significant part of organic molecules associated with fine and ultrafine particulate matter. PAHs have been shown to exhibit numerous health effects and have been implicated not only in carcinogenesis, but also in processes linked with endocrine and metabolic disruption. This is particularly interesting in the light of recent observations that high levels of particulate air pollution are associated with metabolic disease. The aryl hydrocarbon receptor (AhR) is a major molecular target of various PAHs, and it is involved both in their bioactivation/detoxification and in their acute toxic impact on target cells. In our work, we have extensively characterized the AhR-inducing potencies of major PAHs being associated with PMs, including often neglected PAHs, that are rarely included in environmental monitoring. These include in particular high-molecular weight PAHs or various alkylated PAH derivatives. As recent studies also indicated that, apart from AhR activation, PAHs may also interfere with the activity of nuclear receptors contributing to the control of cellular metabolism, we evaluated their impact on selected metabolic pathways. We used two independent models of liver cells, differentiated human hepatocyte-like HepaRG cells and immortalized human MIHA hepatocytes. For our study, we selected two groups of PAHs: those acting as strong AhR agonists (benzo[k]fluoranthene, benzo[b]fluoranthene) and those with low agonist AhR activity (fluoranthene, pyrene), or moderate AhR agonists (benzo[a]pyrene). Our results showed that PAHs acting as efficient AhR ligands exert mostly negative effects on glucose production/transport or levels of triglycerides in the present cell models. Effects of PAHs could be thus linked with disruption of both transport and synthesis of important energy metabolism and storage molecules. Their impact on cellular metabolism and the functional role of the AhR in these effects thus deserve further attention.

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P21-59
Preliminary study on neurotoxic effects and mechanism of TDCPP, a typical organophosphate

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Tris (1, 3-dichloro-2-propyl) phosphate(TDCPP) is one of the most widely used organophosphorus flame retardants and has been widely detected in various types of samples from both the environment and human beings. Therefore, the health toxic effects of TDCPP have become a growing concern, especially neurotoxicity. In this study, human induced pluripotent stem cell-derived neural stem cells were used as an *in vitro* model to study the neurotoxicity of TDCPP and explore the underlying mechanism.

In this study, we examined biological endpoints such as cell viability, oxidative stress, cell cycle, and apoptosis to reveal the toxic effects of TDCPP on neural stem cells. It was found that 50 and 250 μM TDCPP significantly inhibited the viability of neural stem cells in a concentration – and time-dependent manner. TDCPP also significantly increased the level of reactive oxygen species (ROS) in neural stem cells, even after exposure to human-relevant concentrations (0.4 μM). TDCPP also interferes with the cell cycle of neural stem cells, arresting them in the S phase. TDCPP at 50 μM promoted the apoptosis of neural stem cells.

To preliminarily investigate the cell biological mechanism of TDCPP neurotoxicity, transcriptome analysis was performed in this study, which showed that TDCPP caused 204 significantly up-regulated genes and 183 significantly down-regulated genes in neural stem cells. KEGG enrichment analysis showed that the calcium signaling pathway was the most significantly enriched. Therefore, the present study further investigated the effect of TDCPP on the calcium signaling pathway by checking the changes in calcium content and the expression of related genes and proteins. It was found that TDCPP increased calcium concentration in neural stem cells. The expression of calcium signaling pathway-related genes such as RYR2, CALM2, PPP3CC, PPP3R1, CAMK2B, NFATC2, and GSK3β was inhibited, and the content of translated proteins was reduced, thereby affecting the regulation of calcium ion in neural stem cells and the activation of Ca^{2+}/CALM/CaN/CAMK signaling pathway. It also inhibits the expression and activation of NFATC2 and GSK3βproteins, which have been shown to be related to the formation of neuronal synapses, are essential for learning and memory processes, and may also play a role in neurodegenerative diseases and repair after nerve injury.

In summary, the present study confirmed that TDCPP promotes oxidative stress and apoptosis in neural stem cells, hinders their cell cycle, disrupts intracellular calcium homeostasis in neural stem cells, interferes with calcium signaling by inhibiting the expression of Ca^{2+}/CALM/CaN/CAMK, and inhibits the expression of NFATC2 and GSK3β proteins regulated by the calcium signaling pathway, which may affect neurological development and repair and induce neurodegenerative diseases.

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P21-60

Metabolic activities in Rainbow trout (Oncorhynchus mykiss) S9 fractions from liver and extrahepatic organs as an alternative *in vitro* ecotoxicity assessment approach

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Whole body biotransformation rate constants can be calculated using an appropriate *in vitro* to *in vivo* extrapolation (IVIVE) model. These models use CL,*In vitro*$_{,\,INT}$ rates derived with OECD Test Guideline 319B or 319A to estimate liver clearance rates, which are then extrapolated to a whole-body (*in vivo*) biotransformation rate constant. However, beside the liver, extrahepatic organs may also display Phase I and Phase II biotransformation activities and thereby play a role in metabolic clearance and bioaccumulation of compounds.

To adress these questions, we have maintained rainbow trout (Oncorhynchus mykiss) under controlled housing conditions according to OECD 319A/B. Specimens of eight sexually immature animals were harvested and pooled, including liver, gill, intestine, brain, heart and spleen. S9 fractions were prepared to determine the Phase I and Phase II enzyme activities by Liquid Chromatography-Mass Spectometry analysis. Cytochrome P450 activities, glucuronidation and sulfation activities were analyzed.

The liver displayed the highest Cytochrome P450 activities of all organs tested. Chorzoxazone Hydroxylase activity was only detectable in liver, 1-OH-Midazolam Hydroxylase activity was mainly restricted to liver, minor activities could be detected in intestine. However, Phenacetin-O-Deethylation was also detectable in other organs, with intestine, gill and spleen contributing 34, 18 and 11% of the total enzyme activity. Diclofenac-hydroxylase activity was present in all organs, as well as Bupropion-4-Hydroxylase activity, which was more or less evenly distributed among all organs. Phase II activities were detected in the liver, gill, intestine and heart, but not in spleen or brain.

In summary, the liver is the major organ for detoxification of compounds. However, extrahepatic organs, mainly intestine and gill, but also the brain, heart and spleen exhibit certain cytochrome P450 activities. Phase II enzyme activities were also detected in the intestine and gill. Our results suggest that extrahepatic organs, mainly intestine and gill, should also be taken into account when bioaccumulation and *in vitro* clearance rates are determined for IVIVE modeling in rainbow trout.

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P21-62

The copper-based nanopesticide Kocide 3000® worsens development of colitis in mice as a function of sex, and disrupts the intestinal barrier function in an enteroid-derived epithelial cell monolayer model from mouse gut organoids

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Nanoparticles (NPs) can cross gut barrier, interact with immune cells and alter gut microbiota with health concerns. Kocide 3000® (K3) is a copper ($Cu(OH_2)$)-based nanopesticide, but the intestinal effects of Cu-NPs compared to Cu-based conventional (non-nano) pesticide have not received attention. We aim to evaluate in mice the impact of K3 exposure on microbiota-dependent colitis, and the gut barrier tolerance to K3 using an enteroid-derived monolayer (EDM) model and immune cells from mesenteric lymph nodes (MLN) in comparison to Kocide 2000® (K2), a non-nanosized form.

Female mice were exposed to a control, K3 or K2-enriched diet from pregnancy to weaning of pups. All doses were adjusted for Cu content, and mice were exposed to 0, 0.25 and 25mg of K3/kg bw/d, or 0.215mg of K2/kg bw/d. Male and female offspring (F1) were fed as their mother until adulthood and colitis was induced by dextran sulfate sodium (DSS; 2% in drinking water) for 7 days, followed by a 5-day recovery, and disease activity was monitored daily. EDM were cultured from intestinal crypt stem cells of naïve mice and exposed for 24h to Cu-adjusted doses of K3 (0.025–25µg/ml) or K2 (0.0215–21.5µg/ml). LDH release was assessed for cytotoxicity, and gene expression of gut barrier homeostasis markers by qPCR. Genotoxicity was studied by 53BP1 and γH2AX immunostaining. MLN cells from naïve mice were stimulated (PMA/ionomycine or anti-CD3/anti-CD28) in the presence of K3 or K2 for 48h, and cytokine levels measured for direct effects of K3 and K2.

At 25mg of K3/kg/d (Cu NOAEL), F1 males were more susceptible to DSS-induced colitis, while females were protected. Colitis activity at 0.25mg of K3/kg/d and 0.215mg of K2/kg/d (Cu ADI) was not different from controls. To decipher the K3-related mechanisms in support of the colitis flare in males, the direct impact of K3 on EDM and MLN cells was evaluated. A slight cytotoxicity on EDM was noted at 25µg/mL of K3 only. Neither genotoxic nor oxidative stress effects were reported for K2 and K3 regardless of dose. Exposure of the EDM to K3 resulted in a decrease in the stem cell marker *Lgr5*, suggesting impaired epithelial renewal, while an increased mucin-producing gene *Muc2* expression occurred. Downregulation of the antimicrobial peptide genes *S100a8* and (dose-dependently) *Reg3*γ occurred after K3 exposure, possibly promoting gut dysbiosis. Interestingly, EDM alterations were not reported after K2 treatment, highlighting K3 effects linked to nanoformulation. Finally, no effect in cytokine production by MLN cells was reported whatever the treatmentThese data show that exposure to long-term K3 exposure through the diet aggravates colitis in a sex-dependent manner. As the immune cells do not respond to K3, the exacerbation of colitis could be due to Cu-NP-evoked disruption of the gut barrier integrity and its secretory functions. In addition to proper biocidal properties of Cu, this could aggravate the pro-inflammatory dysbiosis in DSS-treated males.

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P21-63

Towards an understanding of the relative toxicity of nanoparticles from different transport sources

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In recent decades the focus has increased on the smallest size fraction of particles found in urban air (less than 100 nm), called ultrafine particles or nanoparticles. A vast variety of health effects have been linked to particulate matter (PM) inhalation, including respiratory and cardiovascular diseases as well as cancer. There is, however, still a lack of understanding regarding to what extent nanoparticles from different sources differ in toxicity and health effects [1]. Within the project called nPETS (nanoparticle emissions from the transport sector: health and policy impacts), we have explored the toxicity of a range of nanoparticles formed in various transport systems. These include laboratory settings in which we generated nanoparticles from rail systems, as well as brake- and clutch wear. We also collected nanoparticles at different sites in Europe including in a road tunnel, subway, harbor, and airport. For road tunnel, subway and some brake materials, we compared the toxicity to larger sized particles (micron sized or PM2.5). We used the lung epithelial cell line A549 as well as differentiated THP-1 (dTHP-1) and explored cytotoxicity, DNA-damage (comet assay) and inflammation (secretion of IL-8, IL-6, TNFα and IL-1β). For some materials we mainly focused on inflammation in dTHP-1 cells.

The results showed that both nano- and micron sized particles from the road tunnel and subway caused DNA strand breaks and secretion of inflammatory cytokines. The cytokine secretion was mainly evident in the dTHP-1 cells and the micron-sized particles appeared more inflammatory. Brake wear particles showed in general low cytotoxicity and little inflammatory potential, except for one material (NAO, Non-Asbestos Organic) in nano-size. The harbor and airport nanoparticles (from Barcelona) showed low cytotoxicity but a high inflammatory potential. We are now exploring the possibility to generate "toxicity scores" of the nanoparticles from the different sources and to link the effects to their chemical composition.

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P21-64
Characterization of the toxic effects by the marine toxin ovatoxin-a on human skin keratinocytes

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Ovatoxin-a (OVTX-a) is the major palytoxin (PLTX) analogue identified in the benthic dinoflagellate *Ostreopsis cf. ovata* from the Mediterranean area. Humans can be exposed to OVTX-a mainly through inhalation of marine aerosol and/or skin contact with seawater during dinoflagellates' blooms, with possible threat to public health. Despite the hazard posed by PLTX has been extensively characterized, very few data are currently available for OVTX-a. Hence, this study was aimed at assessing the cutaneous *in vitro* effects of OVTX-a using spontaneously immortalized HaCaT keratinocytes.

The effects of OVTX-a ($1x10^{-16}$–$1x10^{-7}$ M) in HaCaT cells were compared to those of the reference toxin (PLTX), in terms of cell viability, cell necrosis, reactive oxygen species (ROS) production and mitochondrial depolarization. After 4 h exposure, OVTX-a induced a concentration-dependent cell viability reduction ($EC_{50}=8.3x10^{-9}$ M), with one order of magnitude lower potency than that of PLTX ($EC_{50}=3.7x10^{-10}$ M). Accordingly, OVTX-a induced a concentration-dependent increase of cell necrosis with a potency lower than that of PLTX. Moreover, despite OVTX-a increased ROS production similarly to PLTX, it caused a lower mitochondrial depolarization in keratinocytes with respect to the reference toxin. Then, to investigate the possible mechanisms involved in OVTX-a cytotoxicity, the same cellular parameters were assessed in presence of ouabain (OUA, $1.0x10^{-5}$ M) as inhibitor of Na^+/K^+ ATPase, the molecular target of PLTX, or diphenyliodonium chloride (DPI, $5.0x10^{-6}$), a non-specific inhibitor of flavoprotein-based enzymes, known to be involved in PLTX-induced oxidative stress. On the whole, results suggested that OVTX-a and PLTX share the same molecular target and mechanism of cytotoxicity.

In conclusion, this study provided a contribution in the characterization of the toxic effects of OVTX-a in skin keratinocytes. Although less potent than PLTX, the OVTX-a cytotoxic effects at nanomolar concentrations after a short exposure time rise some concern for humans exposed to this toxin during *Ostreopsis* blooms.

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P21-65
Androgenic and anti-androgenic effects of nanoplastics

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Excessive use of plastic and inappropriate disposal of plastic waste have led to a massive accumulation of plastic particles in the environment. Micro- and nanoplastics can be released into environment directly or derived from larger plastic items by mechanical, biological or chemical degradation. Due to their small size and large surface area, nanoplastics can penetrate various barriers and adsorb chemical substances onto their surface. This property represents additional risk for toxic effects on marine and soil organisms but also human health. [1] Although the presence of plastic particles in the human body was confirmed, the effect of these particles and their mixtures on the human endocrine system has not been clarified. [2]

This study aimed to evaluate toxicity and endocrine disrupting activity of 8 different plastic nanoparticles (PNPs) and their mixtures on cell line AREcoScreen GR KO M1. This cell line was developed to test the effects of various chemicals on the androgen receptor (AR) activity. In order to examine the influence of polymer type and nanoparticle size on their ability to interact with androgen receptor, we used polystyrene nanoparticles (PSNPs) sized 50 nm, 150 nm, 350 nm and polyethylene nanoparticles (PENPs) sized 50 nm and 350 nm. Additionally, we included polypropylene nanoparticles (PPNPs) sized 50 nm and 180 nm and polyethylene terephthalate nanoparticles (PETNPs) sized 80 nm. To examine the effects of PNPs complex mixtures, we combined nanoparticles of approximately the same size and used the same concentration of each polymer to prepare the mixtures. Cytotoxicity of PNPs individually and in mixtures was tested using MTS assay prior to any experiments on AR activity. To determine whether used PNPs and their mixtures were androgen receptor agonists or antagonists, transactivation assay was performed in accordance with the

OECD guideline No. 458.[3] While agonistic activity towards the AR was not observed for any of the tested PNPs or mixtures, results have shown that PPNPs of both sizes were androgen receptor antagonists. The same effect was observed with 350 nm PENPs and with mixture of 50 nm PSNPs, PPNPs, PENPs and PETNPs. While there were differences between observed activity in regards to the type of polymer, it seems that the size of the PNPs had little to no effect.

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P21-66

Soot particles induce cellular stress, inflammation and remodelling responses in human respiratory cells over time

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Soot particles in ambient air $PM_{2.5}$ have been associated with development and severity of chronic lung disorders, as chronic obstructive pulmonary disease (COPD). $PM_{2.5}$ regulatory limits are based on mass concentration and not particulate constituents. To understand underlying mechanisms associated with adverse health effects, there is a need to elucidate relationships between physicochemical characteristics and cytotoxicity of $PM_{2.5}$.The aim was to investigate the effects of airborne soot particles on the cytotoxic responses in respiratory cells, focusing on inflammation and remodelling processes. Human bronchial (BEAS-2B), alveolar epithelial (A549 and TT1) cells and human lung fibroblasts (HFL-1) were exposed at air-liquid interface for 1 hour to either 7 or 70 ng/cm^2 airborne soot particles (88 nm), including soot coated with biogenic alpha-pinene, using the Nano Aerosol Chamber In-Vitro Toxicity (NACIVT). TT1 cells were also cultured and exposed in decellularised rat precision cut lung slices (lung scaffolds). Exposure responses were measured at 24 and 72 hours post-exposure using the colorimetrics assays LDH (cytotoxicity) and WST-1 (metablic activity), luminex immunoassays, mass spectrometry based proteomics, and microscopy. The high dose (70 ng/cm^2) of biogenic coated soot particles reduced metabolic activity and induced cell death in monolayer cultures of HFL-1, BEAS-2B and A549 72 hours post-exposure and in TT1 cells 24 hours post-exposure. TT1 cells cultured in 3D scaffolds showed reduced metabolic activity and induced cell death 72 hours post-exposure. High doses (70 ng/cm^2) of uncoated and biogenic coated soot particles increased the release of the inflammatory cytokines MCP-1, interleukin (IL)-6 and IL-8 and the growth factors vascular endothelial growth factor (VEGF), endothelial growth factor (EGF) and hepatocyte growth factor (HGF) at 72 h in contrast to 24 h where minor alterations in release of inflammatory mediators were observed. Most pronounced effects were observed in the bronchial epithelial cells with markers related to fibrosis. Intracellular proteomic and pathway analysis showed that soot affected proteins related to cellular stress, cellular organisation, angiogenesis and extracellular matrix 72 hours post-exposure. In conclusion, airborne coated and uncoated soot particles induced inflammation and remodelling in human respiratory cells. The results illustrate the importance of longer post-challenge periods when examining cytotoxic responses and underline the importance to consider combustion-derived particle sources when developing air quality quidelines.

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P21-69

How special are nanoplastics: studying the difference between nanoparticle and nanoplastic cellular response under long-term repeated dose exposure scenario's

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The ubiquitous presence of small micro- and nanoplastics (MNP, <1mm) is raising concerns on their negative impact for human health. Understanding the underlying cellular mechanisms involved in nanoplastic effects is imperative for evaluating their environmental and human health risks accurately. One critical question that remains unresolved is whether the observed cellular responses to nanoplastics primarily stem from their general nanoparticle properties, i.e. the presence of a foreign particle, or from specific toxicological attributes intrinsic to nanoplastics. Addressing this question is pivotal for delineating the risks associated with nanoplastic exposure.

In this study, we aimed to explore the underlying mechanisms behind the impact of nanoplastics on cells, specifically focusing on the distinct effects between nanoparticle and nanoplastics exposure. For this we used a mixed exposure scenario using nanoplastics (polystyrene and polydisperse polyethylene nanoplastics (<800nm)) and manufactured nanomaterials, silica dioxide nanoparticles (150nm). Silica dioxide nanoparticles are one type of manufactured nanomaterials with high production volume and wide applicability which were included in the priority list of OECD reference materials for which risk assessment is urgently needed. The three nanoparticles were added in different ratio's per treatment (1:1:1; 1:0:1, 1:0:0, etc.) with two constant total particle concentrations (10^2 and 10^6 nanoparticles/mL). Caco-2 cells were exposed to the particle mixtures during 12 days under a repeated dose exposure scenario. Oxygen consumption rate was determined as a proxy for the bioenergetic state of the cells using

an extracellular flux assay on day 2, 6 and 12 of exposure. Additionally, sulforhodamine B assay was performed to measure total protein content as a proxy for cellular viability.

Particle exposure caused a significant decrease in protein content compared to the untreated cells from 6 days exposure onwards, indicating that particle exposure did cause either decreased cell growth or lower protein production per cell. The bioenergetics of the cells were affected by nanoparticle exposure with an observed shift towards glycolysis. There was no observed difference between the plastic and non-plastic nanoparticles indicating similarities in modes of action or downstream effects.

In conclusion, nanoplastic and non-plastic nanoparticle exposure affect cellular responses of Caco-2 cells in a similar way indicating it is rather a cellular response to a foreign particle then a plastic-specifc response. Importantly, a crucial characteristic of nanoplastics are their heterogeneity with different shapes, sizes and functional groups. This was not yet included in the study and the effect of these characteristics should be studied in future research.

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P21-70

Environment and drug marketing authorization: the new era with ERA

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The ERA is the Environmental Risk Assessment of the active substance of Human Medicinal Product (HMP), based on its release in the environment due to its use and disposal.

Since environmental protection is becoming a global issue, the ERA is more thoroughly defined by the just-revised guideline (2024). It describes in more detail a step-wise, tiered procedure based on the evaluation of the environmental exposure, considering the predicted worst-case scenario use (dosages and posology) of the product. ERA sheds lights on physico-chemical properties, on environmental fate and ecotoxicological effects of the examined HMP. All the required information for the environmental assessment needs to be adequate and accordingly with OECD-compliant like. Furthermore, in 2023, the European Commission has raised the curtain on reform of the new pharmaceutical legislation for HMP, to address environmental challenges, that supports initiatives under the European Green Deal.

To avoid unnecessary study replications, and in particular animal studies, Marketing Authorization Holders (MAHs) are encouraged to share their data with new Applicants. If a new applicant has access to an ERA that was performed earlier by another MAH, this ERA (including study reports) can be submitted only including a letter of access. If the reference ERA is not compliant with the current guideline (e.g. missing studies or expected increase of environmental exposure) the applicant should conduct the missing studies and/or update the ERA.

Nowadays, the rules for data sharing are not fully addressed in the available environmental law and guidelines; additionally, the “if” and “how” a financial compensation for the MAHs should still be defined. A possible strategy in light of the 3Rs (Replacement, Reduction, Refinement) could be setting-up an ERA monograph system for active substances, that would be available to Applicants when conducting an ERA for a new application.

Another approach could be the use of some tools, such as *in silico* and read-across methods, that may be helpful to interpret data and/or design more relevant tests, without replacing the studies requested.

In conclusion, considering that the ERA may need to be performed in parallel with the evaluation of the quality, safety and efficacy of the HMP, improved harmonizing regulatory measures are required for cooperation procedures and in case of data sharing. On the other hand, the strengthening of the requirements for the ERA in the market authorisation of HMP will drive pharmaceutical companies to evaluate and limit potential adverse effects to the environment and public health.

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P21-71

Expanding fish invitrome-based methods as alternatives to animal use in aquatic toxicity testing

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Aquatic environment often becomes a final sink for synthetic chemicals abundantly used in industrial societies, and hence effective risk assessment procedures are necessary to ensure adequate protection from unwanted chemical effects. Among the aquatic inhabitants, fish are particularly important providers of ecosystem services, as they occupy crucial positions in aquatic food webs and comprise a source of human food as well, thus linking environmental and human health. Therefore, environmental risk assessment of chemicals typically requires data on endpoints relevant for fish population health, such as survival, growth and reproduction. With conventional test methods, large numbers of fish are sacrificed each year to perform these assessments. This poses both economical (costs-related) and ethical (animal suffering-related) challenges. As an alternative to testing with live animals, fish cell line-based assays and molecular analyses could be used instead to predict endpoints of relevance. Such assays could further be combined together as individual, computationally-linked modules, to form a so-called fish invitrome framework for toxicity prediction, following the vision to realize an “alternative fish”. The proof-of-principle demonstration has been achieved with the establishment of the acute toxicity test performed with the rainbow trout gill-derived RTgill-W1 cell line, which was adopted in 2019 as the ISO standard 21115 and in 2021 as the OECD Test Guideline 249. Other modules currently being developed focus on growth, bioaccumulation potential, reproduction, and neurotoxicity, as well as on establishing a set of protein markers that can be used to monitor cellular stress responses and molecular mechanisms of toxicity. As an illustration, this presentation will show recent advances in the latter module, including proteomics profiling of several fish cell lines performed to understand their functional capacity and tissue specificity, and the analysis of (phospho)protein responses to growth inhibitors in the zebrafish embryonic cell line PAC2. We are convinced that employment of fish invitrome-based methods offers a means to overcome the limitations of traditional animal-centric approaches and opens up numerous avenues for industry engagement and innovation. Yet, the lengthy and resource-intensive validation requirements placed on alternative toxicity tests currently pose a persistent obstacle to their broader uptake. To address this, we have initiated a collaboration with social science researchers specializing in the study of innovation dynamics and socio-technical transitions. Capitalizing on our team’s collective expertise in natural and social sciences, we engage in a co-design process to further develop the fish invitrome framework with diverse stakeholder groups. With this, we expect to foster its wider acceptance and accelerate its practical implementation for use in regulatory toxicology and environmental risk assessment.

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P21-73
Monitoring of bisphenol A and S in hair during pregnancy

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Purpose: Bisphenols are a group of chemical compounds used in polymers and resins production. Two typical representatives of the species are bisphenols A and S, which are considered as endocrine disruptors. The main target of both bisphenol A and S, are the estrogen receptors (ERs), ERα and ERβ, resulting in affecting the secretion of insulin, diabetes mellitus, obesity, feminization, hypospadias, thyroid dysfunction, asthma and anxiety. Many studies have shown that exposure of pregnant women to these pollutants can affect fetuses.

Materials & methods: Head hair samples were collected from 49 mothers two weeks after delivery during the period 2022–2023. Segmental hair analysis was performed (3 cm per segment), for the proximal to head sample (9 cm), length that corresponds to the period of pregnancy. The samples were washed and extracted by methanolic liquid-solid extraction for 4 hours in an ultrasonic bath. The analysis was performed by liquid chromatography–mass spectrometry.

Results &discussion: Our results showed that the% detection frequencies of bisphenol A and S were 63.3% and 93.9%, respectively for the first trimester, 57.1% and 100.0% for the second trimester, 65.3% and 100.0% for the third trimester. The bisphenol A mean concentration levels for the first trimester were 224.8 pg/mg (median 120.6 pg/mg), for the second trimester 175.2 pg/mg (median 132.9 pg/mg) and for the third trimester 168.6 pg/mg (median 76.0 pg/mg). Bisphenol S mean concentration levels for the first trimester were 208.0 pg/mg (median 107.8 pg/mg), for the second trimester 177.3 pg/mg (median 113.9 pg/mg) and for the third trimester 131.6 pg/mg (median 71.1 pg/mg).

Conclusion: In this study, the percentage of positive samples for bisphenol S was found to be higher than bisphenol A. No observed difference on detection frequencies were depicted between the examined trimesters. A decrease in the mean concentrations of bisphenol A and S was noted during the last trimester of pregnancy.

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P21-74
Assessment of the (anti)androgenic potential of three organophosphate esters using the recombinant yeast androgen bioassay

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Introduction and aim of the study: Organophosphate esters (OPEs) are synthetic phosphoric acid derivatives widely used as flame retardants, plasticizers, and lubricants [1–3]. In recent years, the production of OPEs has rapidly increased. This is due to the withdrawal of brominated flame retardants, as well as the lack of regulation on OPEs usage limits [1]. The presence of OPEs has been detected in various environmental matrices and biota [1,2]. Also, the estimated daily intake of the sum of the eight most common OPEs is high, especially in toddlers (1.547 μg/kg b.w./day) [3]. Earlier toxicological studies have demonstrated that OPEs can cause various adverse toxic effects, including neurotoxicity, genotoxicity, and nephrotoxicity [1,3]. The risk associated with exposure to OPEs is intensified by the fact that these substances accumulate in adipose tissue, which hinders their rapid metabolism. Recent toxicity tests indicate potential reproductive and developmental toxicity and hormonal activity in humans and animals [1,4–5]. Since hormonally active compounds may cause harmful health effects, there is a need to identify their mechanism(s) of action. In this study we examined (anti)androgenic activity of three commonly used OPEs.

Materials and methods: The influence of BPA BDP (CAS No. 5945-33-5), TDCIP (CAS No. 13674-87-8) and TOCP (CAS No. 78-30-8) on the androgen receptor (AR) signaling was measured with the use of the recombinant yeasts, stably transfected with human AR genes and the yEGFP reporter gene under the control ARE sequences [6].The compounds were dosed at 9 concentrations ranging from 0,1 nM to 100 μM alone or in binary mixtures with 17β-testosterone (T, 100 nM, and 500 nM). Each bioassay was performed at least two times, for each concentration 6 replicates were applied. Before and after the exposure absorbance (λ=620nm) and fluorescence (λex/em=485/520 nm) were measured. The cytotoxicity and antiandrogenic activity of OPEs were assessed.

Results and conclusions: In the selected concentration range none of the compounds were cytotoxic for the yeasts above 20%. Neither of the OPEs showed androgenic activity. All compounds showed partial antiandrogenic activities in the presence of T. Their antiandrogenic activity decreased in the order TDCIP>TOCP>BPA BDP. At the concentration of 500 nM TDCIP, diminished the androgenic activity of 100 nM T by 40% and 500 nM T by 31% . At the same concentration, TOCP showed an antiandrogenic effect of 30% and 19% for 100 nM T and 500 nM T, respectively. BPA BDP diminished the activity of 100 and 500 nM T by 8 and 5%, respectively. Our study reveals that the three OPEs possess possible antiandrogenic activity. It is known that androgen functions could be adversely affected by antiandrogens that block the androgenic responses via AR antagonist mode of action [7]. Further research is warranted to fill current knowledge gaps, regarding the hormonal activity and mechanisms of action of other OPEs.

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P21-75
Protective effect of the carotenoid astaxanthin against Pb-induced cytotoxicity in human kidney cells

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Lead (Pb) is a non-essential metal and a major environmental contaminant that causes toxic effects in different target organs, including the kidneys. Different strategies have been considered in the literature to reduce Pb-induced toxicity. In this work we evaluated two complementary approaches to mitigate the cytotoxic effects of Pb in human kidney cells, using the macrocycle [15]pyN5, a pyridine-containing chelating agent, and the marine carotenoid astaxanthin (ATX). ATX displays several beneficial health properties and has been thoroughly studied regarding its action as a recognized potent antioxidant. Several experimental studies have shown that ATX is valuable against the toxicity induced by xenobiotics from different classes, including metals.

Thermodynamic studies of [15]pyN5 were carried out by our group and the protonation and stability constants calculated. The distribution of species of [15]pyN5 in the presence of different concentrations of Pb^{2+} was also carried out and the theoretical calculations revealed that this compound could be effective to decrease free Pb^{2+}, being thus a candidate to be further studied in cell-based assays. Both [15]pyN5 and ATX were evaluated alone and in the presence of different concentrations of Pb (in the form of nitrate) in non-tumoral human kidney cells (HK-2), using the crystal violet (CV) assay to assess cell viability. Pb showed to be cytotoxic in HK-2 cells (72 hour incubation), exhibiting clear morphological alterations at concentrations ≥10 μM, and an IC_{50} value of 26.3 μM. While the macrocycle [15]pyN5 did not reveal to be protective against Pb-induced cytotoxicity, ATX enhanced the viability of kidney cells exposed to this metal, being this increase significant for the maximum concentration tested of ATX (20 μM). In conclusion, the results obtained in this work suggest that ATX could be a promising compound that should be studied in greater depth in the context of Pb-induced nephrotoxicity.

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P21-76
Effects of tobacco heating products on viability, proliferation and cytokine production of mesenchymal stem cells isolated from periodontal ligament

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Effects of smoking on mesenchymal stem cell (MSCs) are not completely investigated and explained. MSCs from periodontal ligament (PDL-MSCs) are within oral cavity continuously exposed to various hazards, including cigarette smoke and tobacco heating products (THPs)-aerosol, that can affect their therapeutic potential. Although tobacco heating products are as preserved as safer tobacco-products, there is lack of evidence that can guarantee this assumption. The methodology required to examine effects of THPs on regenerative and immunomodulatory properties of PDL-MSCs included various experimental methods: NRU, MTS, ELISA and flow cytometry. After aerosol generation and cell exposure, changes in viability, proliferation and cytokine-production of PDL-MSCs were investigated. Regenerative capacity of PDL-MSCs decreased after treatment with THPs-aerosol. However, THPs showed potential to generate immunosuppressive response that can inhibit further cell decay. Even though results show less severe effects of THPs, compared to reuslt obtained after exposure to combustible cigarettes, further research is essential to evaluate their safety and impact on regenerative and therapeutic potential of PDL-MSCs to promote reparation and remodeling of tissue damages caused by long-time exposure to aerosol..

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P22 | Ecotoxicology

P22-02
Environmental hazard assessment of new pesticide formulations

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Studying the ecotoxicological properties of new pesticide preparations and assessing their potential danger to non-target environmental objects is important before their introduction into agricultural practice.

Aim: Summarize the main requirements for ecotoxicological studies and assessment of the ecological danger of pesticides. To determine the toxicity classes of new pesticide formulations for non-target test objects.

Materials and methods: The work uses OECD recommendations for determining the acute ecotoxicity of pesticides for aquatic and terrestrial environments following GLP principles on all provided non-target test objects (fish, daphnia, algae, earthworms, soil microorganisms, birds, bees). The selection of required test subjects for different plant protection products was based on their intended use, environmental exposure and conditions of use.

The studies were conducted only in compliance with the unified OECD guidelines for testing and GLP principles, seasonality of studies, recommended exposure time using appropriate reference substances, and chemical analysis of the content of the active substance in the studied environments.

The results: Ecotoxicological studies of 15 new pesticide formulations recommended for registration in Ukraine were conducted. It has been established that preparations containing herbicides (tribenuron-methyl, florasulam, flumetsulam, terbuthylazine, S-metolachlor, imazamox, desmedipham, phenmedipham, etofumesate) for fish, daphnia, algae, earthworms, soil microorganisms, birds, bees, according to The Global Harmonized Classification (GHS) is "not classified" or belongs to Acute Category 3. The diquat dibromide herbicide is highly toxic to algae (acute toxicity 1). The preparation containing the fungicide tebuconazole was very toxic to fish and daphnia (acute toxicity 2). Insecticidal preparations containing pyrethroids (λ-cyhalothrin, cypermethrin, bifenthrin) and neonicotinoids (imidacloprid, thiamethoxam, acetamiprid) or their mixtures had pronounced toxic effects on fish, daphnia and bees (Acute 1).

Conclusion: Representatives of the aquatic ecosystem are most sensitive to the action of some pesticide compounds. Therefore, when using such drugs, it is recommended to take the necessary measures to prevent direct entry into nearby bodies of water due to possible air and surface transportation. As expected, products based on pyrethroids and neonicotinoids are very toxic to bees. Therefore, it is essential to strictly adhere to the recommended consumption rates, processing frequency and application rules. In addition, it is necessary to inform beekeepers about their use timely.

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P22-03
Concentrations of toxic elements in freshwater fish (*Cyprinus carpio*) blood and eggs: potential health implications

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Toxic metals are among the most prevalent environmental pollutants and are known to cause various health problems in humans and animals. Fish are particularly sensitive to heavy metal toxicity due to their aquatic habitat and the potential for bioaccumulation in their tissues [1,2]. Common carp is still an economically important fish species and is often exposed to heavy metals in its natural habitat. The objectives of the study were to assess the levels of selected toxic elements (Cd, Pb, Hg) in fish blood and eggs, as well as to evaluate possible associations with the blood metabolic panel. The fish were caught using a seine net (locality – Nitra region, Slovakia). 16 females were included in the study. During the slaughtering process the fish were handled by an authorized person who strictly followed all relevant European regulations (Directive 2010/63/EU). After slaughtering (cranial concussion, following decapitation), the whole blood and eggs were collected. Metals concentration in blood and eggs were measured using a flame atomic absorption spectrometer (AAnalyst 200) and CVAAS MA-2 [3]. Parameters of comprehensive metabolic status (Ca, Mg, Na, K, Cl, urea, total proteins, glucose, AST, ALT, ALP, cholesterol, bilirubin, triglycerides, and creatine kinase) were analysed according to our previous study [4]. Concentrations of toxic elements was as follows: Cd >Pb >Hg in blood, and Pb >Cd >Hg in eggs. We can consider the reproductive system as a sensitive barometer of contamination, but the comparison of the monitored matrices showed higher levels of Cd ($p<0.001$) and Pb (ns) in blood according to eggs. Hg levels were significantly higher ($p<0.01$) in eggs. Correlation analysis revealed several associations of toxicants with parameters of metabolic status of fish. Significant positive correlations were observed between blood Cd and Ca, Mg, and AST ($p<0.05$ for all). Significant positive correlations were also observed between eggs Pb and glucose ($p<0.05$), and eggs Cd and triglycerides ($p<0.05$). Significant negative correlation was observed between eggs Hg and chlorides ($p<0.05$). Blood Hg concentration was also positively correlated with ichthyological parameters (total length and weight) at the $p<0.05$, which indicates increased bioaccumulation of this element in larger fish. The presented results represent the possible use of several parameters of the metabolic status of freshwater fish as indicators of freshwater biotope contamination. However, the expected associations with, for example, liver enzymes were not confirmed, which is why we recommend continuing ecotoxicological studies on other species of fish, as well as increasing the number of individuals in experimental groups in the future.

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P22-04
Health risk assessment of toxic metals (Cd, Hg, Pb) in common carp muscle tissue

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The research is devoted to the quantification and comparison of the accumulation of toxic metals – mercury (Hg), lead (Pb) and cadmium (Cd) in the muscle tissue of common carp *(Cyprinus carpio)*. The aim of the research was to identify potential inter-gender differences in

bioaccumulation and consequently to assess the toxicological risk of consumption of the fish to humans. The study was carried out on a sample of 20 individuals. The samples were equally divided into two groups of males and females. Ichthyologic parameters of the fish (total length, weight) were measured before starting the experiment to calculate FCF (Fulton's condition factor). Heavy metals content in muscle tissue was measured using a flame atomic absorption spectrometer (AAnalyst 200, PerkinElmer, USA). Tendency of heavy metals concentration in muscle tissue was as follows Hg<Cd<Pb. The results obtained were used to calculate metal pollution index (MPI), estimated daily intake (EDI) and total hazard quotient (THQ) for each metal. The sum of all THQ values was used to calculate the hazardous index (HI), which serves as an indicator of potential health risk. We concluded that all studied fish were in good condition based on the FCF calculations, which averaged 2.04. A common universal interpretation of FCF identifies healthy condition values of 0.8–1.2, but in the case of carp, an adjustment of these values is necessary due to the specific body shape. Based on the achieved results, statistically significant differences in accumulated heavy metal concentrations between males and females were found for Cd, with higher mean concentration in male´s group; P value: 0.0147. These findings may suggest that gender could play an important role in the bioaccumulation of some of the toxic heavy metals. For the other metals such as Pb and Hg, there were no statistically significant gender-based variations in bioaccumulation. Similarly, no differences were found for the calculated MPI and HI parameters between genders. The highest calculated MPI value was 0.6027, and the lowest was 0.3106, all of which indicate low levels of fish contamination. The highest HI value calculated was 0.4611, with an overall average of 0.25. Based on these HI calculations, it was found that none of the samples posed a health risk from consuming this meat, as none of the calculated values exceeded the threshold of 1, where we can consider the consumption of the product as risky. This research contributes to a better understanding of the bioaccumulation mechanisms of heavy metals in aquatic organisms. The results also provide important information for the development of strategies to monitor and reduce the risks associated with the consumption of fish from contaminated waters. This study was supported by The Ministry of Education, Research, Development and Youth of the Slovak Republic under the project VEGA 1/0571/23; and by the Slovak Research and Development Agency under the contract No. APVV-21-0168.

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P22-05
Associations between blood mercury levels and basic metabolic panel in freshwater fish (*Ctenopharyngodon idella*)

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Mercury is highly toxic element, particularly its organic compounds such as methylmercury that poses serious health issues, including damage to the nervous system, kidneys, and immune system. Mercury accumulates in the environment and moves up the food chain, particularly affecting fish [1,2] and, subsequently, animals and humans that consume fish. Fish absorb mercury from their environment, both through the water they swim in and, more significantly, through their diet [3]. This accumulation can result in mercury concentrations in fish tissue and body fluids that are much higher than those in the surrounding water. The aim of this study is to understand how mercury concentration affects biological systems (fish blood) at the molecular and biochemical levels. Blood samples (n=19) were collected during the autumn season, and the analysis of basic metabolic panel, including glucose, urea, calcium (Ca), sodium (Na), potassium (K), chlorides (Cl), magnesium (Mg), and phosphorus (P), was conducted. Total Hg was measured by cold vapor atomic absorption spectrometry [2]. After standard statistical analysis, we used Pearson's correlation analysis to examine potential relationships between the observed markers. The range of Hg content in analyzed fish blood samples varied from 0.001–0.102 mg/kg with mean Hg content value of 0.017 mg/kg. Analysis of correlation identified multiple relationships between mercury concentration and the metabolic status parameters (Na, K, Cl, Mg Ca, P, glucose, urea) as well as total length and weight of fish. The concentration of mercury in the blood exhibited a negative correlation with ichthyological factors such as total length and weight, with statistical significance at p=0,0002 and 0,006 respectively, suggesting heightened bioaccumulation of this element in smaller fish. For some biochemical parameters, no correlation was observed between them and mercury content. Significant negative correlation was noted between chlorides content and mercury concentration. There was also identified significant ($p<0.05$) positive correlation between glucose concentration and detected mercury content. Obtained results indicate the potential utilization of various metabolic status parameters in freshwater fish as indicators of contamination within freshwater habitats. Monitoring mercury levels in fish populations, including their blood, provides valuable information about the health of aquatic ecosystems and potential risks to human health through fish consumption. This study was supported by the Slovak Research and Development Agency under the contracts No. APVV-16-0289 and No. APVV-21-0168. This work was also supported by The Ministry of Education, Research, Development and Youth of the Slovak Republic under the project VEGA 1/0571/23.

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P22-06
Danio rerio embryos as a model for determining LC_{50}

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The increasing use of chemicals in agriculture requires proper ecotoxicological studies of chemicals introduced into the environment. One of the tests required as part of the registration process for compounds introduced into use is an acute fish toxicity test in accordance with OECD guideline 203. The use of vertebrate animals in studies is ethically controversial and attempts are being made to develop alternative methods to reduce or eliminate the use of these animals in research. One of the way is to introduce early developmental stages into research. Zebrafish embryos are a popular study model and one of their advantage is that they do not require Ethics Committee approval.

The aim of the study was to determine the LC_{50} values for danio embryos after exposure to six plant protection products (PPPs) and to compare them with the LC_{50} values obtained in the acute toxicity test on rainbow trout (historical data).

Initial tests were performed to determine the concentration causing lethality in developing embryos. The main tests were performed using seven increasing concentrations of PPPs, selected so that the lowest concentration did not cause toxic changes, and the highest concentration caused mortality of all embryos.

Developing embryos were observed for up to 96 hours. Three replicates were performed for the selected concentration range in the main tests, allowing 27 individuals to be obtained for each concentration. Additionally, two control groups were conducted: a negative group in which the medium was used, and a positive group in which the embryos were exposed to 3,4-dichloroaniline (3,4-DCA) at a concentration of 4 mg/L.

Embryos exposure to seven increasing doses with a multiplier of 1.5 between doses provides a spectrum from the lowest dose – which caused no changes during development – to the highest dose which caused mortality of all embryos. The Kraber method was used to estimate the LC_{50} value for each compound. Pearson's linear correlation was used to estimate the correlation of the embryo LC_{50} results with trout acute toxicity LC_{50}.

The following LC_{50} values for rainbow trout correspond to the LC_{50} for zebrafish embryos: PPP1 – 2.3 and 6.8; PPP2 – 4.20 and 274.7; PPP3 – 12.2 and 21.4; PPP4 – 13.8 and 28.5; PPP5 – 301.0 and 312.8; PPP6 – >100 and 415.6.The LC_{50} values for embryos are higher than the LC_{50} values obtained in acute toxicity tests on rainbow trout for the corresponding PPPs indicating a lower sensitivity of embryos compared to adult fish. The analysis showed a statistically significant correlation between the obtained results. The zebrafish embryos study is a very inexpensive and rapid method that does not require regulatory approval and allows the determination of the LC_{50} value. Potentially, the presented method in combination with established acute toxicity tests on aquatic crustaceans and cell lines may contribute to the reduction or even complete elimination of adult fish from acute toxicity tests.

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P22-07
High-content imaging to quantify mitochondrial toxicity in aquatic organisms

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New Approach Methodologies are required to elucidate the toxic mechanism in ecotoxicological studies. Alternative endpoints are e.g. necessary to improve chemical risk assessment for ecosystems. The development of automated fluorescence microscopy and specific molecular dyes offer new possibilities to detect changes in biochemical processes due to chemical stressors.

The aim of this study was to assess the applicability of high-throughput fluorescence microscopy methods to quantify sublethal effect in aquatic organisms. We tested the mitochondrial membrane potential dye JC-1 on the water flea *Daphnia magna* and validated the method by exposing our test organism to the model compound carbonyl cyanide 3-chlorophenylhydrazone (CCCP). The image acquisition was coupled with automated image analysis to quantify the JC-1 fluorescence signal as a measure for mitochondrial health. The validated method was then used to investigate the effect of the environmental contaminant 2,4-Dinitrophenol (2,4-DNP) on *D. magna*.

D. magna were exposed to CCCP concentrations between 0.5 and 2500 µg/L, or 2,4-DNP between 0.94 and 40 mg/L. Five daphnids per concentration were used and the experiments were conducted in triplicates. After the exposure, immobilization was measured according to the OECD 202 acute toxicity test and the JC-1 stain was applied. Fluorescence images where acquired in an automated confocal high-content imaging system and the signal intensities were quantified. Dose-response relationships based on the change in fluorescence intensities were calculated.

In all experiments, effects on the mitochondrial membrane potential were detected at lower concentrations than immobilization. Moreover, the *D. magna* that demonstrated altered JC-1 signal were fully mobile, showing that this effect is sublethal. In both experiments, the EC_{50}-values of mitochondrial membrane potentials after 2 h exposure were similar to their corresponding EC_{50}-values of immobilization after 24 h. The mitochondrial effect concentration was up to 30 times lower after exposure to CCCP compared to corresponding values in the OECD 202 acute toxicity test. After exposure to 2,4-DNP the mitochondrial effect concentration was three times lower .

Taken together, these results indicate that the image-based method is able to identify the difference in the test compounds' potency of uncoupling oxidative phosphorylation in mitochondriaof *D. magna*. Furthermore, effects were measured at a much earlier time point and predicted organism death after 24h and 48h. In conclusion, this new approach provides a rapid and sensitive mechanistic method to regular ecotoxicity testing of chemicals. With the application of additional dyes, multiplexed analysis could largely increase the toxicological data and help to understand the mechanistic links by minimal increase in time effort. In combinationwith other species (e.g. algae, fish), the overall understanding of a chemical's effect on ecosystems can be improved.

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P22-08
Reproductive disruption in male fathead minnow exposed to PFAS-contaminated groundwater at a legacy fire-training area

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The use of aqueous film-forming foams (AFFF) at fire-training areas (FTAs) has introduced into ground- and surface waters a complex mixture of per- and poly-fluorinated alkyl substances (PFAS). The toxicity of environmental PFAS mixtures to wildlife is not well understood and presents a knowledge gap that limits accurate risk assessment. To evaluate reproductive biomarker responses to complex environmental PFAS mixtures, we conducted a series of on-site experimentsusing flow-through mobile laboratories exposing fathead minnow (*Pimephales promelas*) to groundwater impacted by a legacy FTA and an adjacent reference site (REF). The measured PFAS concentrations at FTA sites included a high proportion of PFOS, PFOA, and PFHxS. Fish reproductive health was evaluated through assessment of secondary sex characteristics, testis histopathology, immunohistochemical detection of cell-cycle biomarkers (PCNA and TUNEL), sperm count, live cell sperm motility analysis, and analysis of testes transcriptomics. The results indicate exposure to PFAS-contaminated groundwater negatively impacts fathead minnow reproductive health.

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P22-09
TETHYS, new physiological model to help elucidate modes of action of thyroid disruptors

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Identifying endocrine disrupting chemicals is a priority. EU has defined a testing strategy to determine the endocrine activity for EATS (Estrogen, Androgen, Thyroid, Steroidogenesis). Regarding thyroid disruption, three OECD guidelines using *in vivo* non-mammalian models are validated, all of them are based on amphibian metamorphosis. To date no test guideline based on fish are available for the detection of thyroid active chemicals. We aim to fill this gap by developing the Transgenic Eleuthero-embryonic THYroid-Specific assay (TETHYS assay), a new test using medaka eleutheroembryos *(Oryzias latipes)* [1].

This test involves a transgenic line, expressing Green Fluorescent Protein (GFP) under the control of the *thyroglobulin (tg)* promoter. Tg is a precursor of thyroid hormones which expression is negatively controlled by thyroid hormones. A transgene was built by cloning the identified medaka *tg* putative promoter upstream of the *gfp* coding sequence. Series of micro-injections were performed in order to develop the transgenic line. When exposed for 72 h to activators or inhibitors of the thyroid axis, the thyrocytes change their size and express lower or higher levels of fluorescence, respectively. This reflects the regulation of *tg* by the negative feedback loop of the Hypothalamic−Pituitary−Thyroid axis.

A panel of reference chemicals was tested to characterize the different mode of action (MoA) detected by the TETHYS. The natural agonists of the thyroid receptor triiodothyronine (T3) and thyroxine (T4) were detected with lowest observable effect concentrations (LOECs) of 5 and 1 µg/L. Synthetic agonist GC-1 was also detected (LOECs 0.5 mg/L). Inhibitors of the thyroid peroxidase 6-N-propylthiouracil and methimazole and inhibitors of NIS sodium perchlorate and sodium tetrafluoroborate, chemicals that disrupt thyroid hormone synthesis, were detected (LOECs of 8, 10, 10 and 25 mg/L, respectively). The deiodinase inhibitor iopanoic acid was detected (LOEC 0,5 mg/L). Two environmental contaminants, tetrabromobisphenol A and diclofenac, known to disrupt thyroid axis by different MoA including inhibiting plasmatic transport were detected (LOECs of 5 and 100 mg/L). In addition, to investigate the specificity, we tested five chemicals that are inert on the thyroid axis: 17-alpha-ethinylestradiol, methomyl, abamectin, acetaminophen and cefuroxime which displayed no activity in the TETHYS.

Overall, our results show that all tested thyroid MoA are detected by the TETHYS, enhancing the array of thyroid MoA identified by transgenic eleutheroembryo assays by detecting MoA complementary to MoA detected by the XETA (TG n°248). In addition, this new assay using medaka offers the possibility to combine this assay with the RADAR test OECD guideline (TG n°251) and/or REACTIV assays (draft test guideline) using fish models to detect EAS modalities. This strategy could lead to reduce the time and number of eleutheroembryos associated with EATS assessment in fish.

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P22-10
Nitrite and nitrate content in leafy vegetables

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Mediterranean diet high in vegetables is highly advised in order to prevent a number of chronic diseases. The rising demand for vegetables on the market requires more intensive farming practices and the heavy application of fertilizers, the main source of nitrates, which are the basis for the possible formation of nitrites and nitrosamines. Although various studies are being conducted on the possible beneficial effects of nitrates and nitrites, there are still many controversies among the results, leaving them on the IARC list as probable carcinogens.

The legislation of the European Union sets the maximum permissible concentrations for nitrates only for a few types of vegetables, leaving out Swiss chard, which is a very common food in the menu of the inhabitants of the Mediterranean basin. Regarding nitrite content, no maximum permitted concentrations in vegetables have been set at all.

The aim of this research was to determine the concentrations of nitrates and nitrites in different types of vegetables that are commonly represented in the diet of the inhabitants of Split and Dalmatian County. 96 samples of different vegetables were randomly sampled from the local markets.

High-pressure liquid chromatography (HPLC) with a diode array detector (DAD) was used for nitrate and nitrite determination.The nitrate concentrations in the range 2.1–4526.3 mg kg^{-1} were found in 92.7% of the analysed samples. In 36.5% of the leafy vegetables intended for consumption without prior heat treatment, nitrite was found in the range of 3.3–537.9 mg kg^{-1}. The high levels of nitrite in the vegetables intended for fresh consumption and the high nitrate values in Swiss chard indicate the need to establish maximum nitrite limits in vegetables, as well as the broadening of legal nitrate limits to wide varieties of vegetables.

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P22-11
An Image-based toxicity screening method: Calcein AM as an indicator of esterase activity in Daphnia magna to detect sublethal effects *in vivo*

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Freshwater habitats are among the most threatened environments due to the release of a wide range of chemicals into the aquatic environment. These chemicals can induce sublethal changes in aquatic organisms that may affect growth or reproduction. It is therefore fundamental to develop new tools capable of rapidly assessing the adverse effects of chemicals in aquatic organisms by quantifying relevant ecotoxicological endpoints.

Daphniamagna is considered a keystone species in many freshwater habitats and is therefore extensively used as standard test species for testing chemicals. The relevance of *D.magna* for regulatory toxicity testing of chemicals also makes it important to develop additional methods and techniques for detecting negative effects in the species.

Applying the lipophilic dye Calcein AM combined with automated image analysis in the *D. magna* is a novel approach that allows visualization of chemical effects in an intact organism. Esterases are among the main enzymes in the detoxification process in *D. magna*. Intracellular esterase activity is a recognized parameter of cell health and can be used as a measure of cell viability. Calcein AM permeates cells and gets hydrolyzed by the intracellular esterase in viable cells into the fluorophore calcein, which emits a bright green signal.

This study aimed to develop an image-based screening protocol for sublethal adverse effects in *D. magna* using Calcein AM. Esterase activity signal was analyzed and quantified using Calcein AM after 24 hours exposure to three different model compounds known to have specific effects in *D. magna*: triphenyl phosphate and netilmycin sulfate interfering with the esterase activity and the insecticide methoxychlor, known to alter the viability and locomotor behavior of *D. magna*. The signal intensity in each exposure was used to calculate EC_{50}-values, which were compared to corresponding EC_{50}-values obtained from the traditional OECD acute immobilization test.

Results showed a decreasing fluorescence intensity equal to or similar to zero from Calcein AM with increasing concentration of Methoxychlor: 50–200 µg/L. These concentrations did not cause mortality. The EC_{50} obtained by the Calcein AM signal intensity was 28.9 µg/L compared to 171 µg/L from the *D. magna* OECD immobilization test, which demonstrates a higher sensitivity of the image-based screening method.

Taken together, this novel whole-organism image-based screening offers a rapid and sensitive tool to assess sublethal ecotoxicological effects in the aquatic environment.

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P22-12
Assessment of polystyrene nanoplastics toxicity on RTgill-W1 cell line

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Micro- and nanoplastics (MNP) are a multifaceted class of contaminants that include a variety of polymers with different morphologies and sizes. Due to their wide distribution, concerns have been raised over their possible detrimental effects on aquatic environments. Although many studies have been conducted to investigate the cytotoxic effects of MNP in the marine environment, freshwater research is still in its early stages. In this context, the purpose of the study was to assess the cytotoxicity of polystyrene nanoplastics (PS-NPL) on the rainbow trout (*Onchorynchus mykiss*) RTgill-W1 cell line.

PS-NPL were represented by colourless microspheres of PS that measure 100 nm (0.1 µm). RTgill-W1 cells were exposed to various concentrations (1, 10, 50, 100, and 500 µg/mL) of PS-NPL for 24 and 72 hours. Untreated cells were used as control. Cell viability and death were evaluated by the Live/Dead test and membrane integrity was examined by determining the activity level of lactate dehydrogenase (LDH) released into the culture medium. Cell morphology was examined by fluorescent staining of F-actin filaments and cell nuclei. The ability of PS-NPL to cause oxidative stress in cells was explored by measuring the content of reduced glutathione (GSH) antioxidant and the levels of malondialdehyde (MDA), as a lipid peroxidation marker. Moreover, the expressions of Hsp70 and Hsp60 proteins were also examined for an in-depth investigation of the pathways activated by oxidative stress.

The results suggested the cytotoxicity of PS-NPL, as indicated by the presence of dead cells after the treatment with 500 µg/mL for 24 hours and with doses higher than 50 µg/mL for 72 hours. Fluorescence microscopy imaging showed that PS-NPL decreased cell density and caused an altered and elongated cell shape-dependent on dose and exposure, particularly after 72 hours. A dose of 500 µg/mL increased LDH activity level by 17.16% and 39.13% compared to control, after 24 and 72 hours, respectively. Moreover, PS-NPL induced an increase in GSH and MDA concentrations after 24 and 72 hours. The highest levels of GSH (an increase by 104.79% over control) and MDA (an increase by 97.66% over control) were found after 24 hours of incubation with a dose of 500 µg/mL. In accordance with these, the protein expressions of Hsp70 and Hsp60 markers decreased significantly after the treatment with 50 and 500 µg/mL PS-NPL for 24 hours. An increase in Hsp70 protein expression was also noticed after 24 hours of incubation with a concentration of 500 µg/mL.

Overall, the results showed that PS-NPL could determine several toxic effects on the RTgill-W1 fish cell line, including alterations of the cell morphology, loss of cell membrane integrity, and the generation of oxidative stress by increasing MDA and GSH levels and decreasing the expression of Hsp70 and Hsp60 proteins.

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P23 | Occupational toxicology

P23-01
Assessment of pulmonary inflammation induced by cellulose nanofibrils: insights from rat model intratracheal instillation study

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Cellulose nanofibrils (CNFs), also known as cellulose nanofibers or nanofibrillated cellulose, are lightweight and robust materials with a low coefficient of thermal expansion, making them highly desirable for a wide range of applications. However, the inherent inhalation risks associated with CNFs present significant safety considerations. This study aims to examine the impact of intratracheal instillation of three CNFs, each at a dose of 2.0 mg/kg bw, on lung inflammation in rats over a 28-day period. Specifically, we investigated the effects of 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO)-oxidized CNF (CNF1), CNF produced via mechanical defibrillation using needle bleached kraft pulp (CNF2), and ultrashort CNF produced via mechanical defibrillation (CNF3) on pulmonary inflammatory response through histopathological examination and bronchoalveolar lavage fluid (BALF) analysis. Histopathological examination revealed distinct patterns among the groups. In the CNF1 group, a significant portion of the test substance reached the alveoli and was prone to phagocytosis by alveolar macrophages. Conversely, in the CNF2 group, most of the material remained in the terminal bronchioles, often leading to granuloma formation. The behavior of the CNF3 group resembled that of the CNF2 group. BALF analysis showed varying characteristics among the CNF groups. The CNF1 group exhibited a marked increase in the count of total leukocytes and normal macrophages. Conversely, the CNF2 group did not show significant changes in the counts of total leukocytes, neutrophils, lymphocytes, eosinophils, or basophils. In contrast, the CNF3 group displayed a significant elevation in the counts of total leukocytes and neutrophils. Significant increases in total protein and higher levels of lactate dehydrogenase were also observed in the CNF2 group, while no significant differences were observed in the CNF1 and CNF3 groups. The instillation of CNFs in the CNF3 group significantly elevated cytokine levels, including MIP-1α, IL-1β, and IL-18, in BALF on day 28. In contrast, there was no significant increase in these cytokines observed in the CNF1 and CNF2 groups. The results of this study suggest a critical relationship between lung inflammation and several properties of CNFs, including fiber diameter, fiber length distribution, and the methods used for CNF manufacturing. Understanding these associations is crucial for ensuring the safe utilization of CNFs in various applications. Moreover, the study emphasizes the necessity of thorough safety assessments and informed material design strategies to mitigate potential risks associated with CNFs and to unlock their full potential in diverse fields.

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P23-02
Detection of 25 PFAS in human blood by quantitative dried blood spots microsampling for large population monitoring

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Background: PFASs represent a class of synthetical chemicals, widely used in industrial, domestic, and consumer applications since the 1940s. They are ubiquitous in the environment, and they tend to bioaccumulate in tissues and fluids of human body. Following repeated exposure to PFAS, a broad range of adverse effects has been described: immunosuppression, hormones disruption, carcinogenicity, and lipids profile alteration. Therefore, monitoring PFAS levels in human blood is of paramount importance for public health policies. Compared to traditional venous blood, Dried Blood Spots (DBS) is an attractive and reliable sampling technique to assess the individual *exposome*.

Purpose: This study aimed to develop and validate an innovative analytical method based on quantitative DBS microsampling to identify and quantify a selected panel of 25 PFAS. The proposed method is highly promising for a simple, rapid, and cheap monitoring activity in exposed or at-risk population, such as pregnant women, newborns, or certain categories of workers.

Methods: A quantitative volume of 10 µL of fortified blood was deposited on *CapitainerB* card. After 3 hours of drying, 500 µL of methanol and internal standards were added. After 30 minutes of sonication and 10 minutes of centrifugation, the extraction solvent was evaporated under nitrogen flow and then reconstituted with 20 µL of 75:25 aqueous:organic mobile phases solutions. Finally, 3 mL of sample were injected into the UHPLC-MS/MS for targeted analysis. The calibration curve was built at six different concentration levels (2–5–10–20–50–100 ng/mL). The validation for sensitivity, specificity, linearity, accuracy, and precision was performed during three non-consecutive days, with three calibration curves for each session. Additional experiments were performed for matrix effect and recovery assessment. Stability was evaluated under three different storage temperature (-20°C, 4°C, 25°C) and time conditions (1 day, 2 weeks, 1 month).

Results: The developed method enabled to achieve LOD and LOQ values in the range from 0.4 (PFODA, PFOS) up to 1.0 ng/mL (PFOA, 3,6-OPFHpA) and from 0.8 up to 2.0 ng/mL, respectively. Accuracy and precision fulfilled the acceptability criteria within ±20% for each analyte at all concentrations. Extraction process showed high recovery above 80% for all analytes, whereas matrix effect experiment demonstrated ion enhancement for 13 molecules (+50%) and a moderate result for others (<50%). Consequently, the extraction protocol revealed a process efficiency higher than 100%. Real samples collected from *non-exposed* volunteers showed negligible levels of PFAS.

Conclusions: The validation results demonstrate that the proposed workflow, which combines the DBS microsampling with UHPLC-MS/MS instrumentation, is reliable, fit-for-purpose, and easily adaptable in the laboratory routine. The proposed approach provides a straightforward and effective solution to monitor PFAS levels in selected population.

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P23-04
In vivo investigation of the vestibular toxicity of industrial aromatic solvents in rats

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Numerous experimental studies in rats have shown that a limited number of aromatic solvents can cause hearing loss by targeting primarily the outer hair cells of the cochlea. In addition, most epidemiological studies investigating hearing loss in populations of workers have concluded that the risk of developing hearing loss increased when they were exposed to solvents alone or solvent plus noise. Surprisingly, there is no *in vivo* experimental data studying the effect of these substances on the vestibular receptor, an organ that shares many similarities with

the cochlea. Only one study using organotypic cultures of vestibular epithelia suggested that styrene and *ortho*-xylene were toxic for these structures but not *para*- and *meta*-xylene. The objective of this study is to determine, *in vivo*, if rats sub-acutely exposed to these same four industrial solvents develop balance deficit and vestibular impairment.

Rats were exposed for four weeks to styrene (1000ppm) and each of the three xylene isomers (1800ppm) by inhalation. The toxicity of these solvents on both the cochlea and the three vestibular epithelia (utricle, saccule and *cristae ampullaris*) were determined by counting the total number of hair cells in each of these structures. The fact that these different epithelia were dissected from the same animals, and that the same immunohistochemical stainings were used for the different tissues ensured that the comparison between cochlear and vestibular toxicities were unbiased. Hearing performance was assessed by Distortion Product Oto-Acoustic Emissions (DPOAEs), and vestibular function by measuring *post*-rotary nystagmus and two anti-gravity reflexes, the tail-lift and the air-righting reflexes.

Preliminary results confirm the toxicity of styrene and *para*-xylene on the cochlea, through the measurement of DPOAEs and histological assessment, but results obtained with *post*-rotatory nystagmus and the two anti-gravity reflexes tests do not suggest vestibulotoxicity. In addition, the quantification of hair cells in vestibular epithelia does not support a potential vestibulotoxicity in our experimental conditions. This study provides, for the first time, *in vivo* data about the effects of aromatic solvents on the vestibular receptor. Differences between *in vitro* and *in vivo* data are discussed.

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P23-05
Physiological and electrocorticographic pattern changes in adult Long-Evans female rats upon acute exposure to industrial aromatic solvents

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Industrial solvent exposure is known to induce depressant effects on the central nervous system and to affect behavior in both humans and animal models. Nevertheless, the specific effects on brain activity during acute exposure remain insufficiently investigated. The present work aims to evaluate the effects of three commonly used industrial solvents, styrene, toluene and methyl-ethyl-ketone (MEK) on the power of brain oscillations using wireless electrocorticography (ECoG), thereby identifying neurophysiological signatures of such exposures in rats.

Female rats (n=12/group) were implanted with ECoG electrodes soldered to a linear connector. When needed, the animals could be equipped with a wireless ECoG transmitter that permitted the signals to be acquired from inside the exposure chambers. The same animals were first recorded when placed in control conditions [air: 8h/d; 4d], and then during solvent exposure [air/1000 ppm solvent/air: 1h/6h/1h; 4d]. In addition, locomotion, sensory-motor coordination and strength impairment tests were performed at the end of air/solvent exposures, as well as post-rotatory nystagmus (PRN).

Toluene induced an immediate increase of the power of fast oscillations (30–90 Hz), which rose gradually over time, and a concomitant decrease in the power of slow waves (2–12Hz), in accordance with its known ketamine-like dissociative effect caused by NMDA receptor blockade. Styrene caused a global decrease in brain activity's total power, suggesting a strong neurodepressant effect. Toluene and styrene exposures increased the number and duration of saccades measured by PRN. No change in locomotion, sensory-motor coordination or strength was obtained following styrene exposure, while toluene induced a slight increase in motor activity. MEK showed no effects on cerebral oscillations, PRN or behavior, thus confirming the relative harmlessness of this solvent.A subsequent experiment using decreasing doses of styrene revealed that changes in oscillation power were dose-dependent, and remained significant at concentrations as low as 50ppm. These results suggest that ECoG is a quantitative and powerful tool to assess real-time dynamics of solvent's effect on brain activity, surpassing the sensitivity of traditional sensorimotor tests.

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P23-06
Intranasal deferoxamine mesylate alleviates silica-induced pulmonary fibrosis by regulating iron homeostasis

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Silicosis is characterized by chronic inflammation and progressive pulmonary fibrosis. Currently, the specific pathogenic mechanisms underlying silicosis are not completely understood, and no effective treatment methods have been identified. Iron accumulation in the lungs is strongly correlated with pulmonary fibrosis. However, the contribution of pulmonary iron homeostasis to the progression of silica-induced lung fibrosis is still a subject of controversy and the effectiveness of iron chelators as a therapeutic intervention remains uncertain. Deferoxamine mesylate (DFO), an iron chelator, is commonly used to treat iron overload disorders. Therefore, this study aimed to investigate the role of lung iron homeostasis and clarify the effect of the DFO on silicosis. RNA-seq data, pathological observations, and molecular biology experiments were used to analyze alterations in iron metabolism in silicosis models. Notable Prussian blue staining and increased iron content was observed in the lung tissues after silica exposure. Moreover, macrophages are crucial in maintaining iron balance and reusing substantial quantities of iron on a daily basis. Inductively coupled plasma mass spectrometry analysis confirmed elevated iron content in macrophage after silica exposure. These results suggest that exposure to silica can alter lung and macrophage iron homeostasis. Subsequently, through a combination of *in vitro* and *in vivo* experiments, DFO intervention restored imbalanced iron homeostasis in lung and macrophages, and lung fibrosis induced by silica. This study provides a theoretical foundation for understanding the role of iron homeostasis in silica-induced lung fibrosis. In addition, an intervention strategy utilizing DFO for silica-induced lung fibrosis has been proposed.

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P23-07
Occupational exposure to low-level lithium and thyroid dysfunction

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Background: Lithium is the main ingredient in the most preferred medications to treat bipolar disorder. The most common side effects of overdosing on lithium drugs include weight gain, polyuria and tremor[1]. Long-term use of lithium may cause hypothyroidism[2]. The side effects of taking lithium as a medicine are well known, but there is a lack of data on the health effects of long-term occupational exposure to low-level lithium.

Method: Blood and urine were collected from 310 male workers at two lithium-handling workplaces and 75 male workers at one non-lithium-handling workplace, and lithium in urine and thyroid stimulating hormone, free T4, and T3 in blood were analyzed. Logistic regression analysis was performed on urinary lithium concentration based on the median, and thyroid hormone concentration divided into whether it exceeded the reference value or not.

Result: The average age of study participants was 34.3 years, and the average period of current work performance was 26.4 months. The average urinary lithium concentration of workers at two workplaces that handled lithium and one workplace that did not handle lithium was 146.1, 55.3, and 28.5 μg/L, respectively. The average thyroid stimulating hormone concentrations were 2.21, 1.55, and 1.57 mIU/L, respectively, and the rates exceeding the reference value (0.27~4.20) were 18.2% (30/165), 8.3% (12/145), and 4.0% (3/75), respectively. Free T4 and T3 showed no significant differences between workplaces. Based on the median urinary lithium concentration, the odds ratio for exceeding the thyroid stimulating hormone reference value was 2.4.

Conclusion: There were 45 workers who exceeded the thyroid stimulating hormone reference value, and 42 (93.3%) were workers in industries that handle lithium, and the rate of exceeding the reference value was highest in the group with high urinary lithium concentration. Subclinical Hypothyroidism cannot be diagnosed based on a single increase in thyroid stimulating hormone concentration. However, we cannot rule out the possibility that the increase in thyroid stimulating hormone concentration in the study subjects was due to lithium inhaled during work. Additional observations are needed to clearly determine the relationship between long-term occupational exposure to low-level lithium and thyroid function.

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P23-08
A study of cumulative properties of a 10% α-cypermethrin-containing pesticide formulation

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Pyrethroids are a class of insecticides chemically derived from natural substances that have been developed to replace organophosphorus insecticides on the market. One of the widely-used synthetic pyrethroids is α-cypermethrin (CYP) [1]. CYP residues are found in the environment and food, as well as in human urine and breast milk, causing public health concern [2]. Although the main target of pyrethroids is the nervous system, studies have shown that exposure to CYP may be associated with reproductive toxicity, hepatotoxicity, immunotoxicity and genotoxicity [3]. In accordance with the local Russian requirements it is mandatory to assess the cumulative properties of all domestically produced pesticide formulations to protect the health of workers in occupational settings. In preventive toxicology the most used criterion for assessing the cumulative properties of a toxic agent is functional accumulation of effect, which stands for lethality upon repeated exposure [4].

The purpose of the study was to conduct an assessment of cumulative properties of a 10% CYP-containing formulation (emulsion concentrate).

48 white outbred male rats (200–220 g) were used in the study in a GLP OECD certified facility. During acute experiments, animals were randomly assigned to groups (6 animals). The administered doses (orally, fasted) were selected based on literature [1]. Clinical signs of intoxication were monitored in accordance with OECD 423 [5]. The cumulative effect of the formulations was determined in a 2-month study [4]. The treatment and control groups contained 10 animals each (randomization). The daily administered doses were 1/10 of acute LD50. Body weight was recorded weekly, STI (summation threshold indicator) was recorded every 2 weeks. Hematological and biochemical analyses were performed at the end of the study. Data were processed using IBM SPSS Statistics v.22 at $\alpha=0.05$.

Clinical signs in acute oral toxicity testing included: tremor; hypersalivation; decreased motor activity and respiratory rate; body weight loss; comatose state. LD50 for the formulation was 449.88±135.89 mg/kg bw.

During the study of cumulative effect, animals in the treatment group were excitable and aggressive. There was no mortality in both treatment and control groups. No difference in body weight gain was observed. A significant decrease in the concentration of basophils and monocytes was seen in the treatment group as compared to control. The analysis of the obtained biochemical parameters revealed no significant changes.

The study shows that the 10% CYP-containing formulation can be used in occupational settings under controlled conditions with mandatory precautions (individual protective equipment, proper ventilation, etc.), since the studied formulation did not cause lethality when the dose equal to 1/10 of acute LD50 was administered repeatedly for 2 months. Constant exposure to the formulation in normative doses is unlikely to present potential hazards.

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P23-09
Occupational dermal exposure assessment: an overview of the most relevant exposure estimation tools

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The assessment of occupational exposure to substances meeting the requirements of Article 14(4) of Regulation (EC) No. 1907/2006 (REACH), plays a crucial role in implementing risk management measures to ensure worker safety. In many cases, the use of a Tier 1 modelling approach to support the assessment of the uses identified is sufficient. In other cases, higher Tier modelling or appropriate data from measurements are required to conclude that uses are safe[1].

This study focuses on the occupational dermal exposure assessment of the Process Category PROC8a, which is intended to cover general transferring operations of large quantities of chemicals, without dedicated engineering controls in place for reducing exposure[2]. This kind of task is commonly assessed in the context of industrial activities, especially as PROC8a also covers cleaning and maintenance tasks. The dermal exposure estimate for PROC8a is quite high in comparison with other PROCs also frequently assessed, which explains why a higher Tier modelling approach is often required to safely cover this kind of activity. Difficulties may be encountered by risk assessors when evaluating this type of activity, making PROC8a a relevant case study for the analysis of exposure assessment tools.

A Tier 1 modelling approach is often based on limited tool-specific inputs. The ECETOC Targeted Risk Assessment (TRA) worker model, one of the most widely used Tier 1 models under REACH, is a good example of a tool in which the choice of exposure determinants is quite limited, and the exposure estimates are conservative, which is even more true since the release of the tool's latest update in October 2023[3]. Numerous higher Tier models have also been developed (*e.g.* RISKOFDERM), or are under development (*e.g.* dART), to estimate occupational dermal exposure. Models have their limitations but they can be a solution to realistically refine exposure scenarios identified as being at risk.

An analysis of the models' robustness (reliability, accuracy) and limitations to their domain of applicability (substance origin, type of tasks) has been performed based on the case of PROC8a. This study emphasises the importance of having an overall picture of available exposure assessment tools, to be able to select the most appropriate one according to the chemical nature of the substance, its intrinsic properties, and its intended uses. Given the limitations of the tools currently available, it also highlights the need to continue developing models which are as representative as possible of an exposure situation, reflecting as closely as possible the operational conditions and risk management measures implemented on site.

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P23-10
Particle exposure in metal industries and its impact on biomarkers, indicate effects on several biological systems

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Purpose: Occupational particle exposure constitutes a known health hazard in many occupations, while the risks are still largely unknown in more recent industries, like additive manufacturing. The purpose of this study was to investigate how particle exposure in different metal industries affects blood biomarkers known to indicate biological effects on lungs, cardiovascular system, liver, kidneys, and inflammation. The aim was also to understand if particle exposures from different work environments induce similar or distinct biological responses.

Methods: Five cohorts with particle exposure measurement and biomarker data were included in the study: two iron foundry cohorts (40 and 85 participants), one welding cohort (136 participants), one hard metal industry cohort (72 participants), and one additive manufacturing cohort (87 participants). Individual dust exposure levels were calculated based on respirable and on inhalable dust exposure measurements, adjusted for respirator use. Biomarker levels were compared to i) control groups vs. exposed individuals within each cohort, or ii) correlated to exposure across all cohorts. The five cohorts were combined into one comprehensive analysis to find common biomarkers of exposure. Biomarker levels were normalized across cohorts using the z-transform based on the control groups, and the normalized biomarker data were correlated to particle exposure levels. Kendall τ correlation was used without covariate correction, and Pearsson correlation as well as ANOVA analysis was used with covariates (age, BMI, sex, smoking, cohort).

The biomarkers were conceptually categorized into six groups, including biomarkers of lung injury (mucin 1, surfactant protein D, CC16, MMP7), cardiovascular impact (ApoA1, ApoB, ApoB/ApoA1 ratio, sST2, vWF, PON1 activity), liver toxicity (ASAT, ALAT, ALP), kidney toxicity (urinary a1 microglobulin), NLRP3 inflammasome activation (IL-1b, IL-18, IL-1Ra), and general inflammation (CRP, SAA, MIP4, sRAGE).

Results: The highest mean respirator adjusted dust exposure was found in the foundry cohorts, followed by additive manufacturing, welding, and finally hard metal industry. When comparing biomarkers of controls and exposed for the cohorts, cardiovascular markers were the once that were most consistently significantly different using Wilcoxon-test, with three out of five cohorts demonstrating differences in ApoB/ApoA1-ratio and sST2-levels. When comparing dust exposure to biomarkers across all cohorts, ALAT was significantly correlated to exposure using Kendall τ (p 0.03) as well as in ANOVA analysis, including correction for covariates (p 0.03). These results indicate that metal dust exposure may put a stress on the cardiovascular system and liver, regardless of metal industry exposure, and that exposure to secondary organs, during clearing of the particles from the lungs, is an important health aspect to consider in regard to exposure of inhaled metal particles.

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P23-11
Assessment of PAH exposure in trainee firefighters using a PAH CALUX bioassay

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Occupational firefighters, including trainee firefighters, are exposed to PAHs despite wearing personal protection equipment (PPE). As PAHs comprise numerous compounds with diverse toxicological potentials, effect-directed approaches may better assess the overall exposure as opposed to conventional chemical-specific methods. An improved estimation of the effective exposure and toxicity of PAHs can assist in the work towards a safer working environment for firefighters.

This work investigated the application of the PAH CALUX bioassay in assessing PAH exposure and related toxicity in trainee firefighters. In this bioassay, PAHs activate the aryl hydrocarbon receptor in a reporter cell line, and this is recorded by increased luminescence. A repeated measurement study was performed, collecting urine and skin wipe samples in three firefighting sessions: one without fire, and two with wood or gas fuels. The response in the bioassay was expressed as B[a]P equivalents, which was compared to levels of 16 PAHs in skin wipe samples and 8 OH-PAHs in urine samples quantified by chemical analysis.

B[a]P equivalents by PAH CALUX bioassay and PAH levels by chemical analysis in skin wipes indicated that larger exposure to PAHs occurred during the wood session as compared to the gas session. Results from urinary samples were more uncertain, and the effect sizes varied between exposure markers. The urine bioassay showed decreased exposure levels after both the gas and wood sessions, whereas the chemical analysis showed increased OH-PAH levels only after the gas session. Non-significant changes were observed for the session without fire, suggesting that exposure from contaminated PPE was minor. Linear regression showed no correlation between the bioassay and chemical analysis data.

The results indicate that the PAH CALUX bioassay is useful for assessing the toxic potency from skin wipe samples. For urine samples, further investigations are needed to clarify uncertainties related to the response. In conclusion, the bioassay response for skin wipes show that firefighters were exposed to substantial levels of potentially toxic PAHs during the wood session.

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P23-12
Use of minimally invasive matrices for characterization of biomarkers in occupational exposome studies

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Self-sampling can be an important means for continuous remote occupational health research, and offers a potential strategy to optimise data capture when resources and access to workers or health care professionals are limited [1]. In our review[2] we present an overview of different methods that have been or can be used for self-sampling in occupational exposome studies. Of the different matrices, dried blood spots (DBS), urine, saliva and oral buccal cells emerged as matrices of choice for rapid and minimally invasive sample collection. Additionally, exhaled breath condensate (EBC) emerged as a minimally invasive matrix to identify lung-related biomarkers.

Therefore, as part of the EPHOR and INTERCAMBIO EU projects, we set up pilot studies to optimize a suitable workflow for minimally invasive sampling in occupational setting. This study was approved by the Ethics Committee (UZ/KU Leuven- S64599).

DBS samples were collected from 45 participants (18–65 y, researchers & family members, Belgium), based on written and video protocols. A survey allowed us to understand aspects related to ease of sampling and clarity of instructions provided. DBS samples from the pilot study yielded good quality DNA, which are being tested for DNA methylation (LINE-1) and oxidative DNA damage (8-OHdG). In a follow up study with 15 participants, we focused on refinement of protocols to characterize immune markers in DBS and dried plasma spots (DPS), with key emphasis on sample storage and shipment conditions (after 1 day and 1, 2, 4, 6 weeks). A large number of immune markers could be detected in DBS and DPS derived sample using the MSD cytokine panel (IFN-y, IL-8, IL-1B, TNF-a etc). Additional analyses are in progress for better interpretation of the observed results.

We investigated quality and stability of EBC derived markers. With only limited saliva contamination (α-amylase activity), we were able to characterize the EBC for several cytokines, MMP-1, 2, 7, and 9, VEGFR-1/Flt1, E- and P-selectin, CysLTs, LTB_4, and TXB_2. Proteomics analysis of a low volume sample showed presence of proteins involved in several key biological processes, originating from different cellular components, with different molecular functions.

The refined protocols will be made available through the project tool box. Based on the results so far, we can consider minimally invasive techniques for biological sample collection and biomarker analysis in the design of future occupational exposome and intervention studies and can hopefully contribute to an increased participation rate.

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P23-13
Hexavalent chromium exposure induces lung injury via activation of NLRP3 and AIM2 inflammasomes in lung of rats

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Background: Hexavalent chromium [Cr(VI)] is a recognized carcinogen of lung cancer. However, the mechanism underlying Cr(VI)-induced inflammation and its exact role in lung cancer remain largely undefined.

Objective: To investigate the mechanism of lung inflammation and the role of lung cancer induced by Cr(VI) .

Methods: For four weeks, 36 Sprague-Dawley male rats treated weekly inhalable intratracheal instillation of normal saline or potassium dichromate (0.05, 0.25 mg Cr/kg) at a volume of 3ml/kg. After the 4th

exposure, half of the rats in each group were randomly killed, and the other rats were killed 2 weeks later after stopping exposure and self-healing. Blood, urine and lung tissue were collected. The lung tissue sections were stained with hematoxylin and eosin (H&E) for assess the degree of lung injury. Trace element (Cr, Mn, Ni, Cu, As, and Pb) concentrations in the samples were measured by inductively coupled plasma mass spectrometry (ICP-MS). Blood mtDNA copy number of was measured by using real-time quantitative PCR (qPCR).The protein expression of NLRP3, AIM2, ASC, Caspase-1 and IL-18 proteins were detected by Western Blot.

Results: Pathological results showed that Cr(VI) induced slight dilatation and hemorrhage of perialveolar capillaries, pulmonary bronchodilation, and congestion with peripheral flaky-like necrosis accompanied by inflammatory cell infiltration, especially the 0.25 mg Cr/kg group. Lung injury was alleviated in low-dose group after 2 weeks of repair, but not in high-dose group. Blood Cr, blood Cu, blood As, urine Cr in exposure group and blood Cr and blood Mn in repair group were significantly increased. Two weeks after repair, blood Mn, urine Cr and urine Pb decreased significantly, blood Cu increased, but blood Cr did not decrease. The mtDNA copy number of blood in Cr(VI) low-dose and high-dose exposure group was 0.49 times and 0.39 times higher than that in control group. Two weeks after repair, the mtDNA copy number of blood increased. Compared with the control group, Cr(VI) exposure could promote the expression of NLRP3, AIM2, ASC, Caspase-1 and IL-18 proteins, and only the exposure group had up-regulated Caspase-1 expression, only the repair group had up-regulated AIM2 expression. After 2 weeks of repair, ASC was significantly down-regulated in the high-dose group and Caspase-1 was slightly up-regulated in the control group.

Conclusion: Cr(VI) exposure may activate not only the NLRP3 inflammasome through oxidative stress and mitochondrial dysfunction, but also the AIM2 inflammasome in lung tissue, two-week repair may alleviate lung inflammation.

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P23-14
Multimodal pulmonary clearance kinetics of carbon black nanoparticles deposited in the lungs of rats: a role of alveolar macrophages

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Alveolar macrophages (AMs) are predicted to have some effects on the pulmonary clearance of nanomaterials, but their qualitative and quantitative role is poorly understood. In this study, carbon black nanoparticles (CBNPs) were instilled into the lungs of Wistar rats at 30, 100, and 300 μg/rat, and evaluated concentrations of particles in organs, including lung, lung-associated lymph node (LALN), liver, spleen, and kidney, at days 0 (immediately after instillation), 1, 7, 28, 60, and 90 post-instillation. The results showed that CBNPs showed a multimodal pulmonary clearance pattern: slow clearance till day 28, fast clearance at days 28 to 60, and slow again at days 60 to 90. Then, CBNPs at 100 μg/rat were instilled into AM-depleted rats using clodronate liposomes (CLO) to prove the mechanism of this unique clearance pattern. At 28 days after instillation, the CBNP levels in the lungs treated with CLO showed about 31% reduction than those in normal rats. In addition, the concentration of CBNPs in LALN treated with CLO significantly increased on day 28, while those of normal rats showed no detectable levels. This result highlights that prolonged retention of poorly soluble NPs in the lung till 28 days is mediated by phagocytosis of AMs, and fast clearance at days 28 to 60 is due to the turnover time of AMs, estimated around 1 to 2 months after birth. Likewise, new generations of AMs mediate the slow phase at days 60 to 90. However, further studies, such as testing in AM-depleted rats with genetic engineering, are needed to support the conclusion of this study.

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P23-15
Pulmonary toxicity is the most sensitive endpoint for deriving MAK values for aluminium compounds

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Introduction: The German Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK Commission) evaluated the toxicological data for aluminium compounds and re-evaluated their previous occupational exposure limit value (maximum workplace concentration, MAK values).

Objectives: Aluminosis and neurodegenerative diseases have been known for many years in aluminium welding and the aluminium-processing industry. In 2007, a MAK value for aluminium-containing dust of 4 mg/m^3 (inhalable fraction) was set. Recent studies of aluminium-exposed workers have shown the occurrence of subtle neurotoxic effects. In addition, new inhalation studies in rats are available, necessitating a re-evaluation of the MAK value.

Methods: A comprehensive literature search was conducted on the toxicity of aluminium compounds. Reviews by other regulatory agencies and original studies, particularly on neurotoxicity, inhalation toxicity, genotoxicity and carcinogenicity, were evaluated.

Results: In longitudinal studies of aluminium welders, preclinical neurotoxic effects were detected. Pulmonary effects were also observed, however, confounding by co-exposure to ozone is possible and the results were influenced by a high proportion of smokers or ex-smokers. A no observed adverse effect concentration (NOAEC) for lung effects could not be derived.

In rats, bronchoalveolar lavage fluid showed signs of pulmonary inflammation after exposure to various aluminium compounds at very low concentrations, so that pulmonary toxicity is the most sensitive endpoint.

Based on inhalation toxicity studies in rats, the MAK values related to Al in the respirable fraction are: 0.05 mg/m^3 for poorly soluble aluminium compounds, i.e. aluminium oxide, aluminium hydroxide and aluminium oxyhydroxide and for the soluble aluminium compounds: 0.005 mg/m^3 for aluminium chlorohydrate and 0.0002 mg/m^3 for aluminium chloride, aluminium citrate, aluminium lactate, aluminium nitrate and aluminium sulphate.

Lung overload effects were clearly observed with the poorly soluble aluminium compounds, therefore, lung carcinogenicity at higher concentrations cannot be ruled out.

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P23-16

Biokinetics of carbon black, multi-walled carbon nanotubes, cerium oxide, silica, titanium dioxide, silver, gold, copper oxide and zinc oxide nanoparticles after inhalation – analysis of existing data

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Understanding the biokinetics of nanoparticles will support the identification of target organs for toxicological endpoints and guide the selection of materials for drug carriers. In the Horizon 2020 NanoInformatix and FFIKA projects, we investigated the biokinetics of nanomaterials expected to be either poorly-soluble or soluble. Only inhalation studies were included in this review of the existing literature. A total of 56 inhalation studies were included. The expected poorly-soluble materials were carbon black, multi-walled carbon nanotubes (MWCNT), cerium oxide (CeO_2), titanium dioxide (TiO_2), crystalline silica (SiO_2), while the expected more soluble materials were amorphous silica, silver (Ag), gold (Au), copper oxide (CuO) and zinc oxide (ZnO). The data were entered into a database library and illustrated on graphs.

Following inhalation exposure, the most persistent materials were: MWCNT, carbon black, and silica which were present in large proportions more than 8000 hours after the inhalation ended. At the other end of the scale were titanium and copper which returned to baseline values within a few hundred hours.

From the lung smaller amounts of the materials are translocated to most of the investigated organs. Similarities and differences will be illustrated. It should be noted that for some materials and organs, there was limited data. E.g. carbon black was only investigated in the lung.

Data on the biodistribution of the inhaled nanomaterials will be presented. Similarities between the materials' expected dissolution, their persistence, and bio-kinetics will be presented.

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P23-17

Genotoxic effects of styrene exposure as modified by the knock-out of *Aldh2* gene in mice

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Introduction: Styrene is an important chemical widely used in the production of plastics and synthetic rubber. Exposure to styrene has been reported to cause DNA damages as evidenced by increased DNA adducts and chromosomal aberrations. This effect has been correlated with genetic polymorphisms in metabolising enzymes such as CYPs and GSTs and DNA repair enzymes. Aldehyde dehydrogenase 2 (ALDH2) is also involved in styrene metabolism, contributing to the conversion of styrene glycol to mandelic acid, but the enzyme activity is deficient in about 40 percent of the East Asian population due to a mutant allele of the gene encoding the enzyme. In this study, we used *Aldh2* gene knockout mice (*Aldh2–/–*) (KO) to detect any difference in styrene-induced genotoxic effects compared with those in wild-type (*Aldh2+/+*) (WT) mice. We also analysed the difference in the excretion of urinary metabolites of styrene in the two types of mice.

Methods: Male C57BL WT and KO mice were used at 8 weeks of age. The KO mice, generated by Dr Kitagawa, have been shown to have reduced activity towards a variety of aldehydes. The mice were exposed to styrene at 0, 20, 100 and 200 ppm by inhalation, 6 hr per day, 5 days per week, for 6 consecutive weeks. At the end of the last exposure, urine samples were collected for 18 h in the cages and the urinary concentrations of mandelic acid (MA) and phenylglyoxylic acid (PGA) were determined. Peripheral blood was then collected under anesthesia from the postcaval vein, and the alkaline comet assay was performed on WBCs to determine tail intensity (TI), which is defined as the percentage of DNA migrated from the head of the comet into the tail and used as the index of DNA strand breaks in cells. The comet assay was also performed on liver cells.

Results: Urinary MA levels in KO mice in the three exposure groups were only 20–42% of those in WT mice. To a lesser extent, PGA was also lower in styrene-exposed KO mice compared to WT mice. As for the DNA damage index, TI value was 1.36 in WBC cells from WT controls and slightly increased in the 20 ppm styrene group, but significantly high in the 100 ppm and 200 ppm groups. In KO mice, the TI value was 1.45 in controls and was significantly increased in all three exposure groups. The TI results in hepatocytes were similar to those obtained with WBC cells.

Conclusions: These results suggest that styrene metabolism was altered in *Aldh2* KO mice and DNA damage was increased in tissues. The *Aldh2* polymorphisms are another genetic factor that may modify the toxic effects of styrene and may be used as a biomarker of susceptibility to styrene exposure.

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P23-18

Classical photovoltaic semiconductors leverage their catalytic properties to influence the molecular regulation mechanism of immune lung injury by interfering with mitochondrial electron transport

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Aims: This study aims to investigate the structural characteristics and molecular mechanisms of four typical III-V photovoltaic materials (PVs) in terms of their interference with mitochondrial electron transport, leveraging their catalytic-like properties, amidst concerns about potential environmental pollution and health issues in a dual-carbon context.

Methods: GaAs, InAs, CdTe, and CdS were selected as representatives of second-generation PVs. Various parameters, including morphology, catalytic activity, isoelectric point, crystal fine structure, and density of states, were analyzed. Real environmental exposure levels were calculated based on the NIOSH recommended algorithm. Research employed both THP-1 cells and C57BL/6 mice as models: *in vivo* to explore the effects of oropharyngeal inhalation of PV semiconductor nanomaterials on lung function and immune lung damage, and *in vitro* to investigate the impact of particulate matter deposition on THP-1 cell immune activity at environmental exposure levels.

Results: The four PVs exhibited irregular lumps, with the DCF method indicating the ability to induce abiotic ROS. Catalytic-like activity assays revealed POD-like and NOX-like activities for GaAs and CdTe, respectively. K-edge XANES and PDOS analyses highlighted metastable structures within CdTe and GaAs, characterized by low electron cloud density and high electron overflow probability. Mice exposed to environmentally relevant levels of CdTe and GaAs showed reduced lung function and inflammatory infiltration with immune activation in lung tissue. Treated THP-1 cells displayed increased M1 polarization, in-

flammatory factors, and accumulation of total ROS and mtROS. Screening of PV material-related proteins using the GeneCards® human gene database indicated predominant expression in mitochondria. Assessment of mitochondrial mass in PVs-treated THP-1 cells showed decreased TOM20 expression and MMP depolarization. Transcriptome and metabolome analyses suggested that GaAs and CdTe exposure primarily affected glucose metabolism pathways, leading to reduced energy metabolism levels. Expression of mitochondrial electron transport chain-related complexes I-V was reduced, while CoH2/CoQ and NADH/NAD+ ratios were elevated. Treatment with electron transport chain inhibitors revealed a decrease in mtROS, with combined treatment resulting in reduced inflammatory factor expression.

Conclusion: Metastable electronic structures and biosimilar redox potentials may underlie the catalytic properties of PVs. Disruption of lung function by PVs is associated with disturbances in energy homeostasis, driving reverse electron transfer to promote mtROS accumulation. These findings suggest that intervention in mitochondrial electron homeostasis could be a crucial target for preventing immunogenic lung injury induced by PVs.

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P23-19
Cell-specific responses to mild and stainless steel welding particles in bronchial epithelial and macrophage-like cells

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Welding fumes induce lung toxicity and are classified as carcinogenic in humans, but the underlying mechanisms are not fully understood. The aim of this study was to explore the mechanisms of toxicity of mild and stainless steel welding particles generated from commonly used electrodes (Aristorod 12.50 and Autrod 316LSI) during gas-metal arc welding. To this end we exposed bronchial epithelial cells (BEAS-2B) and immune competent cells (THP-1 derived macrophages) to 12.5–150 μg/mL mild or stainless-steel welding particles for 24 h. Cell viability was assessed by Alamar Blue assay. We measured gene expression changes by RNA sequencing at 50 μg/mL of exposure and generated mechanistic hypotheses based on enriched pathways and functions (Ingenuity Pathway Analysis). We then validated the hypotheses using traditional assays (comet assay for DNA damage, multiplex immunoassay for cytokine secretion). Metal cellular dose was measured by ICP-MS. After 24h exposure, both mild and stainless steel welding particles caused dose-dependent cytotoxicity with the THP-1 cells being more sensitive compared to BEAS-2B cells and stainless steel particles being more cytotoxic compared to mild steel particles. The metal cellular dose was similar between the two cell lines. In BEAS-2B, we found 150 and 554 differentially expressed genes (DEGs, FDR-adjusted p-value <0.05) for mild and stainless steel particles, respectively. In THP-1 cells, we found 354 and 2968 DEGs for mild and stainless steel particles, respectively (FDR-adjusted p-value <0.05). The top enriched pathways and functions were similar between mild and stainless steel welding particles and to some extent overlapped in the two cell models. However, we found pathways and functions specifically enriched in THP-1 cells related to cell cycle signaling and DNA damage response. Genotoxicity was confirmed for stainless steel particles in THP-1 macrophages but not in BEAS-2B cells. We also observed cell-specific differences: pathways related to inflammation were predicted to be activated in THP-1 cells and predicted to be inhibited in BEAS-2B cells. The immunomodulatory effect was confirmed in the two cell lines by analysis of cytokine levels for IL-1b, IL-6, IL-8, and TNF-a. Overall, we found that stainless steel particles were more potent compared with mild steel in inducing both gene expression and phenotypical changes. Also, we observed both qualitative and quantitative differences in the cellular response to welding particles, with THP-1 cells being in general more sensitive compared with BEAS-2B cells. Further studies using single cell sequencing on co-cultured THP-1 and BEAS-2B cells are planned to further elucidate these cell-specific profiles as well as identify interactions between the cell types. This will ultimately lead to a better understanding of the mechanisms of toxicity and carcinogenicity of welding fume particles.

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P23-20
Towards safer and sustainable MXene production

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Two-dimensional (2D) materials are under consideration as a new generation of materials for a variety of applications, ranging from sensors to energy storage or biomedical applications, owing to their distinctive physicochemical properties. Over the past decade, MXenes have gathered considerable research interest. The most common method to obtain MXenes involves selectively etching the 'A' layers from MAX phases, followed by delamination and exfoliation to obtain a few-layer MXene sheet dispersion. This process requires the use of solvents such as dimethyl sulfoxide (DMSO), N, N-dimethylformamide (DMF), or N-methyl-2-pyrrolidone (NMP), which pose significant safety, health, and environmental-related issues. In this study, we assessed the use of the *green* solvent Cyrene, a bio-based sustainable solvent derived from renewable sources such as cellulose, combined with a good biodegradability profile that generates only carbon dioxide and water [1], to replace the use of more hazardous solvents (e.g. NMP) to exfoliate and stabilize MXene ($Ti_3C_3T_x$). Dispersion in Cyrene was found to be stable for up to 6 months and less prone to oxidation, compared to NMP dispersions. Furthermore, we investigated the cytotoxic effects of MXenes for both Cyrene and NMP exfoliated samples, and respective vehicles, on HaCaT cells, a commonly used model for skin-related studies. Both types of MXene displayed a similar profile, inducing cellular toxicity in a concentration- and vehicle-dependent way except when cells were exposed to 1 μg/cm^2 ($****p<0.0001$). The impact on viability was significantly attenuated when MXene was re-dispersed in bovine serum albumin (BSA) solution (for concentrations up to 100 μg/cm^2), a more biocompatible vehicle, likely due to the formation of a protective protein corona.

Overall, we were able to synthesize MXene in a more environmentally safe bio-based solvent, which can be used for large-scale production of high-quality MXene dispersions and at a later stage be re-dispersed in a biocompatible solution for biomedical applications.

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P23-21
Workflow for occupational exposure limit determination and banding: focus on skin and respiratory sensitization using NAMs approaches

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Occupational exposure to hazardous substances poses significant health risks with sensitization, whether dermal or respiratory, being one of the most critical concern for workers, and necessitating accurate exposure risk assessments and the establishment of safe Occupational Exposure Limits (OELs). When an OEL is not established, identifying the necessary measures to safeguard workers from chemical exposures becomes complex. Occupational exposure banding (OEB), often referred to as hazard banding or health hazard banding, is a methodical approach that utilizes both qualitative and quantitative hazard data related to specific health-effect endpoints to delineate potential exposure ranges or categories. Specifically, the OEB delineates a spectrum of airborne concentration levels that are anticipated to protect worker health, serving as a tool to support risk management decisions. When existing data is insufficient to fully characterize the hazards of compounds, additional experimental activities are required to fill these knowledge gaps and ensure accurate hazard assessments.

Current scientific advancements highlight a notable disparity in the development and validation of *in vitro* models for dermal and respiratory sensitizations. While validated *in vitro* models for determining dermal sensitization are available, offering robust methodologies for identifying potential skin sensitizers, they fall short of providing dose-response relationships. This limitation underscores a significant gap in our ability to quantitatively assess dermal sensitization risks based on *in vitro* data alone.

Conversely, the landscape for respiratory sensitization presents a different set of challenges. To date, there are no validated *in vitro* models specifically designed for assessing respiratory sensitization. This lack of validated models poses a critical barrier to fully understanding and mitigating the risks associated with respiratory allergens in the workplace. Despite this, there are emerging models that, although not yet validated, offer promising insights into respiratory sensitization mechanisms and potential risks. These preliminary models are instrumental in bridging the knowledge gaps and enhancing our mechanistic understanding, thereby contributing valuable information for risk assessment processes.

This work aims to shed light on the current state of New Approach Methodologies (NAMs) for both quantitative dermal and respiratory sensitization within the framework of occupational toxicology. We will explore the available alternative methods for quantitative dermal sensitization and investigate the potential of emerging, yet-to-be-validated models for respiratory sensitization assessment. By examining these NAMs, we intend to demonstrate their utility as powerful tools in the quantitative evaluation of sensitization risks.

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P24 | Gut microbiota and toxicity

P24-01
Impact of perinatal and subchronic dietary glyphosate exposure on gut microbiota and liver metabolism in mice

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Glyphosate is the world's best-selling herbicide. This massive use leads to contamination of all environmental compartments and exposure of the general population via the diet. There is evidence that glyphosate affects the gut microbiota, but the consequences of glyphosate-induced dysbiosis on the host have been poorly studied. Dysbiosis of the gut microbiota contributes to disturbances in energy and liver homeostasis. Here, we studied the consequences of chronic and dietary exposure to different doses of glyphosate on the gut microbiota and energy and liver metabolism in mice.

Male and female C57BL/6J mice were exposed to glyphosate in utero (via maternal dietary exposure), during lactation (via maternal dietary exposure) and then via the diet for 3 months. Glyphosate was incorporated into the food pellets enabling the mice to be exposed to two European regulatory doses: the Tolerable Daily Intake (TDI, 0.5 mg/kg body weight/day) and the No Observable Adverse Effect Level (NOAEL, 50 mg/kg body weight/day). In a 2nd independent experiment, male and female C57BL/6J mice were exposed to glyphosate (at the TDI) in combination with a diet enriched in fat, sugar and cholesterol (western-diet, WD). Food and water intake and body weight were measured weekly. We studied carbohydrate homeostasis by oral glucose tolerance tests and plasma biochemical analyses, the hepatic transcriptome using microarrays and the hepatic phenotype by histological staining and immunolabeling and neutral lipid analysis by gas chromatography. The composition and metabolic activity (fecal metabolite production) of the gut microbiota were studied by 16S sequencing and fecal NMR metabolomics.

In the 1st experiment, mice exposed to glyphosate showed no significant difference in weight gain, oral glucose tolerance or plasma markers of liver damage compared to controls. Analysis of hepatic gene expression revealed that glyphosate induced expression of genes involved in lipid metabolism, particularly de novo lipogenesis, irrespective of sex and exposure dose. Nevertheless, the amount of hepatic neutral lipids (triglycerides, free cholesterol and cholesterol esters) was not significantly different between groups. The composition of the intestinal microbiota was significantly impacted by glyphosate, in a dose- and gender-dependent manner, in particular there was a significant enrichment of bacteria from the Marinifilaceae family in all glyphosate-treated groups. In the 2nd experiment, glyphosate combined with the WD diet induced dysbiosis of the intestinal microbiota compared to control-WD mice, but did not induce either a deleterious metabolic phenotype or liver toxicity.

Exposure to glyphosate induced significant changes in the composition of the gut microbiota, but no major disturbances in energy metabolism or liver toxicity. The consequences of glyphosate-induced dysbiosis of the intestinal microbiota will be studied for other organs such as the intestine or the brain.

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P24-03
Modulation of glucose-lipid metabolism in Type II Diabetes through butyric acid-mediated epigenetic regulation: a probiotic intervention study in mice

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The intricate relationship between gut microbiota and the metabolic health of the host organism offers promising avenues for therapeutic interventions, especially in the context of metabolic disorders such as type II diabetes (T2D) [1,2,3,4,5]. This study focuses on elucidating the impact of probiotics and their metabolite, butyric acid, on the expression of genes involved in glucose-lipid metabolism and the epigenetic mechanisms potentially mediating improvements in T2D outcomes in a mouse model [6,7,8]. Specifically, the effects of butyric acid on DNA methyltransferases (DNMT1, DNMT3) and ten-eleven translocation enzyme (TET1), which are key players in DNA methylation and demethylation processes, were examined to understand their roles in the regulation of genes critical for glucose and lipid homeostasis [9,10]. Mice with induced T2D were divided into two groups: one receiving a standard diet supplemented with probiotics known to produce butyric acid and the other serving as a control. Over the course of the study, the mice consuming the probiotic-enriched diet showed significant modulation in the expression of DNMT1, DNMT3, and TET1 genes, suggesting an epigenetic influence mediated by butyric acid. This modulation was associated with altered methylation patterns of key genes involved in glucose and lipid metabolism, leading to observable physiological improvements, including enhanced glucose tolerance, reduced insulin resistance, and more favorable lipid profile [1,5,8]. The results of this study highlight the therapeutic potential of targeting gut microbiota and their metabolites, such as butyric acid, for the management of T2D. By influencing the expression of DNMT1, DNMT3, and TET1, butyric acid contributes to the regulation of gene expression patterns critical for metabolic health [9,10]. These findings underscore the complexity of host-microbiota interactions and suggest that manipulating these relationships through dietary interventions could offer a viable strategy for improving outcomes in T2D patients [6,7]. Further research into the specific mechanisms by which probiotics and their metabolites affect gene expression and methylation patterns will be essential for developing targeted therapies aimed at modulating metabolic pathways implicated in T2D.

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P24-04
Quantifying rate and global profile of gut microbiota metabolism *ex vivo* by LC-MS/MS

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Microbes perform many chemical reactions within our gut with direct impact on human health. Sequencing of microbiota communities is insufficient to accurately decipher the chemical reactions occurring within the gut environment and which drive chemical toxicity. Thus, we use anaerobic fermentations of fecal microbiota, time-series sampling, and a merged targeted/untargeted LC-MS/MS analysis to quantify chemical transformation rates of known food, drug, and endogenous host metabolites. Metabolic profiles encompassing deglycation, nitroreduction, sulfoxide reduction, deglucuronidation, bile acid metabolism, benzisoxazole ring reduction, and unique food chemical transformations were generated for 5 human donors. Chemical reaction rates varied dramatically by chemical structure and across donors. Conditions of fermentation were tested including multiple growth media, fresh and frozen samples, and fecal slurry dilutions to determine an optimized protocol for *in vitro* cultivation of gut microbe communities. As expected, dilution of the inoculated microbiota influenced chemical reaction rates that are dependent on microbial metabolism (nitroreduction, deglucuronidation, etc.), but not reactions primarily driven by spontaneous reactions (chemical-matrix interactions). Nutrient rich media increased reaction rates compared to nutrient scarce media for benzisoxazole reduction, nitroreduction, and sulfoxide reduction reactions, while glucuronidation, and bile acid metabolism rates remained unchanged. Fresh compared to frozen microbiota samples did not significantly change chemical reaction rates for targeted chemicals. Untargeted metabolomics analysis provided global insight into metabolic behavior of approximately 400 annotated chemicals. Chemical metabolism products, and microbially generated metabolite profiles revealed complex interactions determined by different growth conditions and interindividual variation of donor samples. Metabolite correlation analysis revealed characteristic metabolism profiles shared by diverse chemical classes linking chemical transformations to donor and growth specific conditions. *Ex vivo* fermentations monitored by LC-MS/MS provide quantitative metabolic profiles and global metabolism measurements applicable for individualized assessment of microbiota function that are not possible to glean from other -omics technologies. Our approach provides a basis to characterize chemical metabolism in the gut to more comprehensively assess and predict toxicity of drug, food, and xenobiotic chemicals.

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P24-05
Evaluation of acute gastroprotective effects of the selected natural clays in rats

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Purpose: Clay minerals are widely used in animal nutrition since their absorption/adsorption properties positively impact the animal's health [1–4]. Clay minerals have also gained special attention as potential materials for biomedical applications (e.g. pharmaceutical excipients in the formulation of various dosage forms or active pharmaceutical ingredients), due to their favourable physicochemical and functional related characteristics along with good biocompatibility [5,6]. It was previously determined that natural bentonite (NBNT) and natural halloysite (NHAL) have low acute oral toxicity [7]. Accordingly, this study tested the gastroprotective activity of these clays in acute gastric injury in rats.

Methods: This study was performed on adult, male Wistar rats using the earlier published procedures [8]. Rats were randomly divided into five groups: 1. Ethanol (1 ml/kg p.o), 2. NBNT (1 g/kg p.o.), 3. NHAL (1 g/kg p.o.), 4. NBNT (1 g/kg p.o., 30 min. before ethanol (1 ml/kg p.o.)) and 5. NHAL (1 g/kg p.o., 30 min. before ethanol (1 ml/kg p.o.)). Animals were sacrificed 1, 3 and 7 days after treatments, and guts were prepared for the pathohistological examination of the gastric mucosa injuries as previously described [5]. Experimental procedures were approved by the Ethics Committee of the Faculty of Veterinary Medicine, University of Belgrade (No. 21/2020) and the Veterinary Directorate, Ministry of Agriculture and Environmental Protection, Serbia (No. 323-07-11720/2020-05).

Results: Acute exposure of rats to ethanol caused gastric mucosal oedema, petechiae and ulcerations associated with neutrophil infiltration. During the 7 days after oral application of ethanol, there was a peak in the mean number of petechiae and ulcerations reached on the first day (6.40). However, this value was not significantly different compared to values established on days 3 and 7. Peroral administration of NBNT or NHAL sporadically induced the occurrence of only discrete oedema and petechiae. In addition, when NBNT or NHAL was given as a pretreatment, both significantly decreased the occurrence of petechiae induced by ethanol, starting with day 1. The majority of these acute gastric injuries were minimized after a week period.

Conclusions: Results supported the hypothesis that NBNT and NHAL have potent gastroprotective activity in a model of acute gastric injury induced by ethanol in rats. It might be the consequence of their functional properties, as well as the potential inhibitory effect on inflammatory cell infiltration. These results may be useful in the definition of specific characteristics of the natural clays, which will be essential for their potential practical application in animal nutrition and biomedicine.

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P24-06
Tackling interindividual differences in toxicokinetics of gut microbial metabolites by microbiome-competent PBK modeling

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Xenobiotic metabolism in the liver is considered one of the most important factors in toxicokinetics. However, the microbiome has recently emerged as a significant contributor to chemical biotransformation. The diversity in microbial community composition leads to significant variations in available metabolite concentrations across individuals and populations. Consequently, there is a need to develop and refine methods that predict microbiome-mediated individual responses to chemical exposure. Thus, we built and evaluated microbiome-competent PBK models for two distinct chemicals of interest and demonstrated their applicability for quantitative *in vitro* to *in vivo* extrapolation, yielding data of direct utilizability for chemical risk assessment. In the first study, we used urolithin A, a gut microbial metabolite with extensive human pharmacokinetic data availability, to test strategies for developing a microbiome-competent PBK model. The model incorporates urolithin formation in the gut, gastrointestinal absorption, partitioning to organs of interest, glucuronidation in the liver and small intestine, urinary excretion, as well as enterohepatic recirculation of UA glucuronides. UA formation rates were derived from anaerobic batch fermentations using stool from 22 healthy volunteers. The model's accuracy was evaluated by comparing predicted plasma concentrations against human plasma values from clinical studies. After evaluation, we utilized the model to understand how concentrations for biological effects, previously reported *in vitro*,

relate to predicted concentrations in tissues. We also present a similar approach that was subsequently applied in a second study, predicting interindividual differences in the gut microbial formation of 8-prenylnaringenin from hop polyphenols as an example of the gastrointestinal activation of a potent phytoestrogen. Estrogenicity of 8-PN in mixtures with precursor polyphenols was assessed in Ishikawa cells. Simultaneously, a microbiome-competent PBK model was established and subsequently used to extrapolate *in vitro* concentration-response data to *in vivo* effects. Taken together, we refined predictive PBK modeling tools for interindividual differences of gut metabolite toxicokinetics.

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P24-07

In vitro reconstructed 3D models of human duodenum, jejunum and ileum

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The study of gastrointestinal (GI) toxicity is limited due to the lack of physiologically relevant *in vitro* models that recapitulate the role of specific parts of the GI tract. For example, traditional *in vitro* cell cultures approach utilizes immortalized human Caco-2 cell line cultured for about 21 days to mimic properties of small intestine mucosa and assess ADME properties of drugs. However, these models are limited by the fact that they originate from cancer cells, have unphysiological expression and/or functionality of major drug transporters and drug metabolizing enzymes, do not have fully polarized structural features, and are not predictive of GI toxicity even though they have been in use for more than 5 decades. To mimic the physiology and functionality of the human gut, we established the small intestinal model from primary human cells, which recapitulates many aspects of small intestine biology. The only pitfall of this model is that it only consists of cells derived from jejunum.

Here we present development of 3 new models of small intestine utilizing primary cells from different intestine regions in order to provide further physiological relevance and expand ability to pinpoint differences between different regions of small intestine mucosa (duodenum, jejunum, and ileum).

The newly developed 3D tissues mimic morphology of normal intestinal epithelium with structural features resembling villi and physiological-like barrier function. Gene expression analyses revealed differences in the expression of genes encoding transporter proteins (ABC family, peptide transporters) and drug metabolizing enzymes in various regions. On the other hand, expressions of some drug metabolizing enzymes such as CYP3A4, CYP2C9, UDP glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1), and Carboxylesterase 1 (CES-1) were maintained in each segment at a comparative level. Our preliminary experiments aimed at drug absorption and metabolism revealed that the permeation of Vinblastine was affected by inhibition of MRP and/or P-gP transporters. The activity of metabolic enzymes (phase II glucuronidase enzymes) was suggested by the presence of raloxifene-6-glucuronide metabolite following the treatment of tissues with raloxifene, which was sensitive to inhibition with glucuronidase inhibitor.

These results suggest that the reconstructed tissues from the three segments of the small intestine may serve as useful tools to predict both investigational and traditional GI drug safety and absorption in the GI tract. In addition, use of these models will reduce animal use and improve the pre-clinical drug development process.

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P24-08

Material- and size-related uptake and effects of plastic particles after oral ingestion

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Plastic polymers are a versatile group of materials and widely used due to their modifiable characteristics, ease of production, and manifold applicability. As a result, plastic products are constantly entering and interacting with the environment, where they break down into smaller particles, known as microplastics (5 mm to 1 µm), submicroplastics (1 µm to 100 nm) and nanoplastics (100 nm to 1 nm). These plastic particles can be orally ingested through contaminated food. Particles smaller than 1.5 µm can cross the gastrointestinal barrier, but potential human health impacts are not fully understood due to incomplete exposure data and hazard characterization.

In this work, several *in vitro* models are used to investigate the oral uptake and effects of polymer particles of different materials and sizes, namely polymethyl metacrylate (25 nm), melamine resin (366 nm) and polylactic acid (250 nm and 2 µm). Microscopic and light scattering techniques were utilized to characterize the test particles. Uptake and effect analyses were conducted using *in vitro* mono- or co-culture models based on the intestinal cell line Caco-2 and HepaRG liver cells after the application of fluorescent particles for 24 h. In addition, the effect of artificial digestion juices on the behavior of the particles at the Caco-2 barrier model was investigated. Fluorescence measurements, confocal microscopy, polymerase chain reaction, oxidative stress measurements, and cytokine secretion assays were performed to investigate impacts on the intestine and liver *in vitro*.

The results of the study emphasize that the behavior of the particles depends on their material and size when applied to Caco-2 mono-culture or co-cultures additionally mimicking M-cells or mucus-producing cells. The submicrometer particles exhibit distinct intracellular localization behavior, suggesting different uptake mechanisms for the two plastic particles. Compared to the pristine particles, the digested particles formed aggregates and showed less interaction with the cells, resulting in slightly increased transport. Using a combined model of Caco-2 and HepaRG cells, we were able to demonstrate that all particle types were taken up by HepaRG cells after passing through the gastrointestinal barrier. This suggests that the particles have the potential to enter the systemic circulation, which could lead to deposition or other health-related effects. Initial *in vitro* toxicity screening studies showed minimal impact on Caco-2 and HepaRG cells in terms of effects on cytotoxicity.

In summary, the results provide evidence for oral bioavailability of submicro- and nanoplastic particles. The study further demonstrates the importance of considering the physico-chemical characteristics of particles when performing *in vitro* studies. These properties can significantly influence the behavior of plastic particles at the gastrointestinal barrier.

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P24-09

Divergent effects of endocrine-disrupting chemicals on common gut bacteria shaped by compound properties and bacterial physiology

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Exposure to endocrine-disrupting chemicals (EDCs) leads to adverse physiological effects in humans. Despite the pivotal role of the human gut microbiota in maintaining homeostasis, its interactions with EDCs remain poorly understood. In this study, we investigated the effects of 36 commonly detected EDCs (8 pesticides, 12 phenols, 8 phthalates, and 8 poly- and perfluoroalkyl substances (PFAS)), individually or in nine synthetic mixtures, on the growth kinetics and phenotypic traits of 40 gut bacterial species (4 Actinomycetota, 10 Bacteroidota, 19 Bacillota, 6 Pseudomonadota and one Verrucomicrobiota) representing core pediatric and adult microbiota species using an *in-vitro* approach. EDCs selection and mixture design were based on exposure patterns observed in the French couple-child cohort (SEPAGES) and epidemiological reports using a clustering approach, at concentrations reflecting exposure levels, maximum levels, and potential cumulative impacts (10 to 1000 mM). Our results showed that the chemical category of EDCs, phylogenetics, and bacterial cell wall structure type were the main factors influencing bacterial response (growth inhibition) to EDCs or their mixtures. Notably, species from Bacteroidota exhibited susceptibility to phenols and PFAS, while Bacillota displayed resistance to phthalates. Actinomycetota species were most sensitive to PFAS, whilst Pseudomonadota species were the least sensitive. Additionally, cell wall structure played a role in mediating response to EDCs: Gram-positive bacteria were sensitive to PFAS and resistant to phthalates, whereas Gram-negative bacteria were adversely impacted mainly by phenols. Overall *Bifidobacterium* species except *B. catenulatum* were most susceptible to all EDC categories. Within Bacillota phylum, the response towards EDCs were mixed: *Enterococcus* spp., *Faecalibacterium prausnitzii* and *Streptococcus salivarius* displayed more resistant phenotype compared to other Bacillota species. Among Bacteroidota phylum, *Phocaeicola* spp. exhibited the most sensitivity to EDCs compared to *Bacteroides* spp. The synthetic mixtures of EDCs belonging to the same chemical category did not generate response considerably different from individual treatments, however incubation with mixtures composed of EDCs from different categories remarkably altered bacterial responses and induced new patterns of sensitivity in a dose-dependent manner. The mixtures containing PFAS (i.e., PFOS) or triclosan overall had greater toxicity on species in particular Bacteroidota taxa. Incubation with mixtures induced distinct bacterial responses, suggesting intricate community reactions in real-life scenarios, implying a complex network of responses to multiple EDCs. In summary, our study highlights the critical impact of EDC exposure whether individually or in mixtures on gut microbial homeostasis.

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P24-10

Human gut microbiota extensively metabolizes the chloroacetamide herbicide metazachlor

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Chloroacetamides, including metazachlor, are globally widely used herbicides. While metazachlor is hydrolytically stable and persists in the food chain, it can be biotransformed in soil and aquatic microbiota. On the other side, it is not known how metazachlor would be metabolized by the human gut microbiome, if this metabolism would be different from that in the environment and what toxicological effects this may lead to. Therefore, we aimed to characterize the potential metabolism of metazachlor by gut microbiota. We exposed complex microbial communities derived from human fecal samples to metazachlor at levels equivalent to, as well as three times higher or lower than, the EFSA-derived acceptable daily intake level. We then characterized its degradation by untargeted LC-MS^2 analysis. As a result, we identified 6 known and 14 previously unreported metazachlor metabolites, with several of these being produced in substantial amounts potentially relevant to physiological distribution. For example, up to 30% conversion was to the disulfide of the metazachlor thiol, arising from a reaction with cysteine. This metabolite is in turn depleted over the course of 24 h, and it appears to be converted to the corresponding thiol and further metabolites. Furthermore, when we quenched fermentations with methanol, hemiacetals were formed, indicative of the presence of reactive aldehyde derivatives of metazachlor, which are of potential concern due to their electrophilicity. Overall, the metabolic processes in gut microbiota, which are dominated by altered sulfide chemistry, appear to differ greatly from those in soil and aquatic microbiota, which involve the formation of corresponding oxalic acid and sulfonic acid metabolites. As a result of this research, metazachlor was found to be extensively metabolized by gut microbiota, and several metabolites were newly identified, providing a basis for further research to understand the influence of the transformations on host adverse responses.

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P25 | Mixture toxicology

P25-01

Investigation of the miRNA levels changes to acceptable daily intake dose pesticide mixture exposure on rat mesentery and pancreas

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Consumers are constantly exposed to a variety of chemical mixtures as part of their everyday activities and lifestyle. Food, water and commercial products are only some examples of the possible ways people get exposed to these mixtures. However, following federal and local guidelines for risk assessment related to chemical exposure, risk analysis focuses on a single substance exposure scenario and not on a mixture, as in real life. Realizing the pronounced gap of this methodology, the real-life risk simulation scenario approach tries to address

this problem by investigating the possible effect of long-term exposure to chemical mixtures closely resembling the actual circumstances of modern life. As part of this effort, this study aimed to identify the cumulative effects of pesticides belonging to different classes and commonly used commercial products on long-term exposure with realistic doses. Sprague Dawley rats were given a pesticide mix of active ingredients and formulation chemicals in a daily acceptable dose (ADI) and 10xADI for 90 days. Following thorough everyday documentation of possible side-effects, after 90 days all animals were sacrificed and their organs were examined. Exposure to pesticides particularly affects the miRNA levels at that point will provide us with more information about whether they can be potential biomarkers.

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P25-02
Cocktail effects in skin sensitization – a case study of a rubber chemical mix

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Dendritic cells (DCs) are crucial for the immune response as they link innate and adaptive immunity. Cell models resembling DCs have been successfully used to develop assays to predict the adverse effects of chemicals on human health, such as skin sensitisation. Improving the understanding and prediction of cocktail effects is of special interest in the field. Cocktail or combination effects may occur upon co-exposure to several chemicals in parallel, and they can aggravate or inhibit adverse effects triggered by single chemicals alone. In this study, we investigated three rubber sensitizing chemicals mainly found in protective gloves, zinc diethyldithiocarbamate (ZDEC), zinc dibutyldithiocarbamate (ZDBC), and 1,3-diphenylguanidine (DPG), constituting the so-called carba mix. The carba mix is routinely used to patch-test patients for diagnosis of allergic contact dermatitis but test results can be difficult to interpret.

To investigate if cocktail effects already on DC level could contribute to explaining these test results, a human DC-like cell model was exposed to the single chemicals and their pre-defined binary and tertiary mixtures capturing the molar composition in the carba mix. After 24 h incubation, cell pellets and cell culture supernatants were harvested and analysed by mass spectrometry proteomics, multiplex cytokine analysis and using protocols adapted from the GARD®skin and GARD®potency assays. In addition, DC activation marker expression, autophagic flux, and reactive oxygen species (ROS) production were assessed by flow cytometry.

From obtained transcriptomics (GARD assays) and proteomics data, effects on autophagy and the antioxidant response were expected and DPG indeed markedly increased autophagic flux while only minor changes were detected after exposure to the other chemicals and mixes. ROS production in response to chemicals was more complex. DPG seemed to induce ROS production most robustly after several time points while mix exposure may lead to inhibitory effects. Interestingly, in response to all mixes containing ZDEC and ZDBC, IL-8 levels exceeded the expected values based on dose addition, indicating potential synergistic cocktail effects.Responses to the dithiocarbamates, despite being chemically related, are clearly distinct, with some indication that ZDBC is less potent than ZDEC fitting to clinical observations when patch-testing patients. Outcomes from mix exposure are more complex even on DC level, which may contribute to the variations seen in human responses, where some patients react with to the carba mix but not to single constituents. Considering that DPG is very rarely found together with ZDEC and ZDBC in protective gloves, it may be more appropriate to test DPG separately. In summary, *in vitro* cell based assays can contribute to a better understanding of underlying mechanisms in skin sensitization and to improving diagnostic patch testing routines.

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P25-03
Exploration of the novel role of NADPH oxidases in the biotransformation of T2 mycotoxin

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Due to global climate change, the quantity, quality, and distribution of mycotoxins in food and feed make them a constant health concern [1]. The formation of reactive oxygen species (ROS) plays a crucial role in the toxicity of many mycotoxins [2,3]. NADPH oxidase (NOX) enzymes, which generate ROS in the body, play a significant role in regulating cellular functions and developmental processes [4]. ROS modulate cellular, physiological processes and in the meanwhile, they are involved in the development of many pathologies [5]. NOX 4, being the most widely distributed isoform, is an important regulator of physiological redox status in mammals by producing ROS *in vivo*. Using a functionally NOX 4-deficient genome-edited knock-out rabbit (NOX 4-KO) created previously by our group [6], the redox regulation of an *in vitro* T2 mycotoxin exposed rabbit embryonic fibroblast model system has been established and investigated with a focus on enzymes relevant in the biotransformation of T2 toxin. RNA sequencing method has been implemented. We identified various participants of the detoxification process in our rabbit model system with similarity to humans, making the rabbit a promising translational model to study the molecular background of T2 toxin exposure. Comparison of NOX 4-KO and wild-type cells revealed different patterns in the dynamics of gene expression of biotransformation enzymes in the early phases of mycotoxin exposure. We characterized the expression pattern of NOX isoforms in response to T2 toxin exposure. The dose-dependent increase of DUOX 1 was detected in the wild-type cell culture, however, in NOX 4 knock-out cells expression was upregulated but the hormesis effect was pointed out in the applied concentration range. Our findings shed light on the role of NADPH oxidases in the biotransformation of endogenous substances like T2 mycotoxin. The interplay between NOX 4 and DUOX 1 in the biotransformation process of T2 mycotoxin is very intriguing and targeting DUOX 1 and NOX 4 and its associated signaling pathways may offer novel avenues for the development of interventions aimed at reducing the adverse effects of mycotoxins on human and animal health.

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P25-04
Antiandrogenic activity of reconstituted chemical mixtures reflecting real-life co-exposure patterns

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At any given point in time, humans are exposed to numerous potentially hazardous chemicals arising from the environment, food, and consumer products. Multiple experimental studies addressing mixture effects have shown that exposure to chemicals combined at low, realistic levels act additively when leading to the same adverse outcome and can, thus, contribute to significant detrimental effects on human health [1]. This includes male reproductive health disorders, for which androgen receptor antagonism is deemed as a key molecular initiating event.

The H2020 EU project PANORAMIX aims to characterize potential risks arising from complex real-life chemical mixtures, with pooled samples representing both external (food) exposure sources (fish and cow milk) and human internal exposure (human breast milk and serum) collected from various sources in Europe [2]. Chemical mixtures were extracted, and samples were chemically profiled and tested for their antiandrogenic activity using the OECD validated AR EcoScreen™ (human AR) transactivation reporter gene assay.

Thanks to a suspect screening approach, several hundreds of organic contaminants from various classes were qualitatively identified in the generated sample extracts, including pesticides, plasticizers, pharmaceuticals, UV filters, industrial chemicals, among others.

A subset of 24 chemicals for which (semi-)quantitative data were produced were tested individually for antiandrogenic activity. Out of these, 21 chemicals displayed a concentration-dependent antiandrogenic activity at non-cytotoxic levels.

Fourteen reconstituted mixtures of these substances were designed based on the chemical composition of the pooled samples, and further analysed for their antiandrogenic activity to assess mixture effects against established mixture toxicity models, such as concentration addition.This work reinforces the need to take into consideration potential mixture effects when dealing with chemical risk assessment and demonstrates the usefulness of reconstituted mixtures mimicking real-life chemical compositions for the assessment of realistic mixture effects.

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P25-05
Applicability of the Cytotoxicity Eye Irritation (CEI) test for classification of mixtures for eye hazard identification

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The Cytotoxicity Eye Irritation (CEI) test *in vitro* was developed [1,2] to classify substances into two categories of eye hazard, i.e. serious eye damage (category 1) and eye irritation (category 2), and to identify substances that do not require classification (no category) in the GHS system (Globally Harmonized System of Classification and Labelling of Chemicals). However, there was no evidence that the method was effective for multi-component mixtures. Unfortunately, there is no database that determines the hazard of a multi-component material according to the concentration of the harmful substance. It was therefore decided to test whether the final classification of the mixture would change under the influence of different concentrations of harmful substances. The response obtained should depend on the concentration of the harmful substance. This would demonstrate the effectiveness of the method in classifying mixtures.

The aim of this study was to verify whether the CEI test is a suitable method for the classification of mixtures.

Sodium dodecyl sulphate (SDS; Chempur) and Triton-X-100 (Sigma-Aldrich) were used as harmful substances at different concentrations (0.02–200 mg/mL) in a solution containing 100 mg/mL glycerol (Avantor). One mixture contained 20 mg/mL SDS, 20 mg/mL Triton-X-100 and 100 mg/mL glycerol. More than 8,000 HDFn cells (Human Dermal Fibroblasts, neonatal; Thermo Fisher Scientific) in three replicate wells were treated with 5 concentrations of the mixture (0.5–50 mg/mL) and neat material. Cells were treated for 30 minutes. After 6-fold dilution in human fibroblast expansion medium (Gibco) with low serum growth and overnight incubation (37±1°C, 5±1% CO_2, 90±10% RH), the cytotoxicity test was performed. The MTT assay was used in the first run and the neutral red uptake (NRU) assay in the second run. Viability and IC_{50} were calculated by comparison with the solvent control. Classification was as follows: category 1 (viability <50% at 0.5 mg/mL or IC50<0.9 mg/mL), category 2 ($0.9 \leq IC_{50} \leq 60$

mg/mL), no category (viability ≥30% in neat material) or cannot be predicted (other options than above).

As the concentrations of harmful substances decreased, the categorisation became less strict. Only the highest concentrations of these substances (200 mg/ml) were classified as seriously damaging to the eyes. A concentration of 0.02 mg/ml of both harmful substances led to a situation where no classification of the mixture was required. In the case of a mixture containing both SDS and Triton-X-100, a reduction in cell viability was observed compared to when these concentrations were tested independently (SDS without Triton-X-100 and Triton-X-100 without SDS).

The results show that the CEI method is capable of responding to the hazardous substance in a concentration-dependent manner and is therefore suitable for the classification of mixtures.

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P25-06
PackSafe: Integrated chemical safety assessment of Food Contact Articles (ANR-21-CE21-0004 project: 2022–2025)

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The environmental impact of food packaging has become a major concern. In line with the directive (EU) N°2019/904, the French law (N°2020-105) on circular economy enforces manufacturers to lighten Food Contact Articles (FCA), recycle and/or reuse them. These new FCA should be as safe as virgin materials, but some scientific articles have demonstrated a greater release of chemical contaminants in food. Contaminants such as Non-Intentionally Added Substances (NIAS) are represented by impurities, decomposition or reaction products and substances resulting from recycling. They are often unpredictable, difficult to identify, to quantify and their toxicity is not always assessed. As consumers are exposed to NIAS through foodstuffs, the health risk must also be assessed (European plastic Regulation (EU) N°10/2011).

The French project "PACKSAFE" proposes a relevant approach combining bioassays, analytical and physicochemical analyses on FCA extracts to consider all the substances able to migrate from packaging to food. The first step of "PACKSAFE" is to optimize a specific protocol of extraction regarding representative FCA extracts (PET, PE, HDPE, PP, PS, paper-board), relevant *in vitro* bioassays to identify genotoxic effects or endocrine activities. Spiking methodologies are performed to determine the specificity and the biological detection level of the selected bioassays.

Forty-one samples were selected from the French market. Among these samples, 34% were recycled, 41% were coloured or printed and 61% had already been in contact with food.

The protocol of sample preparation in a biocompatible solvent was optimised by using dimethyl sulfoxide (DMSO). This protocol, applied on a reference solution containing 93 substances of known concentrations representing the widest possible range of physic-chemical properties that can be found in FCA (polarity, molecular mass, Kovatz index, H donor/acceptor), gave a median recovery rate of 85%.

In terms of genotoxicity, in the mini-Ames test, 2 extracts were positive. With the MultiFlow® DNA damage assay, only one extract was positive for clastogenic activity with the biomarkers γH2AX and p53. In the micronucleus assay, 4 extracts were positive *in vitro*. Positive extracts are on the way to be analysed using fractionation methodologies to try to identify the active substance(s).

Concerning endocrine disruption, 6 extracts were positive in the ER reporter gene assay and 2 in the AR reporter gene assay. In the steroidogenesis test, several extracts were active increasing the estradiol synthesis.

The innovative nature of this project is to use, in parallel, chemical signatures of extracts and robust bioassays to generate a database to facilitate decision-making and packaging security at different stages of their production life cycle. It will also generate data on the toxicity of new NIAS.

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P25-07
Do co-formulants influence PPP genotoxicity? – a case study

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Plant protection products (PPPs) contain one or more active substances (AS) as well as a varying number of co-formulants. In particular, AS are responsible for a PPP's functionality whereas co-formulants support the efficacy of AS. While active substances are subjected to rigorous safety assessment during their evaluation, including genotoxicity assessment, evaluation of PPPs is limited to acute and topological toxicity only. In addition, while co-formulants usually are subject to data-requirements under REACH they do not undergo any particular toxicological evaluation or authorisation as part of PPP Regulation (EC) No 1107/2009. Genotoxicity is as a critical long-term endpoint. While the focus is on the identification of the genotoxic potential of the AS, the potential contribution of co-formulants and their interaction with one another or the AS is not systematically assessed. Firstly, we propose an approach to identify potentially genotoxic co-formulants and prioritise PPPs for further *in vitro* testing. Prioritisation based on genotoxicity *in silico* screening is combined with CLP criteria on the classification of mixtures. Priority is given to those PPPs with potentially genotoxic co-formulants exceeding the generic concentration limits set for the classification of mutagenic and carcinogenic mixtures. Secondly, we prioritize AS with known *in vitro* genotoxic potential to investigate the contribution of kinetics. The aim of this case study is to investigate the potency of micronuclei induction between the AS and the PPP. An *in vitro* micronucleus test is conducted taking into account an AS inducing the formation of micronuclei *in vitro* and its respective PPP. Our results indicate that there is difference in the induction of micronuclei between the AS and its respective PPP in the presence and absence of metabolic activation. In conclusion, based on *in silico* screening, only a few co-formulants were prioritised for further *in vitro* testing. However, experimental data ruled out the need to further investigate the genotoxicity endpoint *in vitro*. Kinetic effects could contribute to increased PPP micronuclei induction or genotoxicity in general and there is a need for systematic investigation to ensure PPP regulatory safety.

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P25-08
Mixture PBK modeling: from the bench to the CPU

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Plant protection products (PPP) are a combination of one or more active substances and several co-formulants. As mixtures, PPPs are susceptible to biological interactions at a toxicokinetic or toxicodynamic level which can lead to increased toxicity. For a realistic and accurate mixture risk assessment, these interactions should be considered. *In vitro* testing can be utilized to determine the qualitative and quantitative characteristics of the toxicokinetic interactions between PPP components as well as PPP-based toxicological threshold values. *In vitro-in vivo* extrapolation (IVIVE) using physiologically based kinetic (PBK) modelling is required to generate the organism-based threshold values for the whole product. So far, PBK modelling is almost exclusively applied to single substances. Therefore, we propose a strategy to generate data for mixture PBK modeling in PPP operator risk assessment. For a proof-of-concept mixture effect study on *in vitro* triglyceride accumulation endpoint, the PPP Revus top containing a steatotic compound, difenoconazole as well mandipropamid was chosen based on probable metabolic interactions between the two active substances predicted by ADMET predictor. For this purpose, a difenoconazole PBK model is built using *in vitro* generated parameters from PPP thus accounting for all possible interactions. Since PPP operators are chiefly exposed via the dermal route, the traditional rapid equilibrium dialysis (RED) for fraction unbound and pooled human liver microsomes or HepaRG cells for hepatic clearance determination are insufficient. Hence, in this study, we coupled the RED, microsomes and HepaRG cells with EpidDerm FT, a skin penetration model. EpiDerm FT was spiked with Revus top for 24 hours to allow for absorption before transferring the receptor contents to either RED, microsomes or HepaRG. The amount of difenoconazole at different time points was measured using LC-MS/MS and the fraction unbound and hepatic clearance were determined. For this study, the physico-chemical parameters as well as partition and diffusion coefficients across skin layers were obtained from literate and an in-house *in vivo* database. The fraction unbound of difenoconazole was not significantly changed by the presence of mandipropamid and other components in the product. However, the hepatic clearance of difenoconazole decreased in the product as highlighted by an increased intracellular difenoconazole concentration. This indicates that mandipropamid inhibits CYP3A4 thereby reducing the metabolism of difenoconazole. Overall, this strategy provides a starting point for PBK modeling for mixtures as it appreciates potential combined effects and toxicokinetic interactions within mixtures.

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P25-09
Investigating the joint action mode of di-(2-ethylhexyl) phthalate and bisphenol A co-exposure on autism spectrum disorder based on adverse outcome pathway

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Purpose: Di-(2-ethylhexyl) phthalate (DEHP) and bisphenol A (BPA), two commonly found plastics-derived environmental endocrine disruptors, have been implicated in the development of autism spectrum disorder (ASD). Using the adverse outcome pathway (AOP) approach, we recently identified abnormal synaptic formation and plasticity as the early common key event (cKE) that links DEHP and BPA co-exposure with the increased risk of ASD. In this study, we aimed to investigate the joint action mode of DEHP and BPA co-exposure on the risk of ASD by using an *in vitro* neuronal cellmodel.

Methods: SH-SY5Y cells were differentiated into neuron-like cells and treated with MEHP (0.2–20 μM), the major toxic metabolite of DEHP, or BPA (0.1-10 μM) alone, or in combination (0.2 μM MEHP+0.1 μM BPA, 2 μM MEHP+1 μM BPA, and 20 μM MEHP+10 μM BPA) for 6 days. Neurite outgrowth parameters including the percentage of neuron-like cells, the number of neurites per neuron-like cell, and the average neurite length were examined, as a surrogate of the early cKE. The joint action mode of MEHP and BPA co-exposure on neurite outgrowth of neuronal cells was analyzed by the CHOU, interaction factor (IF), and independent action (IA) models, which was then applied to predict the possible joint action mode of DEHP and BPA co-exposure on the increased risk of ASD.

Results: MEHP or BPA treatment alone impaired neurite outgrowth of differentiated SH-SY5Y cells as evidenced by decreases in the percentage of neuronal-like cells and the number of neurites per neuronal-like cell, and an increase in the average neurite length. Such changes were also observed in cells treated with MEHP and BPA in combination. For their possible joint action mode, all three models predicted a synergistic effect on neurite outgrowth when MEHP and BPA were treated simultaneously at low- and medium-dose. By contrast, for the combined treatment of high doses of MEHP and BPA, the CHOU model revealed a synergistic effect on inhibition of the percentage of neuron-like cells and the average number of neurite outgrowths, but an additive effect on the increase in average neurite length; whereas the IF model predicted an antagonistic effect on neurite outgrowth, and the IA model showed an additive effect on the inhibition of the percentage of neuron-like cells.

Conclusions: We uncover that co-exposure of DEHP and BPA at relatively low doses synergistically disturbs neuronal cell functions implying that an exacerbated risk of ASD could be possibly seen for a general population who lives in an environment with low levels of these two contaminants.

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P25-10
Update of European Food Safety Authority (EFSA)'s work on cumulative risk assessment of pesticide residues targeting the thyroid

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In line with Regulation (EC) No 396/2005, EFSA has undertaken a long-term plan to assess and update regularly the health risks at organ/system level resulting from co-exposure to different pesticide residues. For each organ/system considered at risk, EFSA is identifying the unambiguous toxicological effects ("specific effects") that may result from the combined exposure to pesticides and may be relevant for grouping pesticides into distinct cumulative assessment groups (CAGs). As regards thyroid, EFSA is currently updating its 2019 cumulative risk assessment (CRA). In the absence of new mechanistic information, (i) Hypothyroidism and (ii) C-cell hypertrophy, hyperplasia and neoplasia

have been confirmed as the two specific effects relevant for pesticides targeting the thyroid and for establishing two distinct CAGs. Changes in one or multiple indicators, i.e. toxicological endpoint(s) that are measurable in regulatory studies, are used as surrogates for detecting each specific effect and characterising the potency of each active substance (a.s.). For hypothyroidism, histological changes (follicular cell hypertrophy, hyperplasia, and neoplasia) are considered as the most suitable indicators, since hormones are not consistently measured in animal studies. C-cell hypertrophy, hyperplasia and neoplasia are taken as indicators of the homonymous CAG. Indicators of these specific effects are being collected from the available toxicological studies on 21 a.s. in plant protection products and 6 metabolites, previously prioritised for thyroid CRA. Finding at least one statistically significant and/or biologically relevant change in an indicator of a specific effect in a regulatory toxicology study is the necessary and sufficient condition for including an a.s. into a CAG. The potency of each a.s. included in a CAG is defined by means of an overall NOAEL and LOAEL for the respective specific effect, considering the most sensitive indicator across all available oral studies, species, and sexes. As the probability of actually causing a specific effect is not the same for all a.s., the strength of the evidence for a causal relationship between an a.s. and a specific effect will be estimated by means of a weight of evidence approach using pre-defined lines of evidence (LoE) carrying a different relative weight. For CAG 'hypothyroidism', examples of LoE include, among others, a known or a presumed relevant mode of action, evidence of damage progression, consistency of indicator changes within the same species and/or across species, decreased T3 and/or T4 levels and increased TSH concentration in serum. No LoE have instead been defined for the CAG on C-cells. This is no longer needed since EFSA's previous work has shown that the CRA for CAG 'hypothyroidism' broadly covers for the combined effects of pesticides belonging to the CAG on C-cells. The establishment and the characterisation of the newly set CAGs on thyroid are still ongoing and will be presented.

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P25-11
Implementation of a novel mouse plethysmography model for measuring acute changes in respiratory parameters during inhaled dosing

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The potential for inhaled compounds to induce adverse effects in the airways is a challenging hurdle in inhaled drug development. Respiratory irritation following acute inhalation in *in vivo* studies is one issue that should be mitigated early in the drug discovery process before progressing to costly studies using large numbers of animals.

Undertaking inhaled respiratory investigative studies early in safety assessment can bring with it many issues, such as the requirements to scale up synthesis to provide enough material during lead optimisation. It also requires having access to the correct equipment both to administer the test material to the lung and sensitive measurement devices to detect subtle changes in the respiratory parameters of rodents.

At AstraZeneca we have adopted a novel approach when it comes to performing early *in vivo* assessment of new chemical entities for pulmonary drug delivery. Our approach ensures we have calm well-trained animals in statistically powered experiments using modern innovative technology.

During lead optimisation it is uncommon to have synthesised the amount of test material needed to perform rodent inhalation studies. It is more commonplace to administer the test material by intratracheal administration (IT). This dose route comes with inherent limitations, it is more invasive towards the animal, the distribution in the lung is less homogenous when compared to inhalation and it is not possible to measure the acute effect of the test material on a conscious animal as IT dosing is performed under anaesthesia.

To mitigate these issues AstraZeneca has designed and built a new rodent nebulisation system that utilises 15 times less test material when compared to commercially available *in vivo* inhalation systems. Along with this new inhalation system we have tested and validated the Buxco® Head-out Plethysmographs and Alley restraint system to measure respiratory parameters during inhaled dosing.

We have also adapted the training of our mice to the Alley restraint with an ethical and 3R mindset using a refined training procedure and positive reinforcement to ensure our animals are handled and trained so they are calm and breathe well in the plethysmograph tube.

With the new inhalation method combined with the sensitivity of the Buxco system and calm well trained animals we can use robust statistical analysis of the respiratory parameters to reduce our animal numbers and can still detect changes in multiple respiratory parameters during inhaled studies earlier in drug discovery.

With this novel approach, we can provide an early robust plethysmography assessment across our diverse inhaled portfolio to de-risk and rank compounds prior to committing to inhaled MTD/DRF studies.

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P25-12
Effects of personalized chemical mixture on endocrine disruption based on estrogen nuclear receptors

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Chemical compounds are wide spread in modern society and exposure to them is linked with a wide range of adverse outcomes such as cancer, infertility, chronic and metabolic diseases. Although toxicology has traditionally focused on the evaluation of individual chemicals, this approach does not capture the reality of the chemical exposome, where individuals are exposed to multiple chemicals during their life-time, creating a unique personalized mixture profile. These chemicals and mixtures can affect many targets and the endocrine system is one of the most sensitive as by the action of endogenous ligands, at very low concentrations, regulates several aspects of the organism homeostasis as development, reproduction, and metabolism. Dysregulation of the endocrine system may therefore have severe health impacts.

In this work, we aimed to evaluate the endocrine disruption potential of real-world exposure to individual chemicals and mixtures on the estrogen receptor (ER) activity. Based on data collected from the epidemiological Västerbotten intervention programme (VIP) study in Sweden serum concentrations of 24 individual chemicals and their consequent respective mixture from the participants were reconstructed using an automated liquid handling system. The individual chemicals and mixtures were tested in an optimized and scaled-up 384-well plate high throughput screening (HTS) version of OECD test guideline No.455 protocol for endocrine disruption, using VM7 cell line as an ER reporter system. The results showed that some individual chemicals as well as mixtures could elicit an agonist activity.

These results highlight the importance of HTS to evaluate large amounts of environmental chemicals in a faster and more consistent manner, as well as a shift of paradigm towards the evaluation of per-

sonalized and realistic-world exposure of chemicals in humans and its prediction of endocrine disruption.

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P25-13

Effects of personalized mixtures of POPs on steroidogenesis and viability in H295R cells

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Humans are ubiquitously and chronically exposed to environmental contaminants that circulate in blood, including complex mixtures of persistent organic pollutants (POPs) such as per- and polyfluoroalkyl substances (PFAS), polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and polybrominated diphenyl ethers (PBDEs). Individual exposomes, including the relative exposure profile and concentrations of these chemicals in blood, depend not only on environmental factors but many personal characteristics and unique behaviors. With regards to potential mixture effects, it is therefore overly simplistic to exclusively construct and study the effects of universally representative contaminant mixtures. Many individual POPs are known endocrine disruptors, having the potential to disrupt steroidogenesis, but the effects of personalized POPs mixtures on these endpoints is not well studied.

By reconstructing real-world 'personalized contaminant mixtures' of 24 POPs, using multiwell chemical library plates and acoustic no-contact liquid handling, we aimed to investigate the *in vitro* effects of human blood POPs mixtures on cell viability and synthesis of testosterone and estradiol in H295R adrenal cells. The chemical mixtures were reconstructed based on plasma levels of POPs quantified in adult participants of a cohort study in northern Sweden, the Västerbotten Intervention programme. The H295R steroidogenesis assay, described in OECD test guideline #456, was upscaled to 96-plate format to increase throughput. Cells were treated for 48h, after which cell viability was measured by MTT, and testosterone and estradiol levels were quantified by ELISA.

After exposure to POPs mixtures, including ten personalized contaminant mixtures and two artificially constructed mixtures representing a worst-case scenario and a median mixture, we have found significant effects on viability and steroidogenesis for both personalized and artificially constructed mixtures. Specific mixtures elicited reproducible and statistically significant effects at concentrations equivalent to those detected in adult plasma, but not always at higher concentration. These results highlight the importance of studying real-world mixtures of environmental chemicals, in realistic proportions and at concentrations relevant to human exposure. Future investigations include mechanistic studies and subtractive screening of mixtures to identify the major chemical sources of toxicity in the mixtures.

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P25-14

Renal disturbances after prenatal and postnatal exposure to a very low dose of glyphosate and its mixture with dicamba and 2-4-D

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The understanding of the effects on human health of prolonged low-dose exposure to glyphosate and its mixtures is a challenge for researchers. The aim of this study was to assess the effects of exposure to low-dose glyphosate and its mixture with 2,4-D and dicamba from prenatal life into adulthood, mimicking exposure to the latest generation of GM crops tolerant to a combination of these herbicides. Four groups of rats (10 male and 10 female) were exposed to 0, 0.5, and 50 mg/kg bw per day glyphosate and a mixture of 0.5 mg/kg bw per day glyphosate, 0.02 mg/kg bw per day 2,4-D, and 0.3 mg/kg bw per day dicamba in drinking water from gestational day 6 to 13 weeks after weaning. The animal study was performed in accordance with the Guiding Principles for the Use of Animals in Toxicology and was ethically approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova. Creatinine and urea levels significantly increased after exposure to the chemical mixture compared with the control group both in males and females. Exposure to glyphosate at 50 mg/kg bw per day resulted in a significant increase in creatinine, urea and uric acid in males and a significant increase in urea in females. At the tissue level, the chemical mixture caused reversible changes such as hydropic degeneration at the tubular level with rare intense eosinophilic multinuclear masses and mild to moderate enlargement of the subcapsular space at the glomerular level. In the glyphosate-only groups, the effects were dose-related, with clear cytoarchitectural changes in all samples, present at the tubular and glomerular levels in the 50 mg/kg bw per day glyphosate group. In conclusion, our results suggest that prenatal and postnatal exposure to low doses of glyphosate and its mixture with 2,4-D and dicamba, at doses at which individual pesticides are considered safe by regulatory authorities, causes renal effects in male and female rats.

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P25-15

Using zebrafish to quantify the biological responses to chemicals found in human and environmental biomonitoring

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Exposure to chemical mixtures is common, yet studies often focus on simple combinations of only 2 or 3 chemicals, overlooking the primary drivers of toxicity among co-occurring chemicals. Previous efforts categorized chemicals from ARCHE Consulting and the Human Biomonitoring for Europe (HBM4EU) initiative, identifying 8 overlapping chemical subclasses. Four of these subclasses, totaling 25 chemicals, were selected for this project. The aim was to assess developmental toxicity and behavioral responses in zebrafish following exposure to chemical mixtures.

To address this gap, this study elucidated mechanisms of action and mixture behavior within chemical subclasses. The chosen subclasses included alkyl fluorides, diphenylmethanes, fatty acid esters, and halobenzenes. Dechorionated zebrafish embryos were exposed to varying concentrations of chemicals individually from 6 to 120 hours post-fertilization (hpf) and assessed using two behavioral assays: the embryonic photomotor response assay (EPR) and the larval photomotor response assay (LPR), alongside morphological evaluations. Initial experiments involved a 7-point concentration curve (n=12) from 0.1–100 μM for each chemical, followed by definitive testing with a 7-point

concentration curve (n=36) from the highest concentration with no observed adverse effects (NOAEL) to the lowest concentration resulting in 100% affected embryos.

Morphological and behavioral endpoints were evaluated to determine lowest effect levels (LELs) and the concentration at which 50% of embryos are affected (EC50) for each chemical. 24 chemicals exhibited activity in at least one assay, while one was inactive across all assays. EC50 values varied widely among chemicals within the same subclass, such as within the diphenylmethanes, while compounds within other subclasses, namely fatty acid esters, showed more similar EC50 values. Behavioral effects also varied among and within chemical subclasses. For instance, each of the 7 diphenylmethanes showed behavioral effects in both EPR and LPR assays, whereas 5 of the 8 alkyl fluorides exhibited effects in the LPR assay.

After identifying behavioral LELs and morphological EC50s, dose additivity or independent action was further investigated within the same subclass. This study enhances understanding of mixture toxicity drivers and mechanisms, enabling a focus on mixtures by chemical classes; in addition, future directions include exploring these drivers across chemical subclasses.

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P25-16
Toxicokinetics in mixture toxicity assessment: screening for potential CYP inhibitors and substrates to form Common Kinetic Groups (CKGs)

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The evaluation of potential mixture toxicity is of long-standing interest but toxicologically challenging. There is ongoing public discussion if and to what extent mixture toxicity might affect public health and hence needs regulatory addressing. Traditionally many approaches have a strong toxicodynamic focus that conceptually relies on the dose addition concept, target organ toxicity and mode of action. However, recent results suggest to concomitantly also pay more attention to the potential contribution of toxicokinetics on potential mixture effects. This was picked up particularly by the work of Braeuning *et al.* [1] who introduced the concept of Common Kinetic Groups (CKGs), that includes the consideration of ADME (Absorption, Distribution, Metabolism, and Excretion) properties and grouping chemicals based on their potential as inhibitors, substrates, or inducers of molecular mechanisms (i.e., enzymes).

This abstract presents a study focusing on the most toxicologically relevant CYP- (Cytochrome P450) dependent enzymes, that is, CYP1A2, 2C19, 2C9, 2D6, and 3A4 to investigate potential inhibitors and/or substrates within mixtures. We conducted a screening of over 200 pesticide active substances utilizing two open-access and one commercial (quantitative/qualitative) structure-activity relationship ((Q)SAR) tools: SwissADME, SuperCYPs Pred, and ADMET Pred, respectively. The results were compiled and a list of potential modulators were made for each enzyme.

By elucidating the interplay between toxicokinetics and mixture toxicity, our research contributes to the understanding of chemical interactions and their impact on human health. Integrating toxicokinetic principles into mixture toxicity assessment not only enhances regulatory strategies for risk assessment and management but also facilitates informed decision-making in chemical safety. Furthermore, employing *in silico* approaches alongside *in vitro* testing can augment the efficiency of screening efforts, potentially reducing the reliance on traditional *in vivo* studies while ensuring reliable and accurate results. This approach highlights the importance of leveraging computational tools to complement experimental methodologies and advance toxicological sciences, in hopes to replace animal testing.

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P25-17
Mixture toxicity of perfluoroalkyl substances (PFAS) in zebrafish larvae and HepG2 cells

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Per- and poly-fluoroalkyl substances (PFAS) are organofluorine compounds widely used in commercial and industrial products due to their unique physical and chemical properties. They are persistent contaminants and can cause various toxic effects on humans and other biological systems. The aim of our study is to investigate the effects of single PFAS substances as well as of PFAS mixtures *in vitro* and *in vivo*. Perfluorohexanesulfonic acid (PFHxS), perfluorononanoic acid (PFNA), perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) were tested as single compounds and then in mixtures after 48 h treatment in Zebrafish larvae and 4 h in HepG2 cells. The dose response curves of the results of the mixture experiment were compared to the predicted effects based on different methodologies. The results of the individual compound tests indicate a clear concentration-dependent increase of the toxicity to zebrafish larvae and cytotoxicity in HepG2 cells for all four tested compounds, and the half-maximal lethal concentration (LC_{50}) for zebrafish larvae and the half-maximal inhibitory concentration (IC_{50}) for HepG2 cells were determined. Mixture composition was based on these toxicity values. Generally, it can be concluded that most mixtures predominantly lead to additive effects, while in some cases complex interactive effects were shown.

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P25-18
Application of *in vitro* New Approach Methodologies to determine whole mixture-based relative cancer potency factors

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Air pollution and airborne particulate matter (PM) are classified as carcinogenic to humans, but their complex composition makes quantitative risk assessment a challenge. Current strategies for cancer risk assessment of air pollution are based on a pollutant-by-pollutant approach. This is clearly a simplification which excludes the possibility of interaction effects and may misestimate the actual cancer risk. Whole mixture-based testing using *in vitro* New Approach Methodologies (NAMs) has been suggested to facilitate the hazard and risk assessment of complex environmental mixtures. We have addressed this issue by developing a NAM for whole mixture-based cancer risk assessment of air pollution. The overall aim is to combine state-of-the-art

methods for analysis of chemical composition of urban, diesel and biomass burning PM with *in vitro* testing of PM samples in order to determine Mixture Potency Factors (MPFs) estimating the carcinogenic potency of whole mixtures. Our results so far show that MPFs based on whole mixtures indicate cancer potency better than looking at single pollutants [1]. Moreover, these MPFs are in good agreement with potency values based on published data from Salmonella mutagenicity and *in vivo* carcinogenicity studies [2]. This research will develop an approach that can be used for assessment of total carcinogenic effects of air PM pollution both for larger city-wide and for smaller site-specific risk assessments. Ultimately, this *in vitro* NAM will improve the cancer risk assessment of airborne PM by including the obtained knowledge about whole mixture potencies in already established models for estimation of lung cancer incidence in polluted environments.

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P25-21
Examining the impact of a realistic mixture of dietary contaminants on the intestinal barrier and compounds absorption

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Through our dietary intake, we encounter a diverse array of harmful food contaminants (FC) such as pesticides, heavy metals, mycotoxins, heterocyclic amines (HAAs), and polycyclic aromatic hydrocarbons (PAHs). Exploring into the effects of FC mixtures on the intestinal barrier health, directly exposed to these compounds, represents a crucial area of investigation. This is especially pertinent given that existing research frequently neglects the complex mixtures consequences, as well as the interactions among different cell types within the human intestine. Integrating these aspects in our evaluations is crucial for a holistic comprehension of the impact of FC on intestinal health.

In this work, we used an *in vitro* triple culture model (Caco-2/HT29-MTX<insert membrane>THP-1 cells) to replicate both healthy and inflamed conditions (1) within the human intestine to investigate cellular responses to a repeated exposure (2 times for 3 hours) to a complex mixture of 41 food contaminants from the classes listed above. This mixture was formulated based on occurrence data (2), aiming to simulate realistic daily exposure levels at the 25th (P25) and 95th (P95) percentile found in a healthy omnivore diet. Cell viability, barrier integrity (TEER), mRNA expression and compounds absorption in both physiological conditions unveiled significant findings. Exposure to the P95 mixture led to a clear reduction in TEER values in the Caco-2/HT29-MTX monolayer (~20%) more pronounced in the inflamed intestine (~60%). Expression of mRNA in intestinal cells was altered for tight junctions and transporters levels, as well as in inflammatory markers of THP-1 cells. The absorption of compounds trough Caco-2/HT29-MTX was also influenced by the contaminants levels and intestinal state.

These results strongly indicate that the mixture of contaminants at realistic concentrations, particularly the higher level P95, significantly impairs the intestinal epithelium's barrier integrity, especially after repeated exposure which occurs throughout diet, and in an inflamed state. This suggests an exacerbation of effects on an already compromised intestinal barrier. Furthermore, the data confirm that mixtures of FC, present at realistic and prevalent concentrations, can exert a disruptive effect on intestinal barriers and subsequent compounds absorption, with implications for the final human exposure.

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P25-22
Insight into the cardiotoxicity in Wistar rats after subacute exposure to a mixture of lead and polychlorinated-biphenyls

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In real-life scenarios, humans are commonly exposed to a mixture of chemicals rather than just single compounds. Thus, the comprehensive assessment of their complex toxicity is of paramount significance. This study aimed to see how a mixture of lead (Pb) and polychlorinated-biphenyls (PCBs) affects redox status parameters in the cardiac tissue of male Wistar rats (nine groups). Animals (six per group) were receiving different nine combinations of investigated substances (Pb in the doses of 0.1, 0.5 and 1 mg/kg b.w./day and PCBs in the doses of 0.25, 0.5 and 1 mg/kg b.w./day for 28 days) by gavage for 28 days. The control group received corn oil only. The results have shown that subacute exposure to environmentally relevant toxic mixture affects cardiac tissue by altering parameters of oxidative stress, resulting in cardiotoxicity that could be linked to oxidative damage. Furthermore, Benchmark dose modelling confirmed dose-dependent changes in one parameter of oxidative stress (ischemia-modified albumin (IMA)) and calculated BMDL was 0.264 mg PCB/kg b.w./day. These findings indicated that IMA may be sensitive marker for oxidative stress and cardiac ishemia in further assessment of adverse effects on human health after combined exposure to a mixture of lead and polychlorinated-biphenyls.

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P25-23
Toxic effects of ethylbenzene and *m*-xylene, individually and as a mixture, on human bronchial epithelial cells exposed at the air-liquid interface

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Benzene, toluene, ethylbenzene and xylenes (*o*-, *m*-, *p*- xylenes) collectively form a widespread mixture commonly referred to as BTEX. This mixture is known for its toxic effects and is frequently encountered in indoor air within residential and professional environments. While most *in vitro* toxicological studies focus on the mechanisms of action of benzene and its substitute, toluene, there are very few experimental toxicology studies that have characterized the mechanisms of action of *m*-xylene and ethylbenzene, and even fewer for the binary mixture. The result is incomplete data on the consequences of human exposure to these two VOCs.

In this research project, our objective was to investigate the acute toxicity of ethylbenzene and *m*-xylene, both individually and in binary mixtures, using human bronchial epithelial cells exposed at the air-liquid interface (ALI). BEAS-2B cells were exposed for 1 hour to VOCs alone or as a mixture at their French Occupational exposure levels for long- and short-term (VLEP-8h and VLCT-15min, respectively). By studying the cells after 5, 23 and 47 hours of incubation, we were able to characterize the kinetic of various toxic effects, including cytotoxicity, xenobiotic biotransformation, the antioxidant defense system, the inflammatory response and apoptosis.

Our findings revealed that biological responses to exposure to ethylbenzene and *m*-xylene are specific, whether encountered alone or in a binary mixture. Ethylbenzene does not appear to be metabolized in BEAS-2B cells, as it inhibited gene expression of the studied xenobiotic metabolizing enzymes (XME). It does not induce antioxidant defense systems or apoptosis. However, a slight inflammatory response was observed but only after exposure to its VLCT-15min (100 ppm). In contrast, *m*-xylene is metabolized in BEAS-2B cells, inducing several XMEs. It also upregulates certain enzymes involved in the antioxidant defense system, as well as markers of inflammation and apoptosis. Co-exposure to the binary mixture resulted in an inhibition phenomenon, affecting the toxic action mechanisms studied. The statistically significant gene inductions observed after exposure to xylene were reduced when co-exposed with ethylbenzene.In conclusion, the results of this study have provided new information on the toxicity of ethylbenzene and *m*-xylene. They also show the importance of conducting ALI exposures to mixtures of toxicants, as the responses observed are not necessarily predictable by conventional hypotheses such as additivity. These results may contribute to a better understanding of the effects of these compounds on human health.

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P25-24
Unlocking nature's arsenal: discovery of *Alternaria* mycotoxins as novel immunosuppressive and gut microbiota-targeting compounds

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Mycotoxins produced by molds of the genus *Alternaria* may pose significant challenges to food safety and public health due to their frequent occurrence in food and wide spectrum of adverse effects observed *in vitro* and *in vivo*. Despite this, critical information on the immunomodulatory and antimicrobial properties of these contaminants is still lacking, underscoring the urgent need for data to evaluate the associated health risks.

The present study aimed to identify the mycotoxins responsible for the *in vitro* immunosuppressive and antimicrobial properties of a complex extract of *Alternaria* mycotoxins (CE) obtained by growing an *Alternaria alternata* strain on rice. Through a toxicity-guided fractionation procedure, involving the production of CE-fractions by supercritical fluid chromatography and the mycotoxin quantification by LC-MS/MS, the mycotoxins alternariol (AOH), tenuazonic acid (TeA), altertoxin I (ATX-I), alterperylenol (ALP), and altersetin (AST) were identified as potentially responsible for the effects exerted by the extract. The immunosuppressive properties of these compounds were assessed in THP-1 Lucia monocytes by applying the NF-κB reporter gene assay in the presence of lipopolysaccharide stimulation. To better simulate the *in vivo* situation, some selected mycotoxins were also tested in a co-culture model of Caco-2 and HT29-MTX-E12 cells, separated from THP-1 Lucia cells by a semipermeable membrane. To ensure accurate interpretation of the results, cell viability was consistently assessed using the CellTiter Blue assay. The mycotoxins AOH, ALP, and AST were further evaluated for their effects on the growth of eight human gut bacterial strains isolated from adults and children (i.e., *Bacteroides vulgatus, Bacteroides thetaiotaomicron, Escherichia coli, Bifidobacterium adolescentis, Clostridium sporogenes, Enterococcus faecalis, Klebsiella michiganensis, Lactobacillus plantarum*). Assessment of the bacterial growth kinetics was performed by measuring the optical density of bacterial suspensions at 600 nm over 24 h.

Results of the study revealed the inability of TeA (up to 250 μM) to suppress the NF-κB pathway, while confirming the immunosuppressive properties of AOH, ATX-I, ALP (≥1 μM), and AST (≥2 μM) in the monoculture model. The suppressive effects of ATX-I and ALP were

also confirmed in the co-culture model, where reductions in NF-κB pathway activation were observed without the occurrence of cytotoxicity or disruption of the cell monolayer. With respect to the antimicrobial properties, the mycotoxins AOH, ALP, and AST were found to affect, albeit with different potencies, the growth of the various bacterial strains tested, except for the selected *E. coli* strain.Given the potential detrimental impacts stemming from alterations in gut microbiota composition and systemic immune responses, further studies are needed to elucidate the underlying mechanisms of action and comprehensively evaluate the health risk posed by these toxins.

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P25-25
Dietary cumulative risk assessment of pesticide residues having effects on kidneys

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The European Regulations (EC) No 396/2005 on maximum residue levels in or on food and feed of plant and animal origin and Regulation (EC) No 1107/2009 concerning the placing on the market of plant protection products (PPPs) require thorough consideration of cumulative effects of residues of PPPs. EFSA applied the methodology that has been developed along the years to group pesticides having effects in the kidney into the so-called cumulative assessment groups (CAGs). To this aim, the following well-characterized and unambiguous toxicological effects (i.e. specific effects) in the kidney were identified as relevant for cumulative risk assessment and for the establishment of CAGs: glomerular injury, tubular injury, tubular crystals, papillary necrosis, interstitial nephritis, pelvis erosion/ulceration, pelvis calculi/crystals, renal preneoplastic and neoplastic lesions and pelvis preneoplastic and neoplastic lesions. Thirty-five active substances (a.s.) used as PPPs and three metabolites, all selected based on indications to cause some effects on kidneys, were considered. The experimental animal studies available for the aforementioned selected substances were analysed to allocate the substances in the different CAGs. To this purpose, a list of histological indicators of the identified specific effects was defined and criteria were established to allow the correct allocation of the substances into each CAG and to define the nature of the hazard (acute or chronic). Finally, EFSA defined a weight of evidence approach to assess the probability of each a.s. or metabolite to be correctly included into a CAG (CAG-membership probability). The establishment and characterisation of CAGs on kidney is still ongoing and upon finalisation it will undergo public consultation. Progress in this activity will be presented at the meeting.

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P25-27
In vitro assessment of PFAS mixtures in a human induced pluripotent stem cell-based 3D model of embryo- and developmental toxicity

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Current efforts to describe the human exposome reveal that all parts of the World are contaminated with per-/polyfluorinated substances (PFAS) and, as a consequence, most humans are exposed. The critical adverse effects of the four PFAS regulated by the European Food Safety Authority (EFSA), namely PFOA, PFOS, PFNA and PFHxS, have been identified as impaired vaccination responses, reduced birth weight and increased cholesterol levels in humans [1]. However, as PFAS are a large class of 10,000 synthetic chemicals, there is an urgent need for screening tools to predict developmental toxicity for the remaining PFAS, for which we are lacking toxicological information.

We have developed the PluriLum reporter gene assay [2,3] which is based on cardiac differentiation of hiPCS in a 3D embryoid body format as a model for embryo- and developmental toxicity. We have previously demonstrated that three PFAS, namely PFOA, PFOS, and undecafluoro-2-methyl-3-oxahexanoic acid (GenX), individually inhibit cardiac differentiation in this assay [3].

In this study, we have investigated potential developmental effects of PFOA, PFOS, PFNA and PFHxS in the PluriLum assay. We found that all displayed detrimental effects in our model, with potencies in the following order (from most to least potent): PFNA>PFOS>PFOA>PFHxS.

We will present the outcomes of the mixture effects of these four PFAS to determine if their combined action follows the principles of dose addition, as well as the effects of realistic mixtures designed based on reported internal blood concentrations.

Lastly, we will investigate the modes of action of the individual PFAS by investigating transcriptional changes upon exposure to benchmark concentrations of the individual PFAS by the use of RNA sequencing.

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P25-28
Assessing the influence of toxic metal mixtures on pancreatic redox imbalance: Wistar rat model

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Toxic metals are still, despite regulatory restrictions, one of the most significant environmental pollutants. In addition to the numerous toxic effects that have been confirmed in various studies, increasing evidence indicates that many of them have endocrine disrupting properties. Also, it is known that one of the main mechanisms mediating the occurrence of toxic effects is the production of reactive oxygen species and the impact on the antioxidant system of the organism. Oxidative cell damage is involved in the pathogenesis of many diseases, including diabetes, and increased oxidative stress in the pancreas has been shown to decrease insulin production and lead to increased tissue insulin resistance. However, the majority of studies addressing this issue have primarily focused on the impact of high doses of individual metals, which is not a real-life exposure scenario. This study aimed to examine the effect of a toxic metals mixture (As, Pb, Hg, Cd, Cr (VI), and Ni) on the parameters of oxidative stress and antioxidant protection (IMA, MDA, SH groups, GSH, SOD) in the pancreas of male and female Wistar rats after exposure for 90 days. The doses were selected based on a previously conducted human biomonitoring (HBM) study and correspond to the exposure of the general population of Serbia. The study included 20 male and 20 female Wistar rats, who were randomly divided by gender into 2 control and 6 treatment groups. The animals were treated with aqueous solutions of toxic metal mixtures where each metal was in the dose mimicking the levels obtained from the HBM study: M1/F1 groups – the lower confidence limit of the Benchmark dose (BMDL) for effects on hormone levels, M2/F2 groups – median concentrations, and M3 /F3 groups – 95th percentile concentrations. After 90 days the animals were sacrificed, tissue samples were collected and pancreas oxidative status parameters were determined by the spectrophotometric method. A significant increase in IMA and SH group levels was observed in the F1 group compared to the control. A significant increase in GSH levels compared to the control was observed in the F2 group, while a significant increase in SOD levels was observed in F2 and F3 compared to the control. No significant increase in MDA levels was observed in female rats if compared to the control. There were no significant changes in any parameters observed in male rats. It has been shown that exposure to toxic metal mixtures may cause certain perturbations in the oxidative status of the pancreas at doses that reflect real-life exposure scenarios of the general population, with the effect and intensity being dependent on gender.

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P25-29
Interactions of plastic nanoparticles with fluoxetine and carbamazepine in estrogen receptor activation *in vitro*

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Plastic value chain has become a central part of modern living. Excessive use and inadequate waste management have led to the emergence of plastic nanoparticles (PNPs) in the environment[1]. Polystyrene, polyethylene and polypropylene nanoparticles (PSNPs, PENPs and PPNPs, respectively) are the most represented. Despite the growing exposure to PNPs, their effects on human health remain relatively unknown, especially their effects on the endocrine system. Pharmaceuticals are another group of emerging pollutants of great concern. Fluoxetine and carbamazepine are among the most frequently detected drugs in the environment with their concentrations reaching up to μg/L[2–4]. PNPs can adsorb pharmaceuticals in the environment, act as a "Trojan horse", and completely change the toxic profile of individual drugs[5]. Despite this, risk assessment of chemicals and materials is still heavily based on the toxicological profiles of individual components.

PNPs and their mixtures were characterized employing transmission electron microscopy (TEM), dynamic light scattering (DLS) and electrophoretic light scattering (ELS). Cytotoxic effects of fluoxetine and carbamazepine were determined using the MTS assay. Effects of PSNPs (25 nm), PPNPs (180 nm) and PENPs (350 nm) on apoptosis activation and cell viability were tested by flow cytometry with annexin-V:FITC and PI. Non-cytotoxic concentrations were used to determine the endocrine-disrupting properties of all three types of PNPs, fluoxetine, carbamazepine and their mixtures on the T47D-KBluc cell line[6]. This cell line is stably transfected with a reporter gene for luciferase, located downstream from the estrogen response element (ERE). ER activity of components and complex mixtures was determined using the luciferase assay according to the protocols developed by OECD[7] and U.S. EPA[8]. Interactions between mixture components were examined using dose addition (D_{DA}) and independent action (D_{IA}) models to determine whether their joint effects were synergistic or antagonistic[9].

Individual components, other than PENPs, have not shown significant effects on ER activity and individually would not be considered as endocrine disruptors. However, their mixtures have shown complex interactions dependent on the qualitative and quantitative composition of the mixture. Mixtures of carbamazepine and fluoxetine show primarily antagonistic activity. On the other hand, mixtures of drugs with PNPs show significant synergistic effects for which dose-response is heavily dependent on the size and type of PNP in the mixture. These results highlight the importance of mixture toxicity investigation as part of an adequate and complete risk assessment of chemicals and nanomaterials in the environment.

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P26 | Toxicology in Life Cycle Analysis

P26-01
Hexagonal boron nitride (hBN) and hBN-reinforced thermoplastic polyurethane composite: an *in vitro* hazard characterization using human skin and lung cells

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Hexagonal boron nitride (hBN) is an emerging two-dimensional (2D) material that is attracting considerable attention in the industrial sector given its innovative physicochemical properties. The potential health risks of 2D materials are associated mainly with occupational exposure where inhalation and skin contact are the most relevant exposure routes for workers. Beside the paucity of toxicological data related to hBN, the hazard potential of hBN-containing products is seldom evaluated. Nevertheless, the risk for human health, as well as for the environment, could be associated not only with the properties of the pristine material, but also with particles released after mechanical degradation during the life cycle of hBN-reinforced products.

Hence, this study was aimed at characterizing *in vitro* the effects induced by a composite made of thermoplastic polyurethane (TPU) and hBN, using immortalized HaCaT skin keratinocytes and immortalized BEAS-2B bronchial epithelial cells as models to study the potential toxic effects after skin contact and inhalation, respectively. The composite was abraded using a Taber® rotary abraser and abraded TPU and TPU-hBN were also subjected to photo-Fenton-mediated degradation to mimic the potential weathering of the material across the life cycle of the product. Cells were exposed at low concentrations of the materials for 24 h (acute exposure) or twice per week for 4 weeks (chronic exposure) and evaluated with respect to material internalization, cytotoxicity, and proinflammatory cytokine secretion. Additionally, comprehensive mass spectrometry-based proteomics and metabolomics (secretomics) analyses were performed to further elucidate the cellular responses to the materials following acute or chronic exposure. Overall, no significant alterations of cell viability or cytokine secretion were observed after acute or chronic exposure of HaCaT cells or BEAS-2B cells, despite evidence of cellular uptake of the material. Similarly, no major alterations in protein expression profiles were observed, identifying only a small number of pro-inflammatory proteins. Interestingly, similar results were obtained for the photo-chemically degraded materials, suggesting that the degradation process does not increase the cytotoxic potential of the materials.

On the whole, these results may support the characterization of the hazard profiles associated with cutaneous and pulmonary exposure to hBN-reinforced polymer composites, also under an end-of-life scenario.

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P26-02
Ensuring pharmaceutical packaging safety: an integrated approach for assessing extractable profiles and toxicological risk

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Extractables are organic and inorganic chemical entities that can be released from container closure systems into an extraction solvent under laboratory conditions. These have the potential to leach into a drug product under normal conditions of storage and use, thus becoming leachables. Extractables are characterized in a concentration equal to or greater than the Analytical Evaluation Threshold (AET), calculated based on the Maximum Daily Dose and of an appropriate Safety Concern Threshold. The extractable and leachable (E&L) workflow for ensuring safety includes two steps:

1. A step-by-step process for extractables identification
2. Toxicological evaluation of extractables

An E&L study is a forced laboratory extraction process aimed at identifying potential leaching of compounds from pharmaceutical packaging. Its purpose is to establish the worst-case scenario. The identification process should ensure to generate an extract that potentially contains:

- all the substances that could eventually leach into the drug product
- extractables at concentrations equal to or greater than the minimum concentration that these chemical entities could reach in the drug product

The drug product's ability to leach chemical entities from a packaging system can be established based on the use of multiple extracting solvents and techniques, each of which addresses one (or more) of the extracting mechanisms related to the drug product under investigation, based on its physico-chemical characteristics. The limits of quantification of the analytical procedures should be lower than the calculated AET. So, this could be considered the "worst case" approach.

The potentially migrating extractables are then evaluated from a toxicological perspective, comparing the amount of extractable that could potentially migrate versus its corresponding safety limit. The safety limit quantification depends on data available for the extractable:

- When experimental toxicological data is available, the Permitted Daily Exposure (PDE) is calculated based on relevant guidelines (ICH Q3C)
- When toxicological data is limited, the appropriate threshold of toxicological concern can be applied based on the *in silico* profile of the chemical; readacross can also support the derivation of higher limits; in such cases a PDE can be calculated for the data-poor extractable using available evidence of a data-rich surrogate

Notably, unknown compounds are considered as mutagens, according to the worst-case scenario.

The toxicological evaluation may prompt further testing, including a target analysis in a leachable study.

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Late Breaking Abstracts

LP-01
The use of an *in vitro* dendritic cells model in the evaluation of polyexposure to two sensitizing substances

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In the workplace, workers can be exposed to various chemical and biological substances, sometimes in a situation of polyexposure. A large number of these substances are known to cause occupational allergies. However, only few data regarding polyexposure side effects on human health are available [1,2]. The European legislation encourages scientists to develop new alternative methods to assess the sensitizing potential of substances [3], but the regulatory tests recognized by OECD have rarely, if ever, addressed the polyexposure issue [4]. In the laboratory, an original *in vitro* murine model of bone marrow-derived dendritic cells (BMDCs) was developed to assess the sensitizing potential of industrial substances [5,6].

Purpose : The objective of this study was to use this model to investigate the effects of a co-exposure to two sensitizing substances.

Methods : BMDCs were exposed to chemical (benzalkonium chloride found in many household products, BAC) or biological (beta-lactoglobulin from cow's milk, BLG) substances alone or in binary mixtures for 48 hours. The first mixture consist of mixing the two substances in a tube during 15 minutes before adding culture medium and exposing BMDCs. Regarding the second mixture, the substances were prepared separately and mixed in the culture well. Then the activation of DCs was evaluated by studying the expression of six phenotypic markers by flow cytometry. A significant increase in the expression of these markers reflected activation of DCs, i.e. a sensitizing potential of the substances or mixtures.

Results : When tested alone, both chemical and biological substances showed a concentration-dependent expression of the six membrane markers. The most overexpressed of them were costimulation markers (CD86 for the both substances, CD80 for BAC, and CD40 for BLG). No differences in the expression of membrane markers were observed with the different types of mixtures. Under co-exposure conditions, the expression of membrane markers seems to be additive compared to the substances alone.These results suggest that the polyexposure issue may be addressed by evaluating the sensitizing potential of mixtures using the BMDC model.

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LP-02
Case study: approaching non-animal preclinical safety testing for pharmaceuticals

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Historically, preclinical tests for new drugs have used animals. Only a few case studies show new therapies reaching human clinical trials without prior animal studies. In these cases, regulators and developers agreed on a non-animal preclinical strategy based on agreement that pharmacologically relevant animal models do not exist(i.e. immuno-oncology drugs). To expand this scope, we developed a non-animal preclinical weight-of-evidence (WoE) safety assessment strategy for a candidate therapeutic that prioritizes modern, human-relevant methods. Most regulatory guidance suggests that our candidate drug should be assessed using animal studies, and this expectation is echoed in discussions with pharmaceutical companies, consultants, and experts' preclinical testing advice. Our case study provides a real-world example of how drug sponsors can approach proposing non-animal testing strategies that diverge from this guidance. We have discussed and refined this WoE strategy in consultations with the US Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the Paul Ehrlich Institute (PEI). This groundwork is essential to support a

transition toward the use and regulatory acceptance of scientifically valid, human-relevant methods in place of defaulting to preclinical animal data. This poster summarizes published case studies of non-animal preclinical testing, and details the nonanimal WoE strategy for our candidate therapeutic. Feedback from FDA, EMA, and PEI is discussed, and next steps focused on extending our candidate-specific regulatory feedback to all drugs in our candidate's same class are explained.

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LP-03

From neurotoxicity DNT hit screening to hit characterization

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Neural crest cells (NCC) play a pivotal role in early embryogenesis and neurodevelopment and they express many targets and signaling pathways that may be affected by developmental toxicants. Here, we used the neural crest cell-based cMINC (UKN2) assay to screen a library (n= 115 compounds) of potential environmental toxicants. We simultaneously measured cytotoxicity and the migration capacity of the cells. This combination of endpoints allowed a classification of compounds as negatives (at ≤100 μM), cytotoxicants and as DNT-specific hits. Primary screen hits were confirmed in an orthogonal, second migration assay. Mitochondrial inhibitors were highly overrepresented among the confirmed DNT hits. The subgroup of strobilurins, suspected inhibitors of respiratory chain complex III, was further characterized. The likely toxicity mechanism of one of the compounds, picoxystrobin, was fully elucidated. Overall, this case study exemplifies how screening of a large library can generate alerts for DNT, and how mechanistic follow-up studies could be used for future DNT testing and risk assessment.

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LP-04

***In vivo* metabolic behavior of nanobiomaterials: bridging the gap between nanostructures and nanosafety**

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Many nanoscale biomaterials have not reached the clinical trial stage owing to the poor understanding of the fundamental principles of *in vivo* behavior. Complex and dynamic interactions of nanomaterials with biological milieu, barriers and molecules, which are denoted by nano-bio interactions, are decisive for their metabolic behavior and fate, such as the distribution, accumulation, transformation and elimination. The nanostructures of the active biomaterials determine the nano-bio interactions and biological behavior, which in turn guide the precise design of the nanostructures and affect their bioactivities and safety. Elucidation of these intricate relationships will contribute to the rational design and clinical translation of nanomedicines. In our research work, the bioavailability, a new metabolic behavior of nanomaterials has been demonstrated. We found that molybdenum elements derived from molybdenum disulfide (MoS_2) nanomaterials were utilized *in vivo* by incorporating into molybdenum enzymes and enhancing their activities. MoS_2 nanomaterials, promising nano-platforms for the drug delivery, cancer theranostics, bioimaging and biosensing, were firstly sequestrated in the liver and spleen mediated by the protein corona, then oxidized to molybdate by phase I enzymes and reactive oxygen species, finally participated into the biosynthesis of molybdenum cofactors to be bioavailable in aldehyde oxidase and xanthine oxidoreductase, two main molybdoflavoenzymes. By studying of interactions of nanoparticles with important biological systems, we systematically clarified the *in vivo* transport–transformation–bioavailability chain of nanomaterials bridged by protein corona. It is the first to prove the process and mechanism of the bioavailability of nanomaterials bearing essential trace elements.

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LP-05

***In vitro* study of the neurotoxicity of series new pyrrole-based azomethine derivatives on isolated rat brain synaptosomes and microsomes**

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The direct or indirect effect of chemicals, causing disruption of the nervous system of humans or animals is known as neurotoxicity. Neurotoxic diseases are often related to numerous of exogenous chemicals and xenobiotics, where a high variety of them is applied as experimental tools to disturb or damage the nervous system of animals. Some act directly on neural cells, others interfere with metabolic processes on which the nervous system is especially dependent. Due to their versatility, synaptosomes have been actively used to study neuroinflammatory and neurotoxicity processes. Because of the preservation of many of the physiological processes such as metabolic and enzymatic activities, synaptosomes have proved to be an indispensable *ex vivo* model system to study synapse physiology when isolated from animal tissues. Their application in this direction is based on the biochemically isolated preparations of detached and resealed synaptic terminals allowing fast and direct evaluation of the effects of the xenobiotic substances on the cellular vitality and certain sub-cellular parameters defining the metabolic status of the cell. This defined the purpose of our investigation aimed to assess the neurotoxicity and neuroprotective properties of a series of newly developed pyrrole-based compound on isolated rat brain synaptosomes and microsomes. The obtained results indicated that 7 of the evaluated substances, when applied alone (at a concentration of 100 micromoles) performed weak statistically significant neurotoxic and pro-oxidant effect. Additionally, in both models of neurotoxicity, all substances, when applied in the same concentration, exhibited good neuroprotective and antioxidant effects. In a model of 6-hydroxydopamine-induced neurotoxicity on synaptosomal fraction, substances **9** and **14** had the best statistically significant neuroprotective effect, relative to the toxic agent. On isolated brain microsomes – in a model of non-enzymatically induced lipid peroxidation, again substances **9** and **14** have the best antioxidant effect compared to the toxic agent. These results defined both molecules as most promising in further development of pyrrole-based azomethine derivatives as CNS active agents.

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LP-06

In Vitro assessment of hepatotoxicity of 15 pyrrole-based compounds on cellular and sub-cellular level in isolated rat hepatocytes and HPLC quantitative evaluation of the least toxic agent

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Hepatocytes, the major parenchymal cells in the liver, are responsible for a variety of cellular functions including carbohydrate, lipid and protein metabolism, detoxification and immune cell activation to maintain liver homeotasis. Drug hepatotoxicity assessment is a relevant issue both in the course of drug development as well as in the post marketing phase. The use of appropriate *in vitro* models in combination with powerful analytical methods is a promising approach to anticipate, as well as to understand and investigate the effects and mechanisms of drug hepatotoxicity. In addition, this can be used to assess the potential hepatotoxicity of new compounds.

The purpose of our investigation is pointed to evaluate the hepatotoxicity of a series of newly developed pyrrole-based compounds on isolated rat hepatocytes and to develop a validated HPLC method for quantitative analysis of the least toxic agents in biological media. The obtained results indicated that from all evaluated compounds substances **12** and **12b** showed the lowest toxicity on cellular and sub-cellular levels in isolated rat hepatocytes, with **12** being the least toxic derivative. The corresponding HPLC analytical procedure for quantitative evaluation of the molecules in isolated hepatocyte suspension was developed and validated according to the ICH guidelines. The methods specificity, linearity, accuracy, sensitivity, precision and repeatability were determined. The chromatographic method was carried out by UltiMateDionex 3000 SD equipped with UltiMateDionex DAD 3000 detector (ThermoFisher Scientific, Milan, Italy). The analysis identified formation of 3 new products during the incubation of compound **12** in isolated rat hepatocytes and 6 new products, when incubating compound **12b** under the same conditions. The procedure indicated one of the biotransformational products of **12b** as molecule **12**. The method defined a decrease in the concentration of the analyzed molecules, suggesting that both compounds undergo biotransformational degradation. The slightly elevated toxicity performed by compound **12b** we believe is due to the formation of compound **12** during the biotransformational changes related to incubation in the isolated hepatocyte culture.

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LP-07

3D lung spheroid model reveals silica-driven fibrosis via oxidative stress and apoptosis inhibition

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Background: Silicotic nodules are typical pathological markers of silicosis, and there is a notable absence of effective *in vitro* models to replicate these structures. This study aims to develop a 3D lung spheroid model that mimics the formation and progression of silicotic nodules. It investigates the temporal dynamics between oxidative stress and apoptosis in myofibroblasts upon silica exposure, elucidating their roles in the pathogenesis of pulmonary fibrosis.

Methods: Mouse lung decellularized matrix (LDM) was prepared via pulmonary artery perfusion. The decellularization efficiency and structural integrity of the LDM were validated using hematoxylin and eosin (HE) staining, Masson's trichrome staining, scanning electron microscopy, and assessments of protein and DNA content. The LDM was subsequently ground into particles by liquid nitrogen. A 3D spheroid model was constructed by combining LDM particles with NIH/3T3 cells in ultra-low attachment plates. The spheroids were subsequently exposed to macrophage supernatants stimulated with silica for 1, 7, and 14 days to simulate prolonged *in vivo* differentiation. Pathological changes within the spheroids were detected using HE and Masson's trichrome staining methods. Immunofluorescence was employed to detect Ki67 as a marker for cell proliferation. High-content imaging technology was used to measure mitoSox levels, indicating oxidative stress within the spheroids. Western blot analysis was conducted to evaluate the protein level changes of BAX, BCL-2, and Caspase-3 in response to apoptosis, as well as Collagen I and III to indicate fibroblast activation. These analyses provided insights into the behavioral alterations of myofibroblasts.

Results: The decellularization process yielded highly efficient removal of cellular content from mouse LDM. Following silica exposure, pathological results showed that the 3D lung spheroid model exhibited increased collagen deposition, reduced central cell density, and enhanced peripheral cell proliferation and polarization. Significant proliferation of peripheral cells and myofibroblast differentiation were observed, accompanied by upregulated expression of type I and III collagen. Bioinformatic analysis of RNA sequencing data revealed that silica exposure modulated oxidative stress, apoptosis, and extracellular matrix remodeling. MitoSox results further confirmed that silica exacerbated oxidative stress levels within the spheroids. Additionally, Western blot results showed that silica exposure led to downregulation of BAX and Caspase-3, and upregulation of BCL-2 in the spheroids, suggesting a potential regulatory relationship between oxidative stress and apoptosis inhibition.

Conclusion: Our 3D lung spheroid model fits the pathologic structure of silicosis nodules well. Silica exposure leads to enhanced collagen deposition in the 3D lung spheroid model, while simultaneously promoting oxidative stress and inhibiting apoptosis in myofibroblasts.

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LP-08
In vitro Phototoxicity validation study using Chlorpromazine Technical

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The 3T3 Neutral red uptake Phototoxicity Assay was designed to detect the Phototoxic potential of test items activated by exposure to the light. Neutral Red is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. NR is not charged at close-to-neutral pH of the cytoplasm but becomes positively charged and trapped in low pH of lysosomal lumen. The low pH of lysosomal lumen is actively maintained, requires ATP, and is dependent on integrity of the lysosomal membrane. Prototoxins can induce cell damage through formation of Reactive Oxygen Species (ROS) and other mechanisms that lead to increased permeability of the lysosomal membrane, reduction in the pH gradient, and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable and damaged or dead cells.

In current study, cytotoxicity and Phototoxicity of the Chlorpromazine Technical to 3T3 cells (in presence and absence of UVA light) is assessed by Neutral Red Uptake method. Two plates containing monolayers of BALB/c, clone 31 cells were incubated at 37°C for 60 minutes in presence of different formulations of test item and vehicle control (DMSO).

Following incubation, the cells were irradiated at room temperature for 50 minutes at 1.7 mW/cm^2 (5 J/cm^2) (SOL 500 Solar simulator, Honle). Another 96 well plate was kept at room temperature in the dark for approximately 50 minutes (without SSL). After exposure, +SSL and –SSL, cells were washed and incubated in culture medium for 24 hours. Following incubation, each plate was examined under a microscope for growth, morphology and integrity of the monolayer. Changes in the morphology of the cells due to cytotoxic effects were observed and recorded. After incubation the uptake of the Neutral Red into the lysosomes of living cells were used as a quantitative indication of cell number and viability. The absorption of the resulting-colored solution was measured at 540nm in microtiter plate reader, using the mean of the outer wells (blanks) as a reference.

Three experiments were conducted. IC50 values, PIF (Photo irradiation factor) and MPE (Mean photo irradiation factor) with and without SSL were determined using Phototox software 2.0 (Recommended by OECD 432).

1. average IC50 value, with SSL and without SLL were 0.516 µg/mL and 33.703 µg/mL respectively for the Chlorpromazine Technical. Thus, the mean value of PIF and MPE factor of Chlorpromazine Technical was 66.994 and 0.432. Based upon the observed results and under the test-conditions chosen, Chlorpromazine Hydrochloride was phototoxic as the PIF was >5 and MPE was >0.15.
2. Based upon the observed results and under the test-conditions chosen, Chlorpromazine Hydrochloride was phototoxic as the PIF was >5 and MPE was >0.15.

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LP-10
Comparative analysis of occupational exposure limits (OELs) for inhibiting RNA therapeutics

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Occupational exposure to emerging therapeutics, particularly RNA-based drugs, introduces novel challenges in toxicological risk assessment. This study conducts a comparative analysis of Occupational Exposure Limits (OELs) for RNA therapeutics from three pharmaceutical companies (Novartis, GSK, and Takeda), focusing on Drugs A, B, C, D, and E, currently on the market or in late-stage development. We scrutinize the Point of Departure (POD) and Adjustment Factors (AF) utilized in OEL calculations for each substance.

Results reveal independently set OELs by the three pharmaceutical companies, clustering within a similar range (110, 70, 23, 200, and 90 µg/m3), derived from human data PODs following subcutaneous administration. Despite the limited availability of substance-specific OELs, this analysis provides insight into the positioning of OELs during late development phases. Moreover, our findings build upon previous research by Bertow *et al.* (2019), supporting the applicability of a default OEL of 10 µg/m3 in early developmental stages of RNA therapeutics.

Adjustment Factors applied uniformly across substances include Intraspecies Variability, with additional factors for Database Completeness (in cases A and C) and Extrapolation from LOAEL to NOAEL (in cases D and E). Variations in POD and AFs among substances underscore differences in toxicity profiles and data availability. Substance D exhibits the highest POD, while substances A and E manifest relatively lower OELs despite lower PODs, suggesting enhanced precautionary measures due to higher AFs or limited data extrapolation.

Overall, our findings support the adequacy of a default OEL of 10 µg/m3, providing a sufficient margin of safety in occupational exposure to RNA therapeutics. These conclusions emphasize the importance of tailored risk assessment methodologies in navigating the unique challenges posed by emerging therapeutic modalities.

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LP-11
In silico metabolomic, cytotoxicity, mutagenicity, and carcinogenicity prediction of givinostat, a histone deacetylase inhibitor

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Histone deacetylases (HDACs) are a class of enzymes that catalyze the removal of acetyl groups from histone proteins, causing them to bind more firmly around the DNA. HDAC inhibitors are therefore considered

a novel treatment strategy for a wide range of diseases, from neurodegenerative and psychiatric diseases to cancer. Givinostat, a potent HDAC inhibitor, targets pathogenic processes to decrease inflammation and alleviate muscle functions. Givinostat was approved by the US Food and Drug Administration on March 21, 2024, for the nonsteroidal treatment of Duchenne Muscular Dystrophy. Herein, our objective is to use various in silico methods, including MetaTox (v.2.0), SwissADME, PASS online (v.2.0), VEGA (v.1.2.3), US EPA TEST (v.4.1.2), and ProTox (v.3.0), to illuminate further the metabolomics profile as well as the cytotoxic and mutagenic potential of givinostat. The compound satisfied Lipinski's rule of five. The highest cytotoxic effect was found in human colon carcinoma cells (HCT-116) with a probability of activation (Pa) and inactivation (Pi) of 0.636 and 0.016, respectively. That was followed by human acute myeloid leukemia cells (OCI-AML2) (Pa=0.529, Pi=0037), human acute monocytic leukemia cells (MONO-MAC-6) (Pa=0.513, Pi=0005), and breast adenocarcinoma cells (MDA-MB-231) (Pa=0.486, Pi=0041). The mechanism of action of givinostat is mainly related to HDAC inhibition, as supported by our in silico PASS analysis. The compound has shown the highest inhibitory activity against HDAC-1, -6, -2, -8, -5, and -9, with Pa values of 0.625, 0.426, 0.373, 0.295, 0.231, and 0.221, respectively. Our in silico analysis indicates that CYP2J2 and HDAC-1 enzymes are direct protein targets with 0.4905 and 0.4144 confidence values, respectively. The ProTox has predicted givinostat to be respiratory toxic, mutagenic, and nephrotoxic with a probability of 0.82, 0.67, and 0.65, respectively. Moreover, the compound was predicted to be mutagenic, with a consensus score of 0.25 regarding the VEGA mutagenicity (Ames test) consensus model. While the CAESAR, ISS, and SARpy predicted the compound as mutagenic (moderate/low/low reliability), the KNN-read-across model estimated it as non-mutagenic (moderate reliability). The EPA TEST consensus method predicted the compound as mutagenicity-positive with a predicted value of 0.65. Givinostat has been reported as Ames-positive, and our in silico mutagenicity results support this finding. Currently, there are no studies that have assessed the carcinogenic potential of the compound. According to the VEGA CAESAR, IRFMN-ISSCAN-CGX, and IRFMN-Antares carcinogenicity models, givinostat was predicted to be carcinogenic, but ISS did not. All carcinogenicity assessments have low reliability. Our findings suggest that while givinostat holds promise for treating many diseases by inhibiting histone deacetylation, further studies are needed to assess its mutagenic and carcinogenic potential.

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LP-12

Behavioral alterations by micro(nano) plastics: insights from Zebrafish and *Caenorhabditis elegans*

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As plastic debris breaks down in environments, it forms micro(nano) plastics that threaten organisms across ecosystems. These tiny particles are potentially harmful due to industrial additives ingested by organisms. Given the growing concern related to micro(nano) plastics, this study compared the toxicity of leachate from tire wear particles (TWPs) and a bioplastic alternative (Mater-Bi®) using two model organisms: zebrafish (*Danio rerio*) and nematodes (*Caenorhabditis elegans*). We investigated embryotoxicity and behavioral changes (photomotror response and tapping test) in zebrafish, alongside survival rates and swimming activity in nematodes. Interestingly, in zebrafish embryos, TWPs caused significant depigmentation (0.6 and 1.2 µg peq/mL, $p<0.05$ reducing visibility and decreasing activity under dark conditions in the larval photomotor response. Notably, activity in the tapping test increased across all tested concentrations of TWPs in zebrafish ($p<0.05$). In nematodes, the leachate was inducing hyperactivity (1.5 to 5 µg/mL), approximately a 1.5-fold increase in activity at 1.5 µg/mL ($p<0.0001$) without significantly impacting their survival. Although seemingly harmless to zebrafish at all tested concentrations, bioplastics significantly reduced nematode activity (approximately 1.2 fold, $p<0.008$) at higher concentrations (>5 µg/mL). These findings emphasize the need for multi-species studies to understand the complex and variable impacts of micro(nano) plastics on various ecosystems.

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LP-13

Neuronal-derived iPSCs cell to evaluate neurotoxicity after 48 or 72 hours in high-through put screening format

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Neurotoxicity is one of the major concerns in central nervous system (CNS) drug discovery and is a frequent cause of attrition. Typically pipelines involve compound testing in immortalized cell lines followed by *in vivo* investigations and extrapolation. Induced pluripotent stem cells (iPSCs) have widened the possibility for new model systems to study adverse toxicities. Moreover, changes in regulatory guidance and the rise of new *in vitro* models provides high-throughput screening (HTS) access and a more relevant environment for compound testing. In this study we have performed screening of 8 compounds. Bortezomib (proteasome inhibitor), gemcitabine (treatment of ovarian cancer), ivermectin (treatment of some parasitic diseases), flavopiridol (first generation of CDKs inhibitors), SNS-032 (CDKs inhibitor), mefloquine (anti-malaria drug), digitonin (nonionic detergent) and tamoxifen (a hormone therapy used to treat hormone receptor-positive breast cancer). These molecules were tested on neuronal-derived iPSCs Dopaneurone, GABAneurone, Glutaneurone, Motorneurone, Microglya and Astrocytes to test for neurotoxicity in HTS.

The cells were thawed and plated in different coating plates, using different seeding conditions and 384 well/plates. DMSO tolerance was performed on the 6 cell models by the addition of different concentrations of DMSO (0, 0.1, 0.3, 1 or 3%) to the cell culture medium. Three hours after plating, the drug was added. Then, after 48 hours or 72 hours, total protease and protease activities was measured using Cytotox Glo. Bortezomib, gemcitabine, ivermectin, flavopiridol, SNS-032, mefloquine, digitonin and tamoxifen were tested at 7 concentrations. After the treatment, 15µl of cytotox Glo was added to each well and incubate. Luminescence was read 15 min later. Then 15µl of lysis reagent was added to each well and was incubated for 15 min, followed by a luminescence read.

Culture conditions were validated. The signal window range allows us to perform neurotoxicity assays after 48 and 72hours of treatment. A DMSO tolerance was measured, and no toxicity was found until 1% DMSO. Neurotoxicity was cell-type dependent. A specific neurotoxicity signature was measurable for each drug tested. Digitonin, our positive control, induced toxicity in all cell models after 48h and 72h of incubation. On the contrary, Tamoxifen, our negative control, did not induce any neurotoxicity. The other approved drugs generated a signature of severe to mild neurotoxicity. These results are in agreement with the known side adverse effects of these drugs.

Conclusions: This neurotoxicity panel, which uses different mature IPCSs, has demonstrated that it is a good tool to anticipate possible neurotoxicity – at the same time respecting the 3Rs. It can be used in early drug de-risking as well as for screening for neuroprotection, with the aim of preventing/reducing/curing neuropathy in at-risk populations or for specific therapeutic areas.

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LP-14
The neurotoxic effects of polystyrene microplastics and nanoplastics on *Drosophila melanogaster*

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Plastics play an important role in maintaining the quality and comfort of everyday life due to their ease of use and attractive price/performance ratio. However, after the use of single-use plastic products, plastic pollution occurs, which the world is struggling to deal with due to rapid consumption habits and poor waste management processes. The increasing presence of these plastic wastes in the environment and their long-term presence pollute most terrestrial and aquatic ecosystems. In addition to the visible part of this pollution, large pieces of plastic are broken down into smaller sizes of nanoplastic (NP) and microplastic (MP) particles by various mechanisms such as abrasion, wave action, photo-oxidation, biological, mechanical wear and degradation, thus creating an invisible environmental pollutant. MPs and NPs have been found in tap water, beverages, salt and samples collected from the oceans. Plastic pollution, especially of aquatic organisms, has reached levels that threaten the environment and human health. The neurotoxicity of plastics has mainly been studied in aquatic organisms, and studies at the molecular level in neurons in terrestrial organisms and comprehensive studies of the nervous system are limited. In this study, the toxicity of round-shaped micro (200 nm, PSMP) and nano (100 nm, PSNP) sized polystyrene particles were investigated in *Drosophila melanogaster* brain cells and eye development. Concentrations were determined from the data obtained according to the pupal emergence success of *Drosophila* exposed to plastic at concentrations of 10–1000 µg/ml in line with the literature information. Pupal emergence success was observed in *Drosophila* exposed to 10–250 µg/ml PSMP and PSNP. DNA damage was assessed by single cell gel electrophoresis in *Drosophila* brain cells at concentrations of 10–250 µg/ml, at which 70% viability was observed by the acridine orange ethidium bromide (AO-EtBr) test. The results indicated that PSMP (100 and 250 µg/ml) and PSNP (10, 50, 100 and 250 µg/ml) were associated with statistically significant DNA damage. In addition, a dose-dependent morphological damage was observed in the evaluation of eye facets in PSMP (10, 50, 100 and 250 µg/ml). However, this damage was not observed in PSNP. A human being is exposed to approximately 1 g of plastic every day, depending on living conditions and habits. The daily intake of micro- and nano-plastics by humans and animals and the associated toxicological effects are not yet fully understood, and studies in this area have become one of the most important research topics in recent years. The findings presented herein contribute to the body of knowledge regarding the neurotoxic effects of MPs and NPs on *Drosophila melanogaster*.

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LP-15
Low-dose hexavalent chromium induces mitophagy in rats liver via AMPK related PINK1/Parkin signaling pathway

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Background: Hexavalent chromium (Cr(VI)) is a hazardous metallic compound commonly found in industrial processes. The liver is a vital organ responsible for metabolism and detoxification, and is the main target organ of Cr(VI).

Objective: To investigate the impacts of low-dose exposure to Cr(VI) on rat livers and the mechanism of liver injury induced by Cr(VI).

Methods: Rats received weekly inhalable intratracheal instillation of potassium dichromate ($K_2Cr_2O_7$) (dissolved in sterile 0.9% sodium chloride solution) at 0, 0.05, or 0.25 mg Cr/kg body weight for 28 days (in total 5 times). The rats were sacrificed and samples were taken 24 h after the final instillation. The serum was used to determine the level of oxidative stress. The entire blood specimen was applied to detect liver function of the rats. The damage of hepatocyte mitochondria was observed by electron microscope. The protein expression of P-AMPK, P-ULK, P-mTOR, PINK1, P-Parkin, P62, LC3II, LC3I were detected by western blotting.

Results: Liver tissue oxidative stress assay results showed that SOD activity was decreased in the 0.05mg/kg Cr(VI) group and 0.25mg/kg Cr(VI) group, MDA was increased in 0.25 mg/kg Cr(VI) group. These suggested that the continuous accumulation of Cr(VI) in the livers induced liver cell oxidative stress. There is no significant difference in biochemical indices between saline group and exposed groups. Electron microscopy showed that chromium exposure caused a significant increase of mitophagy and the destruction of mitochondrial structure. Western blotting showed that exposure of 0.05 mg/kg Cr(VI) and 0.25 mg/kg Cr(VI) obviously increased the expressions of P-AMPK, P-ULK, PINK1, P-Parkin, and LC3II/LC3I while significantly reduced the P-mTOR and P62 expression levels in liver. These revealed the increase in mitochondrial autophagy in liver tissue and the activation of AMPK related PINK/Parkin signaling pathway.

Conclusion: This study simulates the poisoning mode of Cr(VI) workers in the workplace by means of intratracheal instillation of Cr(VI), and confirms that the autophagy of hepatocytes is caused by the poisoning of low concentration Cr (VI), and the liver damage caused by Cr(VI) may be related to the AMPK related PINK/Parkin signaling pathway.

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LP-17
Neuroprotective efficacy of polyphenols against Acrylamide induced toxicity

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Acrylamide (AA), is commonly found in popularly consumed food like potato fries and crisps. Due to its ability to form polymer, it is frequently used in laboratories and industries. AA can potentially affect both central as well as peripheral nervous systems as it is a potent neurotoxin. AA toxicity results in tremors, ataxic gait, illusion, or cognitive dysfunction in humans. These discoveries emphasize the need for effective

measures to reduce neurotoxicity caused by AA exposure. Plant-based polyphenols have antioxidant properties that can help in alleviating oxidative stress. Therefore, this study was designed to evaluate the potential protective effect of certain polyphenols (linoleic acid, kaempferol, hesperidin, and caffeic acid) against AA-induced neuronal damage in rats.

Two different sets of experiments were conducted in this study. The initial experiment involved comparing the efficacy of different test drugs (20mg/kg for 7 days for each polyphenol tested after AA administration) against sub-chronic exposure to AA (20mg/kg for 28 days). The second experiment confirmed the molecular mechanism of recovery by using specific polyphenols selected (20mg/kg for 2 days a week) from the first experiment against chronic AA exposure (11.8mg/kg for 5 days a week). The second experiment last for 90 days.

Our study found that the group exposed to AA displayed reduced motor coordination and learning function as evidenced by reduced latency for falling off the rod. Additionally, increased lipid peroxidation and alteration in brain tissue's antioxidant defense mechanism were observed after Subchronic AA exposure. During histological observation, AA-treated rats showed degenerated pyramidal cells and diminished nerve fibers in the cerebral region. Treatment with four polyphenols (linoleic acid, kaempferol, hesperidin, and caffeic acid) significantly restored the altered parameters compared to the AA-treated group alone, with caffeic acid showing superior protective efficacy followed by kaempferol, linolic acid and hesperidin. Chronic exposure to AA resulted in a significant alteration in neurotransmitter levels (ALAD, ALAS, BuChe, Ache, monoamine oxidase, GABA and serotonin) and myelin sheath damage. Caffeic acid demonstrated the ability to improve the level of neurotransmitters, reduce DNA damage, and restored brain biochemical and electron microscopic cellular organization, indicating its potency to protect brain cells against AA-induced alteration.

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LP-18
Simultaneous determination of 5 kinds of virulent mushroom toxinsin human whole blood by ultra performance liquid chromatography- tandem mass spectrometry

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In this study, a comprehensive analytical method based on ultra performance liquid chromatography-tandem mass spectrometry(UPLC-MS/MS) was developed for the rapid screening of 5 highly toxic mushroom toxins including α-amanitin, β-amanitin, γ-amanitin, phallacidin, phalloidin in human blood. 3 mL of formic acid-acetonitrile (1:99, v:v) solution was added in 1 mL of blood to precipitate proteins. After centrifugation, the supernatant was concentrated at 60°C. The residue was dissolved in 200μL methanol-water (1:9, v:v) solution and separated by 0.22 μm microporous filtered membrane. The components in the purified extract were separated by ACQUITY UPLC® BEH C18 chromatographic column (2.1 mm×100 mm, 1.7μm) with a gradient elution programfor separation. The mobile phase A was consisted of 0.1%(v/v) ammonia in water, and phase B was methanol. ESI ion sourcein positive modes was used for the multiple reaction monitoring (MRM) scanning. The detection limit of 5 toxic mushroom toxins in blood can be less than 10 ng/mL. The calibration curves of 5 toxic mushroom toxins showed good linearity over the range from 20 ng/mL to 300 ng/mL (R^2>0.995) in spiked blood matrix. The extraction recoveries of the 5 toxic mushroom toxins was 78.3% to 88.3%. The established method is operationally simple, with high sensitivity and broad applicability, and it is suitable for screening and testing mushroom toxins in whole blood.

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LP-19
Effects of synthetic insecticides on the toxicity of natural organophosphate guanitoxin in fish

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Guanitoxin ((5S)-5-[(Dimethylamino)methyl]-1-{[hydroxy(methoxy) phosphoryl]oxy}-4,5-dihydro-1H-imidazol-2-amine; $C_7H_{17}N_4O_4P$), formely described as anatoxin-a(s), is a neurotoxin produced by cyanobacteria, considered the only naturally occurring organophosphate reported in the literature, with potential action similar to synthetic insecticides. Despite significant concerns about its presence in aquatic ecosystems, there are few studies on its toxicity in aquatic animals. Therefore, this study aimed to assess the toxicity of guanitoxin extracts derived from the cyanobacterium *Sphaerospermopsis torques-reginae* (ITEP-024 strain), as well as its association with malathion and trichlorfon, two organophosphate insecticides, in specimens of *Oreochromis niloticus* (Nile tilapia). Extracts containing guanitoxin from cyanobacteria were used, with its presence first confirmed by LC-MS/MS, since there are still no commercial standards for this cyanotoxin. For the toxicity assay to be conducted, *O. niloticus* specimens were exposed to different treatments for 96 hours: control group (C), guanitoxin aqueous extract 250 mg/L (T1), malathion 1 mg/L (T2), trichlorfon 0.5 mg/L (T3), guanitoxin + malathion (T4), guanitoxin + trichlorfon (T5), and guanitoxin + malathion + trichlorfon (T6). Genotoxic and histopathological biomarkers were analyzed in the fish to verify the toxic potential of guanitoxin and its interaction with insecticides. Our study demonstrated that the most genotoxic treatment was for fish exposed to T4, exhibiting higher frequency micronuclei, segmented and lobulated nuclei in the erythrocytes. These genotoxic findings are concerning, as this damage can directly impact chromosomal integrity, potentially resulting in deleterious effects on fish. Regarding histopathology, a total of 13 different damages were found in the gill tissues, including hypertrophy, capillary congestion, hyperplasia, fusion of the lamellae, displacement, loss of structure of the lamellae, edema, vasodilation, dilation of the proximal canal, proliferation of mucous cells, rupture of the lamellar epithelium, total lamellar fusion, and lamellar aneurysm. Changes in gill structures may arise as a defense mechanism or as a direct result of the presence of contaminants. The damage found is concerning since alterations in this essential organ can result in the inability of the gills to actively carry out gas exchange, a vital function for fish. In conclusion, our findings highlight the hazardous interaction between natural and synthetic organophosphates in fish, with treatments T1, T4, and T6 standing out as inducing the most significant damage for gills and T4 the most genotoxic treatment. Additionally, our study is the first to investigate interactions between guanitoxin-producing cyanobacteria and insecticides, highlighting environmental risks and urging further research.

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LP-20

Evaluation of the effect of perfluorohexanoic acid on selected cytotoxic and oxidative parameters in human peripheral blood mononuclear cells

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Per- and polyfluoroalkyl substances (PFASs) are synthetic compounds widely used in industry (manufacture of food packaging, waterproof textiles, furniture, cosmetics and other everyday products) [1]. A wide range of applications is associated with physical chemical properties of these substances such as amphiphilicity and strong carbon-fluorine bonding. Simultaneously, PFASs reveal high mobility in soil and water, high and extreme persistence [2]. The long-chain PFASs (carbon chain with C≥7) commonly used for many years (such as perfluorooctanoic acid PFOA), due to reports related to their toxicity, are currently use. These actions have led to the of substitute with shorter carbon chains, such as perfluorohexanoic acid (PFHxA), potentially exhibit . However, reports on their safety are also of concern [3].

The aim of this study was to assess the effect of PFHxA on viability, size (forward side scatter, FSC) and granularity (side scatter, SSC), ATP level and reactive oxygen species (ROS) formation in human PBMCs. The cells were incubated with the compounds in concentrations ranging from 0.0001 to 200 μg/mL for 1 h. A viability was performed using calcein-AM and propidium iodide staining, changes in ATP level were using luciferin-luciferase assay and ROS level using 2',7'- dichlorodihydrofluorescein acetate. ATP level was analyzed by luminometer, other tested parameters were by flow cytometry.

The results of the study have revealed that PFHxA at the tested concentrations did not statistically significant changes in cell viability, granularity and ATP level. was shown that PFHxA only in highest concentration of 200 μg/mL induced significant changes in PBMCs size. increased ROS levels in

In conclusion, in our research model, PFHxA did not change examined parameters at concentrations related to environmental occupational exposure (0.001 and 0.01 μg/mL, respectively). However, it is important to note strong potential of PFHxA to generate ROS in PBMCs after a short incubation time, which may indicate the ability of oxidative damage to cell molecules after prolonged exposure. Further research is needed to clarify the mechanism of action in PBMCs.

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LP-21

Chemical-induced disruptions to behavioral stress responses investigated using the zebrafish model: Deciphering risk for stress-related disorders

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Zebrafish behavioral assays have emerged as effective tools for identifying chemical-induced developmental toxicities due to their ease, sensitivity, and high throughput capacity. These assays quantify zebrafish behavioral responses to acute stimuli and observe how these responses are altered following developmental chemical exposures. However, the recorded behavioral motor responses rely on multiple systems, such as the nervous system, sensory organs, and musculoskeletal system, making it challenging to pinpoint contaminant-induced deficits in a particular organ or system. To address these challenges, we optimized a high-throughput zebrafish-based platform that incorporates three behavioral motor response assays: a visual motor response (VMR) assay, an acoustic motor response (AMR) assay, and a peripheral motor response (PMR) assay. The VMR assay relies on visual input, neuronal processing, and a locomotor response, with chemical-induced alterations in any of these systems potentially affecting the associated behavioral stress response. To pinpoint changes related to visual deficits, we developed the AMR assay, which uses acute acoustic stimuli to elicit a behavioral motor response. Additionally, we developed the PMR assay to assess the locomotor capacity of larval zebrafish through chemical irritants that elicit a peripherally driven motor response. Using this platform, we are able to pinpoint chemical-induced adverse effects in the stress circuitry, detect chemical-induced stress behavioral phenotypes, and identify chemical-induced risks for stress-related disorders.

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LP-22

Regulatory and industrial acceptance of non-animal pyrogen test methods in India: challenges and opportunities

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Purpose: For decades, the rabbit pyrogen test (RPT) was considered as a gold standard for pyrogenicity testing. However, the RPT has been critically scrutinized for its scientific and ethical shortcomings, and in majority of cases, it has been replaced with the bacterial endotoxins test (BET) also known as Limulus Amoebocyte Lysate test. However, BET assay is also associated with scientific and etheical issues, such as variations in sensitivity and specificity to endotoxin. Moreover, BET assay is based on the blood collected from the horseshoe crabs which puts this species under danger.These methods are not capable for detecting alll types of pyrogens, endotoxin, non-endotoxin and material mediated pyrogens. To overcome these scientific and ethical concerns, the interest of global regulatory bodies is shifting towards alternative non-animal pyrogen test methods, such as Monocyte Activation Test (MAT) and Recombinant Factor C (rFC). However, the global adoption of these non-animal pyrogen testing methods within regulatory frameworks and industrial practices is still a subject of debate and scrutiny.

Methods: Scientists at the people for ethical treatment of animals (PETA) India is working with regulators, policy makers, government testing laboratories, scientists and stakeholder companies for the ad-

vancement of alternative non-animal methods and to maintain regulatory harmonization. PETA India participate in regulatory expert working group meetings, submit technical recommendations , build collaboration with scientist and stakeholder companies to understand the challenges and build the roadmap.

Results: Indian regulatory bodies have taken significant steps to build roadmap for the application of new safety testing methods and to maintain global regulatory harmonization. Indian Pharmacopoeia Commission (IPC) accepted PETA India proposal and established an expert working group for alternative to animal testing. In 2018, IPC included MAT as a general chapter (2.2.25) in IP. In 2022, IPC has replaced RPT with BET in most IP monographs. PETA India submitted recommendation to regulators for replacement of BET with alternative non-animal pyrogen test methods, MAT and rFC. The IPC has agreed to include non-animal test methods in IP on submission and approval of their validation data. PETA India is working with non-animal pyrogen testing technology providers and pharma companies to perform validation study of MAT and rFC. PETA India organizing expert session on non-animal pyrogen testing at upcoming conference organized by Society of Alternative to Animal Experiments-India and a hands-on workshop on pyrogen testing in collaboration with IPC to adress issues faced by regulators and industry end user. In conclusion, this poster provides insights into the current challanges faced by regulators and industry, and identifies opportunities to promote the adoption of these alternative methods.

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LP-24

Towards safer insect-based feed and food: toxicokinetics of cadmium, arsenic and lead in the black soldier fly larvae

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Insects are rich in protein, lipids, and other nutrients and possess an exceptionally efficient bioconversion capacity [1,2]. Besides these benefits, a significant advantage, particularly within a circular bioeconomy, is their ability to utilize a wide range of food sources in rearing facilities, including residual biomass, thereby avoiding competition with key food commodities (e.g., wheat and soybean) [3–5]. However, before utilizing alternative feedstocks such as bio-waste, it is crucial to ensure the safety of these insects as food and feed [6–8]. This study aimed to assess the uptake and elimination of common contaminants – cadmium (Cd), arsenic (As), and lead (Pb) – by the larvae of black soldier fly (*Hermetia illucens*) from feed substrates. A two-phase bioaccumulation study was conducted: a 5-day uptake phase where larvae were fed a contaminated substrate and a 5-day elimination phase with a clean substrate. The substrate used was the Gainesville diet, spiked with cadmium chloride ($CdCl_2$), sodium arsenite ($NaAsO_2$), and lead nitrate ($Pb(NO_3)_2$) at concentrations of 2 mg/kg for Cd and As, and 10 mg/kg for Pb, based on EU regulatory limits for feed [9]. The control group consisted of organisms exposed to clean substrate during the 10-day experiment. During the exposure, a 16-hour light/8-hour dark photoperiod and a temperature of 25±2˚C were maintained. Daily sampling was carried out, followed by a 12-hour depuration time, and lyophilization. Inductively coupled plasma mass spectrometry was used for metal quantification. Data from both the uptake and elimination phases were used for toxicokinetic modelling. The kinetic bioaccumulation factors were 1.21 for As, 1.45 for Pb, and 6.13 for Cd. Results indicated that the larvae accumulated these metals at concentrations exceeding the permissible levels (2 and/or 10 mg/kg dry substrate). The highest uptake rate was for Cd, followed by As and Pb. However, larvae reached safe internal concentrations for food for other animals after being moved to a clean substrate during: 2 days for As, 4 days for Pb, and 5 days for Cd. From the obtained results, we recommend implementing this depuration period after exposure to bio-waste, although the duration should be adapted depending on the particular contaminants involved. Toxicokinetic studies are valuable for assessing the safety of insects for feed and food, and they can be useful tools to define the conditions that will assure sustainable use of insects in bio-waste management [6].

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LP-25
Evaluation of the Caco-2 permeability assay as NAM, New Approach Methodologies, for *in vivo* repeated dose toxicity NO(A)EL assessment

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Chemical safety assessment has traditionally been based on animal testing. From an ethical perspective and to improve animal welfare, there is a demand for the development of animal free safety assessment methods. Despite many efforts, non-animal method for predicting *in vivo* repeated dose NO(A)EL has not been established. Read-across is expected to be a useful approach, and various examples cases have been reported, on the other hand, case-by-case expert judgment is required, making evaluation challenging. The ADME is important for *in vivo* toxicity, intestinal absorption has a largely contributes on toxicity, especially when administered orally.

In this study, we examined the usefulness of the Caco-2 permeability assay, which is used for the prediction of intestinal absorption, as NAM for *in vivo* oral repeated dose toxicity.

We developed the regression model using the H2O machine learning library on KNIME, based on the experimental data of Caco-2 permeability assay collected from literature. The coefficient of determination achieved R^2>0.7 for test set. Next, using the developed model, we calculated the Caco-2 permeability prediction value, i.e. logPapp, for the compounds in the Munro dataset, which is the basis for TTC, Threshold of Toxicological Concern. Analysis of the relationship between the predicted logPapp values and the NOEL revealed that the threshold of the NOEL of each Cramer class is dependent on the logPapp predicted value. Our results support the usefulness of in silico Caco-2 permeability model as well as the Caco-2 permeability assay itself as NAM for *in vivo* repeated dose toxicity.

In recently, TTC derived from NOEL distribution of Cramer classification are applied to various regulatory fields, but the TTC values are highly conservative for some chemicals. From the results of this study, we propose the setting of sequential TTC by combining the conventional Cramer class classification and the Caco-2 permeability prediction value.

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LP-26
Revealing hidden challenges: an in-depth evaluation of Anogenital Distance (AGD) and Areola/Nipple Retention (NR) assessments in EOGRT Studies, with implications for regulatory toxicology

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The Extended One-Generation Reproductive Toxicity Study (EOGRTS) (OECD 2018, TG 443) represents the current state-of-the-art in reproductive toxicity testing and is also a vital tool in assessing endocrine-disrupting effects of chemical substances. In a collaborative effort between the European Chemicals Agency (ECHA) and nine EU Member States, a comprehensive review of the EOGRTS was conducted to evaluate its performance in supporting EU hazard assessment. A satellite project dealt with analyses of anogenital distance (AGD) and areola/nipple retention (NR), two critical endpoints in assessing endocrine disruption. In 72 full study reports (FSR) all F1 generation offspring were investigated for AGD and NR. An additional 25 datasets for AGD and 21 datasets for NR were available from the F2 generation, resulting in a total of 97 datasets analysed for AGD and 93 datasets for NR.

In the AGD assessments, good or acceptable performance was seen in 63% of the datasets, while 25% demonstrated insufficient quality, due to either low measuring precision, high coefficients of variation, or a clear overlap between male and female AGD values in the control group. The 20 different test facilities demonstrated variable proficiency, with approximately one-third of facilities excelling, one-third showing mixed results, and one-third consistently underperforming. The analysis of the NR datasets showed that only 17% showed good or acceptable results, while 83% had limited sensitivity, either because no areolae/nipples were reported in any of the male offspring, or because an unexpectedly high number of areolae were seen in the control males. This high percentage of studies with limited sensitivity raises concerns about the general reliability of NR assessments in the EOGRTS and emphasises the importance of improving these assessments in future studies. This could be achieved by providing updated recommendations for the assessment of both endpoints from the regulatory authorities, and by prioritising standardised training, protocol improvements, and increased time allocation for more accurate evaluations at the test facility level. Addressing these challenges is crucial for improving the usefulness of the EOGRTS and consequently contributing to more informed decision-making on the potential hazards and risks associated with exposure to endocrine-disrupting chemicals.

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LP-27
Use of histopathological and biochemical data of Anthracyclines cardiotoxicity in animal studies – an in-depth review analysis

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Background: Human health risks and hazards from chemical substances are well regulated internationally. However, cardiotoxicity, is not defined as a stand-alone hazard and therefore there are no defined criteria (histopathological, functional, biochemical) for classification of substances as cardiotoxic. In fact, in the last 10 years cardiotoxicity is very rarely recognised on a regulatory level at least in Europe. Identifying and regulating substances, which cause cardiovascular adverse effects would undoubtedly strengthen the national health systems.

Methods: To overcome the aforementioned gap, a weight of evidence approach is proposed for identifying regulatory criteria using New Approach Methodologies (NAMs) and data from existing animal studies and endorse them into the legislation in order to classify substances as cardiotoxic. The approach consists of (i) assembling the evidence into lines of evidence of similar type (i.e. cardiac animal models from known cardiotoxicants, Adverse Outcome Pathways (AOPs), In silico data, Omics, Read-Across, QSARs, biochemical and echocardiographic indices and histopathological data) (ii) weighing the evidence using well established scientific methods and (iii) integrating the evidence using statistical analysis and meta-analysis for each line of scientific evidence.

Results: Preliminary in depth review analysis indicate a clear distinction from normal values to biochemical indices (biomarkers of oxidative stress and inflammation)) and histopathological findings.

Conclusions: Further research should be conducted both from the scientific and regulatory community aiming to clearly define the cardiotoxicity hazard caused by chemicals and develop a full set of scientific criteria based on echocardiography data, biochemical indices, histopathological data and NAMs.

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LP-28
Risk assessment approaches for nanomaterials in medical devices

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Nanomaterials (NMs) are defined as materials with solid particles, either standalone or as part of aggregates or agglomerates, where 50% or more of the particles in the size distribution meet certain conditions: dimensions between 1 nm to 100 nm, elongated shapes with two dimensions under 1 nm and one above 100 nm, or plate-like shapes with one dimension under 1 nm and others above 100 nm.

The growing number of medical devices (MDs) incorporating NMs highlights the significant promises offered by their unique properties of the nanoscale. At the same time, the distinct characteristics of NMs pose complexities in evaluating their safety and biocompatibility. It is indeed recognized that nanomaterials often exhibit toxicity profiles distinct from their bulk counterparts. Similarly to other substances, NMs can potentially be safe or harmful. The standard risk assessment process, comprising exposure assessment, hazard identification, dose response characterization, and risk characterization, is applicable to NMs as well. However, due to their nano-scale size and the possibility of unique differences in their physical, chemical, biological, and toxicological properties, NMs might pose distinct or additional safety concerns regarding consumer health.

The aim of this work is to illustrate a practical approach for the toxicological risk assessment of NMs in MDs. This approach uses specific methodologies, prioritizing *in vitro* technologies in line with the principles of Replacement, Reduction, and Refinement (the 3Rs), and follows ISO 10993-22 and Medical Device Regulation (EU) 2017/745. In particular, it will show testing for the raw material analysis for the potential presence of nanomaterials, methods to evaluate the potential release from the MD and methods to evaluate the exposure assessment and hazard analysis by using alternative methods. Finally, a weight of evidence approach is used to determine Margin of Safety according to ISO 10993-17 applied to a specific case study.

The aim of this work is to illustrate a practical approach for the toxicological risk assessment of nanomaterials (NMs) in medical devices (MDs). This approach uses specific methodologies, prioritizing *in vitro* technologies in line with the principles of Replacement, Reduction, and Refinement (the 3Rs), and follows ISO 10993-22 and Medical Device Regulation (EU) 2017/745. In particular, through a specific case study, this work will show: 1) Methods for analyzing raw materials for the potential presence of nanomaterials; 2) Techniques to evaluate the potential release of NMs from medical devices; 3) Approaches for assessing exposure and conducting hazard analysis using alternative methods. 4) Finally, a weight of evidence approach will be applied to determine the Margin of Safety according to ISO 10993-17.

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LP-29
Development of human pluripotent stem cell-derived liver organoids for liver toxicity testing

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Purpose: The liver is the most important metabolic organ in the body, which is responsible for the metabolism of substances, including pharmaceuticals, industrial chemicals, pesticides, and food additives. Some of these substances, or their toxic metabolite generated through the detoxification process, can lead to acute liver failure or chronic liver diseases. Drug safety issues continue to occur despite the approval of drugs following comprehensive clinical studies. Therefore, a human cell-based liver model capable of long-term expansion and mature hepatic function is a fundamental requirement for safety assessment.

Methods: Previously, we developed proliferative and functional liver organoids from pluripotent stem cells. However, the protocol requires improvement for standardized and reproducible mass production. Here, we optimized the method for scalable expansion and aimed to determine the essential factors for the self-renewing potential of liver organoids. Knockdown of target gene using short hairpin RNA was performed. We have been developing a toxicity evaluation method based on the cytotoxicity of liver organoids. We used the DILIrank dataset (drug induced liver injury rank dataset) from the US FDA as a reference, which categorized 1,036 FDA-approved drugs into most-, less-, and no-DILI concern drugs. From this dataset, we selected 108 drugs for toxicity testing on our liver organoid platform.

Results: Three medium components, bFGF, OSM, and ITS, were found to be essential for the efficient generation and long-term expansion of organoids. Transcriptome analysis revealed enrichment of fibroblast growth factor signaling, crucial for hepatocyte proliferation during liver regeneration, in proliferative liver organoids. Knockdown of FGFR4 impaired organoid generation and proliferation. Since liver metabolism varies among individuals, we plan to use at least 50 individual organoids from diverse age, gender, and ethnic groups for toxicity testing to account individual differences in response to drug toxicity. So far, we have acquired data from 8 individual liver organoids, and the accuracy of toxicity prediction was obtained over 83%. Notably, liver organoids can completely detect the toxicity of drugs that were withdrawn from the market due to severe hepatotoxicity, such as causing patient deaths or fatal liver failure requiring liver transplantation. We are currently conducting validation studies for liver organoid-based toxicity evaluation methods in accordance with OECD GD34. This organoid-based evaluation method holds potential for various applications, including preclinical liver toxicity screening, hazard identification, and chemical risk assessment prior to human application.

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LP-30
Leveraging pulmonary nanotoxicological discoveries for the design of therapeutic inhalable nanotherapeutics

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Extensive research in pulmonary nanotoxicology has elucidated several critical toxicological paradigms, including frustrated phagocytosis and NALP3 inflammasome activation in lung-resident macrophages. These findings are complemented by a detailed understanding of the size-dependent lung deposition principles of inhaled nano- and micro-particulates. Moreover, resident macrophages, which play a crucial role in the professional removal of particulates from the lungs, are intensively involved in various occupational exposures to engineered materials. By integrating these insights, we leveraged nanotoxicological discoveries to design inhalable nanotherapeutics aimed at addressing pathologically activated NALP3 inflammasome activation in various pulmonary scenarios.

Specifically, we developed an inhalable lipid-based nanoparticle encapsulating a resolvin precursor, along with phosphatidylcholine and polyethylene glycol to enhance particle permeability and mucosal barrier crossing. These resolvin-laden nanoparticles, which efficiently biodistributed in the lungs following oropharyngeal aspiration or inhalation, improved anti-inflammatory status, histological characteristics, and pulmonary function in fibrotic lungs induced by bleomycin, engineered nanomaterials (graphene oxide or rare earth particles), and air pollution ($PM_{2.5}$). This included beneficial outcomes such as mitigating lipid peroxidation-triggered NLRP3 inflammasome activation and regulating the NF-κB pathway in macrophages. Additionally, we observed effective regulation of ROS-mediated TGF-β/Smad and S1P signaling in epithelial cells, even at ng/mL dosimetry. Recently, scaled-up synthesis of these resolvin-laden particles demonstrated high stability upon storage, optimal anti-fibrosis efficacy, and *in vivo* biocompatibility, which were validated in murine models, including mice and rats.

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LP-31
A straightforward protocol to generate human stem cell-derived astrocytes for the *in vitro* strategy to predictive neurotoxicity profiles

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New Approach Methodologies (NAMs) that can be applied as tools for Neurotoxicity (NT) testing, are required. Primary cells of human origin are strongly recommended as relevant *in vitro* cell models to predict toxicological profiles including NT assessment and there has been a continuously growing interest on stem cell-derived systems in particular on human mesenchymal stromal cells (hMSCs) for the screening evaluation of potential CNS toxicity of compounds in humans. Due to the brain's complexity, NT summarizes various modes-of-action one of which involves the impaired function of astrocytes (astrocyte neurotoxicity). Our protocol starts with the generation of high purity and large quantity number of hMSCs (from umbilical cord) which are stable, expandable, and can be cryopreserved, thawed out when needed, and grown for several passages. The complete system involved 6 distinct steps in the hMSC cell-to-neural workflow, including neural induction, expansion, and differentiation to astrocytes obtained via formation of embryoid bodies (EB), as follows: **Step 1.** C*ulturinghMSCs (0-4d)* in mesenchymal growth 2 medium. **Step 2.** G*eneration of EB (4-10d)* by seeding hMSCs in plates pre-coated with Poly-D-Lysine to facilitate cell adhesion and viability, normal cell growth, and EB formation. **Step 3.** *EB and cell proliferation (10-16d)* using predifferentiation medium, for expansion of human neural stem and progenitor cells, by NeuroCult NS-A Basal medium (serum-free) plus Proliferation supplement further supplemented with bFGF and EGF to promote neural lineage differentiation and impair the mesodermal differentiation ability of MSCs. **Step 4.** *EB and astrocyte progenitors (16-22d)* obtained by medium replacement with NeuroCult NS-A Basal medium (serum-free) plus Differentiation supplement further supplemented with human PDGF-BB, to expand immature astrocytes pool, promote the efficient differentiation of hMSCs/EBs and inhibit the unwanted differentiation of non-CNS-type cells. **Step 5.** *Astrocyte differentiation (22–28d)* obtained with the addition of the STEMdiff™ Astrocytes Differentiation medium at the EBs/cells seeded in plates coated with PDL plus Laminin until the EBs/cells are differentiated in astrocyte-like cells (hALCs). **Step 6.** *Astrocyte maturation* obtained by using STEMdiff™ Astrocyte Maturation medium (for up to *16 days*). This workflow yielded cells with an astrocyte-like morphology, and specific astrocyte markers (GFAP and S100β) expression. The set-up protocol was a reproducible, straightforward and relatively rapid (about 40 days) method to generate a population of hALCs transdifferentiated from human primary cells (hMSCs) without re-programming or gene transfection. We demonstrated that the population of hMSCs, obtained from the lining membranes from healthy mothers' Umbilical Cord, can be induced to differentiate into cells of interest including astrocytes that can serve as a source of human "healthy" cells.

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LP-32
Meta-analysis-based gene expression profiling in rodents exposed to respirable crystalline silica

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Introduction: New technology has allowed dedicating RNA expression patterns to more than 70 cell populations in lung linking unique changes to toxic insults and pathology. Inhalation of respirable crystalline silica (RCS) causes approximately 7000 cancer deaths every year in the EU. RCS exposure is still common in mining, tunnelling, agriculture,

and in the construction and stone production industries. Besides lung cancer, RCS exposure is responsible for silicosis and bronchitis cases. Crystalline silica (CS) is virtually insoluble in human tissues, and its toxicity mechanisms remains unclear. Over two decades of RNA expression data have been accumulated in public databases. We illustrate the potential of these data for understanding the mechanisms of CS toxicity.

Methods: An in-silico workflow was developed to integrate gene expression profiles from rodent toxicity studies of CSexposure. A systematic literature search following the PICO framework was conducted in PubMed and Web of Science databases from 2011 to the present. The search included MeSH terms and corresponding entry terms "silica," "gene expression profile," and "lung inflammation" to identify relevant studies. Obtained datasets were then extracted from the Gene Expression Omnibus. We included *in vivo* studies with non-transgenic rats and mice, with a minimum of three rodents per exposure group. Normalization and batch correction steps were applied to ensure comparability across datasets. Differentially expressed genes were identified, and functional enrichment analysis was performed to elucidate the biological processes and pathways associated with observed gene expression changes.

Results: Thirty-six papers were identified in the literature search. After applying inclusion criterias, 11 papers were eligible for data extraction and 7 datasets passed quality control. In total, 1901 genes were significantly regulated by CS exposure, with 1001 upregulated and 900 downregulated. Functional enrichment analysis provided insights into biological pathways affected by RCS exposure, including innate immune system activation, neutrophil degranulation, cytokine signaling, and Toll-like receptor signaling, suggesting a complex interplay of molecular responses to RCS exposure.

Conclusions: This study presents an effective in-silico pipeline for investigating molecular mechanisms underlying health outcomes related to crystalline silica exposure. The findings underscore the potential of in-silico tools in advancing mechanistic toxicology research and highlight their role in facilitating the transition towards animal-free models.

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LP-33
Environmental modeling of trifluoroacetic acid (TFA) from pressurized metered-dose inhalers releasing HFO-1234ze(E)

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Background: HFO-1234ze is a near-zero global warming potential, next generation medical propellant in development for use in pressurized metered-dose inhalers (pMDIs). HFO-1234ze-containing pMDIs have a similar carbon footprint to a dry powder inhaler. The chemical structure of HFO-1234ze has the $-CF_3$ moiety, which is associated with formation of TFA after atmospheric degradation. However, the yield of TFA from HFO-1234ze and the contribution of HFO-1234ze propelled pMDIs to the formation of environmental TFA are undefined.

Methods: To quantify the contribution of these novel pMDIs to the formation of environmental TFA, we performed an extensive study using a global atmospheric model and detailed watershed model. In the atmospheric model, we included all known pathways that may form TFA worldwide from atmospheric degradation of HFO-1234ze and HFO-1234ze emissions were assumed to originate solely from pMDI usage based on sales data. The spatial distribution of pMDI emissions was estimated using the distribution of NOx emissions, i.e., urban areas would have more usage as compared to rural areas. We used the modeled TFA deposition rates around the Rhine River region as an input to our watershed model, yielding the consequential rainwater TFA in different subbasins of this economically important river.

Results: Our state-of-the art modeling reveals that the formation of TFA due to global pMDIs usage is negligible. Interestingly, even though pMDI sales are the highest in regions within the northern temperate zone, TFA deposition rates are higher in regions within the tropical zone. As per our atmospheric model, in the temperate zone, the photolysis reaction of the trifluoroacetic aldehyde (TFAA: the major breakdown product of HFO-1234ze) is dominant which doesn't yield TFA; whereas in tropical regions the hydroxy radical pathway is the dominant pathway which forms TFA. We coupled our global modeling results with a detailed watershed model of the Rhine River and estimated the amount of TFA expected to accumulate due to 30 years of continuous pMDI usage. Our watershed model predicts that the concentration of TFA in Rhine River subbasins would vary between 0.001 ppb to 0.003 ppb. Using the German EPA's "precautionary measure" for TFA in drinking water, i.e., 10 ppb, this suggests that the Margin of Exposure (MoE) for TFA due to usage of these novel pMDIs is greater than 3000-fold.

Conclusions: Our results demonstrate that atmospheric degradation of HFO-1234ze from medicinal usage, would lead to minimal rainwater TFA (0.003 ppb) and does not present a human health concern due its 3000-fold MoE. Furthermore, HFO-1234ze is the only pMDI propellant option not subject to future restriction under the Kigali Amendment due to its near zero global warming potential.

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LP-34
Is the incursion of the Asian toad *Duttyphrynus melanostictus* a possible biological threat in Madagascar? Determination by UHPLC-MS/MS of 5-OH-DMT (Bufotenine) in toad samples

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The incursion of the Asian toad *Duttyphrynus melanostictus* in 2010 poses a threat to Malagasy biodiversity, as well as to human health and the country's economy since biological invasions are major factors in global environmental change and species loss. *Duttyphrynus melanostictus* belongs to the Bufonidae family that produces an alkaloid poison that contains 5-hydroxy-N,N-dimethyltryptamine (5-HO-DMT), known as bufotenine. This is a tryptamine derivative related to the neurotransmitter serotonin that in large amounts can cause psychoactive effects. In this context, this study seeks to shed light on the toxicity of the species *Duttyphrynus melanostictus* found in the city of Toamasina in Madagascar. This work aimed to develop an easy method for the detection and quantification of bufotenine in eggs, larvae and toad's skin samples of the *Duttyphrynus melanostictus* as well as the possible levels of the substance in its predator *Hoplobatrachus tigerinus* and *Haemopis sanguisiga*. Two extraxtion protocols were implemented: one to determine the toxin and one to determine tadpoles, individuals and toad's skin. For the toxin samples, extraction occurred by adding 500 µL acetone/H_2O (70/30 v/v) together with 2.5 µL of internal standard (concentrated at 500 ng/mL), followed by intense stirring and ultrasonication for 10 minutes at room temperature (approx. 25°C) while for tadpoles, individuals and toad's skin, extraction occurred by adding 500 µL of methanol: H_2O (50/50 v/v) together with 2.5 µL of internal

standard (concentrated at 500 ng/mL) followed by intense stirring and ultrasonication for 30 minutes at room temperature (approx. 25°C). Then, all samples' types were centrifuged at 13000 rpm for 10 min, the solvent was collected and stored. For the toxin samples the extraction was repeated. Finally, after diluting 1/10 with mobile phases, 5 µL were injected into the UHPLC-MS/MS system. Spiked calibrators were prepared in distilled water and keratin matrix to mimic the samples nature (eggs/tadpoles and toad's skin respectively) at six concentration levels, analysed similarly and performance parameters were evaluated. The method showed linear calibration in the 10–1000 ng/mL range, LOD values obtained are: 1.9 ng/mL (toxin), 4.9 ng/mL (tadpoles) and 11.8 ng/mL (toad skin). Average CV% and bias% were within 20% for all concentration levels. The proposed protocol is deemed simple, easy, and fast while the UHPLC-MS/MS method for the detection of the analyte showed sufficient sensitivity. The target analyte was detected in all samples, apart from the eggs of *Duttyphrynus melanostictus*, in increasing concentrations as the development of the animal is ongoing, with the highest being in toxin. These results indicate that the presence of bufotenine differs and depends on the species evolution, likewise its toxicity, degree of harmfulness and the impact on its predators and their digestion.

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LP-35
Use of high-content screening technology for toxicological testing

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A central challenge in toxicological testing is the overwhelming number of chemical compounds existing in our environment that need to be assessed. Many of these compounds have inadequate data about potential toxicological liabilities. With advances in technology emerging, high-content screening has become a popular tool in capturing many cellular features at a large scale by use of automated microscopy and image analysis platforms. This presentation will overview various high-content screening assay technologies that have been used for toxicological testing at the US Tox21 (Toxicology in the 21st century) program and will focus on a homogenous neurite outgrowth assay using GFP-labeled iPSC-derived neurons, which has been used to identify neurotoxic compounds after screening an environmental chemical library.

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LP-36
Xenobiotic stress-responsive Gdf15 protects gut-kidney axis via autophagic and microbiota reprogramming

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The integrated stress response (ISR) is crucial in managing cellular stress by primarily halting global protein translation and enhancing the production of molecules that aid in cellular adaptation. Growth differentiation factor 15 (Gdf15) emerges as a significant biomarker indicative of stress responses, particularly in various inflammatory and metabolic diseases. This study investigates whether cellular stress driven by ISR influences pathophysiological outcomes by regulating Gdf15. Transcriptome analysis from clinical samples reveals a positive correlation between PKR (protein kinase R) and Gdf15 expression in patients with renal injuries. In mouse models experiencing acute renointestinal distress, Gdf15 expression is reliant on the ISR pathway mediated by PKR, and the absence of Gdf15 exacerbates chemically-induced damage in renal tissues and the intestinal barrier. Further analysis of gut microbiota shows a connection between Gdf15 and the presence of bacteria involved in mucin metabolism and their associated enzymes. Additionally, stress-induced Gdf15 promotes mucin production and enhances cellular survival by restructuring the autophagy regulatory network. Overall, the activation of Gdf15 through ISR mitigates pathological conditions by beneficially reprogramming the autophagic network and the microbial ecosystem, offering promising biomarkers and therapeutic targets for renointestinal distress

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LP-37
Surveillance of pharmaceutical and transformation compounds in coastal waters of the Iberian Peninsula

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Currently, 41% of the world population live within coastal limits. This increase in human presence is accompanied by an increase in anthropogenic pollution, including contamination with pharmaceutical active ingredients, and their derived transformation products. Some pharmaceutical products are now listed as priority substances in the new proposal for the European Water Framework Directive, while others are considered in the European Watch List. The objective of the study was to provide an assessment of the ocurrence and exposure levels of pharmaceuticals and transformation products in the coastal waters of the Iberian Peninsula. In this study, the presence of 37 pharmaceutical active ingredients and transformation products (TPs) were monitored across 46 beaches, 16 beaches were selected along the Portuguese coast, mainly in the regions of Lisbon and the Algarve and 30 in Spanish coasts, 18 in the Cádiz-Málaga area and 12 in Murcia (Mar Menor). Sampling locations included natural protected areas but also metropolitan beaches in heavily touristic and urbanized places. Monitored substances belong to the main therapeutic groups: anti-infective, sex hormones, cardiovascular and digestive systems, analgesics, nonsteroidal anti-inflammatory drugs, among others. Four of them are listed as priority substances for transitional surface waters in the new Directive proposal: clarithromycin, erythromycin, diclofenac and carbamazepine. Besides this, venlafaxine and O-desmethylvenlafaxine are included in the 4th European Watch List. In total, 61 grab water samples were collected from the different sampling areas during the spring and summer of 2021. A total of 31 from the 37 substances (including five transformation products) were detected at least in one sampling location. Within the three regions analyzed, the Portuguese coast (Lisbon-Algarve) showed the highest cumulative concentration and the greatest number of detections, while the Mar Menor area in Murcia was the least contaminated site. The most contaminated sampling point was located in the Tagus estuary near Lisbon (Arcos Beach) with a total concentration of 2,071 ng/L of pharmaceuticals. The highest concentration of any detected pharmaceutical was 1,916 ng/L for the diclofenac in the same beach. Apart from diclofenac, the concentration of two additional priority substances (clarithromycin, and carbamazepine) could be quantified. In this study, we show that pharmaceuticals are widespread in the southern Iberian Peninsula coastline, including compounds that are regulated under the Water Framework Directive. Furthermore, the presence of several transformation products in marine waters are described. Further research will be dedicated to assessing the ecological risks of these compounds for marine ecosystems.

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LP-38
DockTox: a molecular docking platform for screening small molecules targeting Molecular Initiating Events (MIEs) relevant to liver, kidney and brain adverse events

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In modern toxicology, Adverse Outcome Pathways (AOPs) outline the sequence of events from MIEs to final adverse outcomes after chemical exposure, providing mechanistic insights into toxicological effects [1]. The ONTOX project is at the forefront of developing New Approach Methodologies to predict toxicity in the liver, developing brain, and kidney. Our work focuses on computational methodologies targeting AOP elements, particularly MIEs involving key protein interactions.

We developed an automated workflow for docking small molecules onto pre-processed protein structures using co-crystallized ligands to generate docking box coordinates and a reference interactions list. This workflow, integrated into the online tool DockTox (https://chemopredictionsuite.com/DockTox), generates conformers from input molecules and performs docking. Outputs include binding energy, interaction lists, and 2D/3D interaction maps. The tool also compares the interaction of query ligand in comparison to reference ligands, generating an interaction fraction value that measures the similarity of their behaviour.

For validation, we conducted a case study on Peroxisome Proliferator-Activated Receptor α (PPARα) using known PPARα ligands from the ChEMBL subset of the NURA database [2]. Results showed that binding energy alone is insufficient to distinguish PPARα binders from non-binders. On the contrary, the interaction fraction metric provided a more informative measure, with non-binders showing lower interaction fraction values. This feature offers critical insights into protein-ligand interactions, absent in other docking tools.

DockTox, with its user-friendly interface and a collection of 23 pre-processed proteins, facilitates virtual screening of small molecules targeting MIE-associated proteins. It provides valuable data on binding energies, interaction profiles, and interaction maps, serving as a useful tool for tiered risk assessment approaches to anticipate adverse outcomes from chemical exposure.

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LP-39
Investigating the neurotoxic potential of endocrine disruptors chemicals through miRNA and pathway dysregulation

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Endocrine disruptor chemicals (EDCs) are widespread compounds recognized for their capacity to interfere with endogenous hormones via transport, metabolism, and receptor binding. Emerging evidence indicates that EDC exposure can disrupt the precise mechanisms regulating neuronal cell proliferation, thus impacting brain health and potentially increasing the risk of neurodevelopmental and neurodegenerative disorders.

This study examines the effects of subtoxic concentrations of diethyl phthalate (DEP), commonly used as a plasticizer, and 17-alpha ethinyl estradiol (EE2), a synthetic estrogen found in oral contraceptives and hormone replacement therapy, on differentiated SH-SY5Y cells. Cells were exposed to different concentrations of EDCs for 48 hours to establish the conditions that did not induce cytotoxicity or oxidative stress. The results indicated that the EDCs under investigation could interfere with the epigenetic machinery. Bioinformatic analysis revealed that the deregulation of miRNAs associated with neurotoxicity, including hsa-miR-200a-3p, hsa-miR-18b-5p, and has-miR-653-5p, affected the expression of numerous genes involved in the EGFR/Ras/p53 and PI3K/Akt/mTOR pathways, such as AREG, EGFR, IGF1R, BTG2, and SH3BP4.

Although preliminary, these analyses provide insight into the capability of certain EDCs to differentially modulate pathways implicated in neurodegeneration and tumor development. Understanding the com-

plex interplay between EDC exposure, miRNA dysregulation, and proliferation deregulation in neuronal cells is essential for assessing the neurotoxic potential of these compounds. This knowledge is crucial for developing targeted interventions and regulatory measures to mitigate adverse effects on brain health.

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LP-40

The role of estrogenic endocrine disruptor chemicals in modulating neuronal signaling pathways and cell fate

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In recent years, there has been growing concern about the potential adverse effects of environmental contaminants on human health. According to the U.S. Environmental Protection Agency (EPA), endocrine disruptor chemicals (EDCs) include primarily human-made chemical agents that disrupt the synthesis, secretion, transport, binding, or elimination of natural hormones, which are pivotal for maintaining homeostasis, regulating reproduction, fostering development, and influencing behavior. Estrogenic EDCs have been the subject of numerous studies over time, and their potential to disrupt various signaling pathways and cellular functions remains a matter of contention.

The present study investigates the effects of subtoxic concentrations of diethyl phthalate (DEP), a common plasticizer, and 17-alpha ethinyl estradiol (EE2), a synthetic estrogen used in oral contraceptives and estrogen replacement therapy, on differentiated SHSY5Y cells. Cells were exposed to various concentrations of these EDCs for 48 hours to establish experimental conditions that did not induce cytotoxicity or oxidative stress.

Western blotting analysis revealed a significant shift in cellular response toward a pro-survival state through the activation of EGFR/Ras/p53 and PI3K/Akt/mTOR pathways. Although these findings are preliminary, they suggest that EDCs can differentially modulate pathways involved in neurodegeneration and tumor development. Understanding the complex interactions between EDC exposure and the deregulation of neuronal cell proliferation is crucial for assessing the neurotoxic potential of these compounds. This knowledge is essential for developing targeted interventions and regulatory measures to mitigate their adverse effects on brain health.

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LP-41

iPSC-derived intestinal organoids offer a physiologically relevant *in vitro* platform for large-scale predictive toxicity and absorption studies.

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Background and Aims: Drug induced gastrointestinal (GI) toxicity is a common adverse effect responsible for a range of conditions that affect GI tract health. Despite the severity of the problem, adverse drug effects are poorly predicted during early-stage drug discovery, mainly due to the lack of appropriate *in vitro* models able to fully recapitulate human intestine physiology. We hypothesized that human induced pluripotent stem cell (iPSC)-derived intestinal organoids can overcome this issue, offering a suitable and physiologically relevant *in vitro* 3D platform for predictive drug toxicity and absorption studies.

Methods: Healthy iPSCs were differentiated towards intestinal lineage and subsequently embedded into extracellular matrix to form intestinal organoid structures (Def-INT). Upon formation, Def-INT were disrupted to generate intestinal epithelial monolayers. Characterization of Def-INT and monolayer functionality were assessed, including quantification of key intestinal, drug metabolism, and transport marker expression by qPCR, immunocytochemistry (ICC), and RNA-sequencing. CYP450 activity was evaluated by luciferase assays, whilst transporter activity, monolayer integrity, drug detoxification and permeability by ICC, colorimetric assays, transepithelial electrical resistance (TEER), and mass-spectrometry.

Results: Def-INT and intestinal monolayers expressed comparable mRNA and proteinlevels of key intestinal (e.g., CHGA, Villin, MUC2*)*, Phase I (e.g., CYP3A4*)* and transporter (e.g.,MDR1*)* markers to those seen in human small intestine. ICC analysis revealed the presence of different intestinal cell types within Def-INT that are physiologically detected in human intestine (e.g., Paneth/goblet/stem/enteroendocrine cells). Importantly, Def-INT, primary small intestine, and colon tissues had a more similar transcriptional profile compared to Caco-2 cells, as shown by RNA-sequencing. MDR1 activity was confirmed by intracellular Rhodamine 123 (MDR1 substrate) accumulation following 48h of treatment, which was reversed when cells were co-treated with Verapamil (MDR1 inhibitor; 20uM). Glutathione-S-Transferase activity was comparable between Def-INT and Caco-2 cells, highlighting the detoxification capabilities of our model. Measurement of TEER showed high monolayer integrity with resistance values closer to those seen in small intestine compared to Caco-2 cells. Intestinal monolayers demonstrated enhanced reproducibility and stability with comparable TEER values across >15 experiments, whilst drug treatment with high, medium, and low permeable compounds revealed their ability to accurately predict drug permeability.

Conclusion: Def-INT are a superior *in vitro* model to Caco-2 cells with similar functionality to that seen in human intestine. Our study demonstrates the robustness and high predictivity of our model and showcases the spectrum of opportunities this can offer in the fields of drug toxicity, absorption, and efflux transporter studies.

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LP-42

A role of aurora A tyrosine kinase inhibitor, Alisertib, in the resistance of cancer cells to Daunorubicin

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The significance of aurora kinases (AURKs) inhibitors in diverse settings has been emphasized by clinical trials, prompting us to examine their effectiveness in isolation as well as in conjunction with anthracyclines (ANT), commonly referred to as standard chemotherapy regimens. These trials are crucial because of the considerable efficacy of anthracyclines, which is limited by the potential development of drug resistance. Alisertib (ALI), a selective inhibitor of Aurora kinase A (AURKA), is now being studied in clinical studies to treat various cancers, including acute myeloid leukemia. Aldo-keto reductase 1C3 (AKR1C3) is overexpressed in numerous malignancies and influences the metabolism of chemotherapy regimens, particularly ANT, by converting them to less potent hydroxy metabolites. Our project is based on intriguing preliminary data that indicates the ability of ALI to coun-

teract AKR1C3-mediated drug resistance. We aim to determine whether ALI impacts the AKR1C3-catalyzed reduction of daunorubicin (DAUN). In this study, ALI reduced DAUN inactivation mediated by AKR1C3 at both the recombinant and cellular levels. Our findings showed that clinically relevant dosages of ALI selectively inhibited AKR1C3 with a Ki of 1.59 μM at the recombinant enzyme level and an IC_{50} of 10.5 μM in cellular model. In addition, ALI has been demonstrated to reduce AKR1C3-mediated resistance to DAUN in AKR1C3 overexpressed HCT116 cell line. In drug combination studies, ALI sensitized AKR1C3-expressing HCT116 and A549 cells to DAUN treatment. There were no significant changes in AKR1C3 mRNA level in HepG2 cells treated with ALI; hence, enzyme induction does not diminish the impact of the drug combination. ALI has been reported to interfere with the DAUN efflux mediated by the ABC transporters in A549 and KG1α cell lines, suggesting its contribution in combination with DAUN. Our findings identified the utilization of ALI in ANT-based therapy, suggesting a novel effective strategy for cancer treatment.

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LP-43
SkinEthic™ is a good alternative for testing phototoxicity *in vitro* using reconstructed human epidermis

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Introduction: The phototoxicity (PTT) is an acute toxic response elicited by topically or systemically administered photoreactive chemicals after the exposure to environmental light. Nowadays, the use of reconstructed human epidermis (RHE) models for the *in vitro* identification of phototoxic substances (OECD TG 498) has become the golden-standard test for safety assessment of cosmetics and chemicals. Despite the efficiency of the method, one point of concern is that there is only one manufacturer (Epiderm™ Mattek) producing the tissues that is validated and approved by OECD for performing the assay for regulatory purposes. This is a problem since it holds the monopoly of one manufacturer for all PTT studies performed in GLP-compliant conditions. For this reason, it is important to test and validate new RHE models for PTT assay to offer alternatives for the safety assessment of substances.

Objective: The present project was conducted in order to verify the performance of RHE tissues produced by Episkin (SkinEthic™) and validate the method using the same conditions that were employed for the OECD TG 498 validation

Methods: Reference chemicals (five phototoxic positive references and five phototoxic negative references) were topically applied (range of 5 concentrations) on SkinEthic™ and incubated during 24 hours. Then, the tissues were exposed to ultra-violet (UV+) light while the controls were kept in the dark (UV-). After 24 hours of recovery, the cell viability was assessed using the MTT viability assay test. For each chemical, 3 runs were performed in triplicate and the relative cell viability of UV-exposed tissues was calculated to classify the chemicals according to their phototoxicity.

Results: The phototoxic chemicals (chlorpromazine, acridine hydrochloride, bergamot oil, neutral red, and tetracycline free base) were correctly classified on SkinEthic™ RHE by following the OECD TG498 protocol Besides, the chemicals that are negative phototoxic chemicals (penicillin G, SDS, Octyl salicylate, 4-Methylbenzylidene, and Octyl methoxycinnamate) were classified as non-phototoxic substances. Overall, the performance of SkinEthic™ RHE was equivalent to the observed using Epiderm™ from Mattek.

Conclusion: The SkinEthic™ showed a good performance to classify phototoxic chemicals using the method described on the current guideline (TG498). Given that the Epiderm™ is the only test system validated so far for regulatory studies, our results offer valuable arguments for employing the SkinEthic™ as alternative for safety assessment of phototoxicity. In conclusion, our study provides strong evidences highlighting the good performance of SkinEthic™ as alternative for *in vitro* phototoxicity testing.

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LP-44
Enhanced interpretation of secondary pharmacology data through automated visualization tools to support safety assessment

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Secondary pharmacology data are typically included in investigational new drug applications to define the selectivity profile of clinical candidates and assess risks from off-target activities. Understanding potential adverse events (AEs) related to modulation of identified targets is crucial but challenging. The IQ-DruSafe *In Vitro* Secondary Pharmacology Working Group recently published an analysis of current *in vitro* pharmacology practices, highlighting new targets used in screening by leading pharmaceutical companies, complementing the existing 44 most consensual targets (Brennan *et al.*, 2024; Bowes *et al.*, 2012).

We aimed to develop a solution for supporting the interpretation of secondary pharmacology data, enabling better and earlier consideration of potential adverse events.

Relevant compounds with known adverse drug reactions and documented pharmacological profiles were tested in the SafetyScreen™ 18 Core Panel (binding and enzymatic assays panel based on new recommendations by Brennan *et al.*, 2024), at 10 μM. Compounds showing at least 50% of inhibition of the control activity at these targets were then tested at 8 concentrations to determine IC_{50} values. The results were processed using our new online automatic visualization and interpretation tools, developed to display pharmacological results and indicate organs at risk. Identification of the organ(s) at risk is supported by AEs information from proprietary and public databases. Pharmacological results and hit profiles were consistent with expectations based on literature and proprietary historical data. Our automatic interpretation tool allows rapid and clear identification of organs at risk, in agreement with documented AEs.

The tool supports fast overview and reporting of secondary pharmacology results. Combining this with a routinely used, time-effective target profiling could significantly improve early stages of drug discovery. For accurate risk assessment, the tool needs inclusion of more translational quantitative information and contextualization to refine the interpretation and adapt the risk mitigation strategy.

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LP-45
Application of a unified probabilistic framework to the dose-response assessment of selected nitrosamines and derivation of health-based exposure limits (HBELs)

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N-Nitrosamines, including N-Nitrosodimethylamine (NDMA) and N-Nitrosodiethylamine (NDEA), are potent carcinogens. Recent data indicate the existence of a threshold for their genotoxicity. Deterministic Health-Based Exposure Limits (HBELs) for an occupational setting, known as Occupational Exposure Limits (OELs), were recently established for NDMA and NDEA to protect workers, with assessments available in the published literature [1].

In this case study, a probabilistic approach was used to derive safety limits for both substances, employing the Approximate Probabilistic Analysis (APROBA) [2] – a tool developed by the World Health Organization's International Programme of Chemical Safety (IPCS) [3,4].

The derivation of both deterministic and probabilistic HBELs is based on the incidence of malignant liver tumors observed in rats, considered as the most relevant endpoint for human health. Benchmark Dose (BMD) modeling was performed, statistical lower bounds (BMDLs) were computed and used as Points of Departure (PoDs). Two population incidence goals (I) were applied: 0.001 and 0.01%. The OELs computed using the probabilistic approach were as follows: NDMA: 0.063 μg/m^3 (I=0.001%); 0.154 μg/m^3 (I=0.01%); NDEA: 0.021 μg/m^3 (I=0.001%); 0.056 μg/m^3 (I=0.01%).

The probabilistic OELs for NDMA are slightly lower than the deterministic OEL of 0.5 μg/m^3, with the risk-specific dose distribution showing an uncertainty factor of 182 (I=0.01%) and 340 (I=0.001%) (95th-5th percentile ratio). Similarly, for NDEA, the probabilistic OEL is lower than the deterministic OEL of 0.2 μg/m^3, with an uncertainty factor of 183 (I=0.01%) and 343 (I=0.001%) (95th–5th percentile ratio). Additionally, probabilistic method yields higher limits compared to the standard method using linear extrapolation (risk-based approach). Ultimately, deterministic and probabilistic OELs were in a similar range for the selected incidence levels, with 3.25–7.93-fold differences for NDMA and 3.57–9.52-fold differences for NDEA.

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LP-46
Leaky gut causes inflammatory stress: a determinant of acute liver injury induced by coadministration of rhein and LPS

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Polygonum multiflorum Thunb. is a widely used traditional Chinese medicine, and its hepatotoxicity has been received widespread attention. Rhein, an anthraquinone substance, is considered to be one of the toxic components of Polygonum multiflorum Thunb. It has been discovered to have multiple pharmacological activities including immune regulation. Furthermore, oral administration of rhein can be metabolised by intestinal flora to rheinanthrone, which has a laxative effect. Based on this evidence, we hypothesize that rhein may lead to intestinal barrier and local immune disorders, increase intestinal endotoxins entering the blood, and cause an inflammatory microenvironment of intestinal-blood-liver tissue, which may be the basis for acute liver injury caused by secondary or cumulative endotoxin attacks. To verify this hypothesis, 18 Sprague-Dawley rats were administered rhein (500 mg/kg) by oral gavage for 15 days daily. Subsequently, 1.5 mg/kg of lipopolysaccharide (LPS) was administered by intravenous injection, and blood and organ samples were collected six hours later.

The results showed that the animals had loose stools after administration of rhein. Compared with the control group, plasma total protein (TP) and globulin (GLB) increased in the rhein group and the albumin/globulin (A/G) ratio decreased. In the rhein-LPS co-treatment group, plasma GLB, ALT and AST increased, ALB and A/G decreased, the proportion of neutrophils increased, the proportion of lymphocytes decreased. Hepatic indices increased in both the rhein and co-treatment groups, and the expression of the hepatic inflammatory factor IL-1β increased significantly in the co-treatment group. Histological changes included atrophy of the ileal and colonic mucosa and dilatation of the rectal crypt in some animals in the rhein group, whereas dilatation of the colonic and rectal crypts was seen in most animals in the co-treatment group. At 14 days after administration, the plasma endotoxin (LPS) levels and hepatic TBA increased significantly in the rhein group, both showing dose-dependent changes.

In conclusion, rhein can induce intestinal abnormalities, compromise the intestinal barrier, and facilitate the entry of LPS into the bloodstream, thereby precipitating inflammatory stress in the liver. In such a scenario, subsequent exposure to lower doses of endotoxin can result in liver damage. Our findings provide direct experimental evidence for the inflammatory stress hypothesis of idiosyncratic drug-induced liver injury by Polygonum multiflorum.

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LP-47
Comparison of the repetitive oral dose toxicities of Okadaic acid and Dinophysistoxin-1

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Okadaic acid (OA) and dinophysistoxin-1 (DTX-1), produced by certain dinoflagellates, are marine biotoxins responsible for diarrhetic shellfish poisoning (DSP) in humans, leading to gastrointestinal symptoms such as nausea, vomiting, diarrhea, and abdominal pain. Current toxicity equivalency factors (TEFs) for these toxins are based on acute lethality, which may not adequately reflect the sub-lethal, chronic effects of these toxins. This study aims to reassess the relative toxicities of OA and DTX-1 by evaluating their chronic effects through repeated oral administration at sub-lethal doses in mice over a seven-day period. In this study, three sub-lethal doses of OA or DTX-1 were administered to mice daily for seven days. Results showed that both toxins led to significant physiological and pathological changes. Mice treated with either toxin experienced body weight loss, with the severity being dose-dependent and more pronounced in OA-treated mice. Additionally, the disease activity index (DAI), which accounts for stool consistency and the presence of blood in stools, increased dose-dependently for both toxins. However, the increase in DAI was significantly higher

in OA-treated groups compared to DTX-1-treated groups, indicating a more severe diarrheagenic effect of OA. Histopathological examination of the small intestine revealed that while the villus height remained unchanged, the crypt depth increased significantly in a dose-dependent manner for both toxins, leading to a reduction in the villus to crypt length ratio. This change was more severe in OA-treated mice, suggesting a higher proliferative activity in the intestinal crypt cells due to OA. Moreover, the study observed the presence of ascites, a condition characterized by fluid accumulation in the abdomen, in mice treated with moderate to high doses of either toxin. The severity of ascites was greater in OA-treated mice, which was correlated with increased serum biomarkers of liver and kidney injury, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, and blood urea nitrogen (BUN). Despite these biochemical changes, no significant histopathological damage was observed in liver or kidney tissues, suggesting a non-cirrhotic origin of the ascites, potentially linked to intestinal fluid retention and cardiotoxicity. These findings suggest that OA is at least as toxic, if not more so, than DTX-1 when administered repeatedly at sub-lethal doses, highlighting the inadequacy of current TEF values based solely on acute toxicity data. This study is the first to report the comparative chronic effects of repeated oral administration of DSP toxins and underscores the need for sub-chronic and chronic toxicity evaluations to establish more accurate TEF values. Such assessments are crucial for proper risk management and regulatory measures to protect public health from the toxic effects of DSP toxins in shellfish.

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LP-48

Derivation of a Maximum Allowable Dose Level (MADL) for Bisphenol S and associated challenges

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Bisphenol S (BPS) is a bisphenol A (BPA) analog often used as a replacement for BPA. In 2023, the State of California's Office of Environmental Health and Hazard Assessment (OEHHA) added BPS as a female reproductive toxicant to the State of California's Proposition 65 list of substances known to the State to cause cancer or reproductive toxicity, noting ample evidence relevant to multiple key characteristics (KC) of female reproductive toxicants: (KC1) Alters hormone receptor signaling, (KC2) Chemical or metabolite is genotoxic, (KC8) Alters direct cell-cell interactions, (KC10) Alters microtubules and associated structures. Under Proposition 65, parties causing consumer exposures to listed substances are required to provide specific label warning information on affected products, but if an exposure to the listed reproductive toxicant can be shown through a quantitative exposure assessment to be less than the chemical-specific maximum allowable dose level (MADL), the responsible person has "safe harbor" from the warning requirement. OEHHA has not established a safe harbor level for BPS; therefore, ToxServices applied OEHHA's published methodologies and conservative assumptions to BPS's toxicological dataset in order to calculate an MADL for BPS. OEHHA's listing of BPS on Proposition 65 is based on human and animal data demonstrating that BPS causes effects on ovarian histology, uterine effects, endocrine effects, effects on reproductive performance, effects on mammary gland development, and alterations in puberty onset. The dataset presented a number of challenges for risk assessment, including non-guideline studies testing limited dose ranges, non-monotonic dose responses, use of non-traditional species displaying varying sensitivities, and uncertain adversity of some effects. ToxServices reviewed the dermal (subcutaneous) and oral studies evaluated by OEHHA in its determination, focusing on studies and effects that OEHHA considered as strongest support of reproductive effects. ToxServices selected a subcutaneous LOAEL of 1 µg/kg/day from a 21-day study in mice, based on reductions in LH and FSH levels, to derive a dermal MADL and an oral NOAEL of 0.1 µg/kg/day from a 28-day study in mice, based on reduced fertilization rate, to derive an oral MADL. The resulting dermal MADL is 0.058 µg/day and the resulting oral MADL is 0.0058 µg/day. These MADLs can be used by manufacturers, retailers, or other stakeholders to assess compliance with Proposition 65 requirements.

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LP-49

Deriving tolerable intakes for data-poor medical device extractables: 3-Prop-2-en-1-yl propanedioate as a case study

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Medical devices undergoing extractables testing under ISO 10993-18:2020 often extract chemicals with relatively limited toxicological datasets. In order to establish that devices extracting such chemicals are biocompatible, tolerable intakes (TIs) must be established following ISO 10993-17:2023. A TI is an estimate of the average daily intake of a substance over a specified time period, on the basis of body mass, that is considered to be without appreciable harm to health (ISO 10993-1:2018). For low-level extractables, use of the Threshold of Toxicological Concern (TTC) is often sufficient to establish derive default TIs; however, use of the TTC approach is often not possible to establish safety of extractables from prolonged (24 hours-30 days) or permanent (>30 days) contact medical devices because TTC-based TI values are too low. This poster presents a framework used to derive TIs for data-poor chemicals that extract at levels above TTC-derived TIs. The extractable 3-prop-2-en-1-yl propanedioate (CAS#Unassigned)(SMILES OC(=O)CC(=O)OCC=C) is used as a case study to example the utility of the framework for a parenteral TI. Three online platforms are used to identify potential surrogates (Ambit, PubChem, and U.S. EPA CompTox Chemicals Dashboard), with surrogate chemicals sorted by similarity metrics such as Tanimoto coefficients. A dose-response analysis of candidate surrogates and their underlying constituents and putative metabolites, including allyl alcohol (CAS#107-18-6), with selection of a NOAEL for the most sensitive species and critical health effect as a Point of Departure (POD) is exampled. A TI for 3-prop-2-en-1-yl propanedioate is shown where the POD is divided by a composite Modifying Factor (MF) made up of three Uncertainty Factors (UF1, UF2, and UF3) that are optimized to minimize the overall MF, including use of the Danish QSAR database to adjust for bioavailability. In conclusion, the framework facilitates a consistent approach to assessing health risks of data-poor chemicals and fulfills ISO 10993-17 requirements to assess health risks from extractable chemicals.

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LP-50

Chemical alternatives assessment of selected alkylbenzenes using the Greenscreen® Tool

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Substituted benzene compounds such as alkylbenzenes occupy important positions in terms of chemical relevance, since they are produced in large quantities and are released into the environment as a result of their wide use in agriculture and consumer products (electronics). However, there is an increased concern for the human health and environmental safety of alkylbenzenes due to the primary compound benzene being a human carcinogen and a developmental toxicant. Therefore, assessing the hazards of benzene derivatives has an important significance for regulating their uses in consumer products and

avoiding regrettable substitution. Towards this we used the GreenScreen® hazard assessment tool to assess and compare the hazard properties of four alkylbenzene derivatives that are used as industrial solvents with currently no regulatory evaluation or restrictions in Europe. These substances belong to two subcategories: 1) methylbenzene derivatives: toluene (CAS #108-88-3) and xylenes (CAS #1330-20-7) and 2) ethylbenzene derivatives: ethylbenzene (CAS #100-41-4) and 1,4-diethylbenzene (CAS #105-05-5). The GreenScreen® for Safer Chemicals is a hazard assessment tool used to rank chemicals and select a preferred chemical alternative. The GS assesses chemicals for 18 human health, environmental toxicity and fate, and physical hazard endpoints to assign an overall benchmark (BM) score ranging from 1 (High concern) to 4 (Safer). A GreenScreen® summarizes endpoint scores in a hazard summary table to allow for ease of visualization. Our results show that all four investigated alkylbenzenes, regardless of the carbon length chain, are associated with a high hazard for aspiration toxicity, a common concern for such type of solvents, as their measured kinematic viscosity parameters are below the GHS Guidance value for Category 1. In addition, all four chemicals are toxic to the aquatic environment based on available experimental data. In terms of developmental toxicity and carcinogenicity, the properties vary with no clear trend that could be established. Ethylbenzene, was the only compound associated with high hazard for carcinogenicity while the other three compounds are not expected to cause cancer. In contrast, methylbenzene derivatives are associated with high hazard for developmental toxicity while ethylbenzene is a moderate concern and no developmental hazard was identified for 1,4-diethylbenzene. Accordingly, 1,4-diethylbenzene seems to be the safest alternative as it is assigned a BM-2 while the remaining compounds were all identified as high concern. These results can be utilized to allow for hazard extrapolation at the group level (aquatic toxicity, aspiration hazard, neurotoxicity) and provide informed choices for selecting less hazardous benzene derivative solvent. To this end, this poster will outline the GS methodology and present in detail the results of the GS hazard classification for each endpoint for the assessed benzene derivatives.

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LP-51
Application of NAMs to the assessment of reproductiveand developmental toxicity: a case study

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For certain product types, animal testing is not only not desirable, but is also prohibited by law. In the EU, new cosmetic ingredients may not be tested on animals, and the same is true in the state of California and a small number of other states. This necessitates the use of new approach methodologies (NAMs) to fill data gaps for critical human health endpoints. Due to the complexity of human reproduction and development in general, the development and validation of NAMs for these endpoints has been challenging. Herein we explore available NAM-based tools for the assessment of reproductive and developmental toxicity and provide a case study of their application to the safety assessment of a cosmetic ingredient, isopropyl cloprostenate. This case study highlights the ways in which NAMs can – and should – be integrated into read-acrossto increase confidence in analogue selection to support next generation risk assessment for cosmetic ingredients.

In this case, our ingredient is a member of the prostaglandin F2a analogue chemical class. Robust reproductive and developmental toxicity data are available for other class members, including latanoprost, travoprost, bimatoprost, and cloprostenol. As assessment of structural similarity alone is not sufficient to support read-across, we performed a detailed analysis of ADME properties as well as a mode of action analysis, including receptor binding profile, to identify an appropriate analogue. Specifically, we utilized OPERA's estrogenic activity and androgenic activity prediction modules; VEGA's CAESAR developmental toxicity prediction model; Danish (Q)SAR Database's dermal absorption, receptor binding, and developmental toxicity models; and *in vitro* prostaglandin receptor binding data to determine which potential analogue is most suitable as a surrogate for isopropyl cloprostenate. In addition, to assess potential bias with respect to our reliance on a previously-identified chemical class, we performed an independent analogue search using the U.S. EPA's Analogue Identification Methodology (AIM) Tool as well as the Long-Range Research Initiative's AMBIT read-across tool.

In a weight of evidence approach, we identified travoprost as a suitable analogue for isopropyl cloprostenate. We also considered cloprostenol, based on our assessment of ADME properties, as a second potential analogue. A detailed dose-response assessment of both potential analogues identified travoprost as the more conservative choice. Therefore, we utilized a LOAEL from a multi-generation study with travoprost as the point of departure for the margin of safety calculation for the intended use of isopropyl cloprostenate in cosmetics. The resulting MOS was >100, indicating acceptability of the evaluated ingredient use.

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LP-52
In vitro percutaneous absorption of dehydroacetic acid and benzoic acid, preservative cosmetic ingredients, from mini-pig skin using the Franz diffusion cell system

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Dehydroacetic acid and benzoic acid are used as preservative cosmetic ingredients at a maximum concentration of 0.6% and 0.5% in Europe and Korea, respectively. Before the percutaneous absorption study, analytical methods were developed for quantitation of dehydroacetic acid and benzoic acid in various matrices of swab (SW), stratum corneum (SC), skin (dermis + epidermis, SK), and receptor fluid (RF). These developed methods of dehydroacetic acid and benzoic acid showed well-fitted linearity (r^2=0.9972–0.9998, r^2=0.9996–0.9999), accuracy (87.5–111.70%, 96.04–101.47%) and precision (1.20–10.16%, 1.30–5.48%) in accordance with the validation guideline, respectively. Franz diffusion cell was used to determine percutaneous absorption of dehydroacetic acid and benzoic acid using dorsal pig skin. The lotion formulation containing dehydroacetic acid (0.12%, 0.24%, and 0.6%) and benzoic acid (0.1%, 0.2%, and 0.5%) was applied to the skin at 10 mg/cm^2, respectively. After 24 hours, dehydroacetic acid and benzoic acid in each matrix were measured by liquid chromatography-mass spectrometry (LC-MS/MS). Finally, the total percutaneous absorption rates of dehydroacetic acid (0.12%, 0.24%, and 0.6%) and benzoic acid (0.1%, 0.2%, 0.5%) in lotion formulation were determined to be 96.18±7.59%, 91.42±4.05%, 101.18±7.82%, and 65.85±5.80%, 51.54±8.97%, 80.31±5.85%, respectively. These data can be applied to perform exposure and risk assessment of cosmetics containing dehydroacetic acid and benzoic acid.

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LP-53
Combining statistical approaches with MIE Analysis for interpretable developmental toxicity prediction

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Purpose: We aim to enhance the performance and interpretability of predictive models for developmental toxicity utilizing statistical and knowledge based approaches, mainly focusing on explanation of possible pathways involved in toxicity of statistically derived structures alerts.

Methods: A molecular fragment tree was constructed to identify structural alerts (SAs) using two databases: drug-induced developmental toxicity (DIDT) dataset (997 chemicals, 76% positives) and aryl hydrocarbon receptor (AhR) antagonists (AhRA) dataset (2274 chemicals, 87% positives), as AhR activation is a MIE in the AOP for developmental toxicity. The identified SAs were examined for overlap and mechanistic interpretation. Additional rules were applied to identify modulating factors that suppress the toxic effects of SAs.

Results: The 156 and 32 SAs were identified in the DIDT and AhRA databases, respectively. Among detected SAs there was only one that was found in both sets. The identified SMILES was "NC=C", which was a part of the pyridine ring in all positive compounds. Modulating factors were estimated to be 9 in the DIDT database and 4 in the AhRA database. The SMARTS pattern for the modulating factor of the common alert was "CSCCNC", indicating that the sulfur atom in the pyridine ring, with an amino group in the side chain, neutralizes the toxic effect associated with the alert. The developed model based on the DIDT dataset, which included 156 SAs and 9 SA/modulating factor pairs, demonstrated an accuracy of 0.66 on the test set. This model exhibited higher predictive ability than the OECD QSAR Toolbox (accuracy 0,58) on the same test set. The positive predictive value was 0.75, surpassing that of the OECD QSAR Toolbox. Thus, out of 156 SAs, our approach enabled us to identify one SA responsible for developmental toxicity, leading to the AOP by initiating the AhR activation pathway. Further analysis of overlaps with MIEs of other AOPs related to developmental toxicity will increase the number of statistically detected SAs with associated MIEs. The primary advantage of this approach is its speed and interpretability, as predictions are supported by structural alerts, modulation factors, and AOP elucidation based on MIE activation.

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LP-54
Protective effect of aryl hydrocarbon receptor (AHR) against high-fat diet-induced obesity in female mice

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Obesity is a global health problem and a major risk factor for cardiovascular disease, type 2 diabetes, and cancer [1]. However, the regulation of body weight requires a clear understanding.

The aryl hydrocarbon receptor (AHR) is an emerging player in energy balance. AHR is a ligand-activated nuclear receptor that regulates various genes that are involved in multiple functions [2]. Recent studies have shown that AHR may participate in energy metabolism [3,4], but its role remains obscure.

The present study aimed to shed further light on this by delineating how AHR deficiency affects energy balance at the whole-animal level. Moreover, the gene expression of the critical factors involved was also studied in select tissues.

To this end, we bred 40 female AHR knockout (AHRKO) and wild-type (WT) mice (derived from C57BL/6J×AHR-/-) that were fed either a standard diet (SD; 10% of energy from fat) or a high-fat diet (HFD; 60% of energy from fat) and randomly assigned to four groups: WT-SD, AHRKO-SD, WT-HFD, and AHRKO-HFD. All diets were purchased from Research Diets (USA), and exposure lasted for 24 weeks. During weeks 16-17, the mice were subjected to glucose and insulin tolerance tests (GTT and ITT, respectively), and during weeks 18–20, their energy expenditure was determined using indirect calorimetry. Whole-body composition measurements were performed using a mini-spec analyzer. Upon study termination, total RNA was extracted from the relevant tissues and reverse-transcribed for RT-qPCR analysis.

At termination, the WT-HFD group (~40 g) weighed significantly more than the AHRKO-HFD (~33 g) and SD-fed groups (~22 g each). This pattern was reflected in the percentage of total body fat concentration, as well as the relative weight of the periovarian fat pad. Relative liver weight was lower in AHRKO than in WT mice and was further reduced by HFD feeding in both genotypes. Surprisingly, the relative weight of the brown adipose tissue (BAT) did not differ significantly among the groups. As expected, Cyp1a1 mRNA abundance (an index of AHR activity) in the liver was significantly higher in WT vs. AHRKO mice with diet showing no modulatory effect on the levels. However, the same was true for hepatic peroxisome proliferator-activated protein α (Ppara), a major regulator of oxidative metabolism, and BAT Ucp1 (uncoupling protein 1), which encodes the critical uncoupling protein of oxidative phosphorylation in BAT. The analysis of data obtained from indirect calorimetry and ITT/GTT is still ongoing, and the results will be presented at the meeting.

The present study substantiates the view that AHR deficiency can protect against excessive body weight gain in HFD-fed mice, suggesting a facilitating role for it in the development of obesity. The data from our ongoing analyses may help unveil the underlying biochemical mechanisms.

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LP-55
Characterization and quantification of fine and ultrafine particles from ilmenite smelting process

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Aim: Characterize fine and ultrafine fractions during the smelting process of Ilmenite ore.

Problem: In 2021, ACGIH adopted exposure limits (TLV-TWA) of 2.5 mg/m^3 for fine-scale TiO_2 and 0.2 mg/m^3 for nanoscale TiO_2. Both TLVs are expressed as respirable particulate matter.

Method: An aerosol sampling project was carried out to assess titanium (Ti) concentrations in the air of a smelter processing ilmenite.

Three site where sampled: area 1 - conveyor, area 2 - reduction furnaces, area 3 - slag processing representing the different stage of the product through processing.

We used different methodologies to capture the fine (respirable) fraction by using a cyclone (SKC Inc.) and a Particlever (ITGA, France) and the ultrafine fraction by using a SIOUTAS cascade impactor (SKC Inc.). Gravimetric analysis, metal determination analysis, and electron microscopy were conducted to characterize aerosols.

Results: Respirable dust concentrations ranged from 1.78 mg/m^3 to 2.15 mg/m^3 (area 1), 1.59 mg/m^3 to 6.59 mg/m^3 (area 2) and 0.66 mg/m^3 to 1.38 mg/m^3 (sector 3). Elemental titanium concentrations ranged from 0.03 mg/m^3 to 0.2 mg/m^3 (sector 1), 0.03 mg/m^3 to 0.27 mg/m^3 (sector 2) and 0.1 mg/m^3 to 0.31 mg/m^3 (sector 3), which represents a proportion between 0.6% and 16.3% of the dust sampled in the finest fraction (<250 nm). Microscopy analysis showed the presence of non spherical particles ranging from 250 nm to 10 µm containing Titanium and Iron. Ultrafine particles (less than 100 nm) were present but are agglomerated to larger particles and their shape and composition did not correspond to those expected for ultrafine TiO_2 particles.

Conclusion: Results suggest that processing ilmenite ore generate significant fine respirable dust containing Titanium. Considering a stoichiometric factor of 1.67 to express the TiO_2 concentrations, the reported concentrations of elemental titanium remain below the ACGIH recommendations.

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LP-56
Toxicological effects of 6PPD in *Caenorhabditis elegans*

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N-(1,3-Dimethylbutyl)-N'-phenyl-p-phenylenediamine (6PPD) is a synthetic antioxidant commonly used in rubber-based products, such as tires. The release of 6PPD into the environment during tire wear has been shown to pose a threat to wild salmon populations. However, its underlying action mechanism is poorly understood. Here, we evaluated the potential toxicity and targets of 6PPD using the nematode *C. elegans* as an *in vivo* model. Exposure to 0.5 mM 6PPD in *C. elegans* resulted in various adverse effects, including delayed development, decreased body growth, and reduced reproduction. Furthermore, 6PPD exposure negatively impacted healthspan parameters, such as body motility and stress tolerance, ultimately leading to a shortened lifespan. Notably, 6PPD-exposed *C. elegans* exhibited disrupted mitochondrial function, characterized by reduced mitochondrial membrane potential, lower oxygen consumption, diminished ATP levels, and decreased reactive oxygen species (ROS). 6PPD exposure influences the activity of SKN-1/Nrf2, a key transcription factor involved in stress response and longevity. Loss of SKN-1 attenuated the reductions in lifespan and tolerance against paraquat, but not mitochondrial disturbance suggesting the involvement of SKN-1/Nrf2 in mediating the toxic effects of 6PPD. Taken together, our findings suggested the detrimental impact of 6PPD on developmental processes, overall health, and aging *in vivo*. Furthermore, our study identifies mitochondria as a key target organelle affected by 6PPD exposure. The association with conserved SKN-1/Nrf signaling highlights a potential molecular mechanism underlying the toxic effects of 6PPD.

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Exposure to 5G and its effects on human physiological functions – a study from the GOLIAT European project

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Fifth generation (5G) of radio frequencies (RF) was implemented worldwide to offer faster connections among other benefits. The band of 3.4 GHz to 3.8 GHz was the first to be deployed in Europe. Higher frequencies up to 26 GHz will be later introduced. However, the health impacts of 5G networks are under speculation since the published studies tackling this subject are limited and likely don't represent the current regulatory and safety limits imposed by health organizations. Accordingly, this project seeks to explore the electrical brain activity along with other parameters of the autonomous nervous system (ANS) of healthy human participants when exposed to a generated 3.5 GHz frequency within the current outdoor exposure levels.

For this purpose, 34 to 44 young adults were included under strict criteria. They partook into two blinded, and randomised sessions, containing a baseline and a post-exposure period with no RF. Both were separated by either a "genuine" or a "sham" exposure phase. The frequencies were pulse-modulated and antenna-emitted in an electrically shielded and dim-lit room. Electroencephalograms (EEGs), Electrocardiograms (ECGs), and body temperature were continuously acquired in a resting awake and seated position. In addition, electrodermal activity (EDA) was also recorded during repeated 150 second vocal stimuli throughout the sessions. Moreover, salivary samples were collected before and after each exposure period to assess the stress levels in cortisol, alpha-amylase, and chromogranin-A biomarkers.

According to our data analysis, 3.5 GHz exposure did not reveal any significant differences in the explored ANS parameters. Therefore, according to our study conditions, we conclude that this frequency band do not seem to have an impact on the physiological functions studied. However, longer exposure periods and higher 5G frequencies are still to be evaluated in the future.

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