

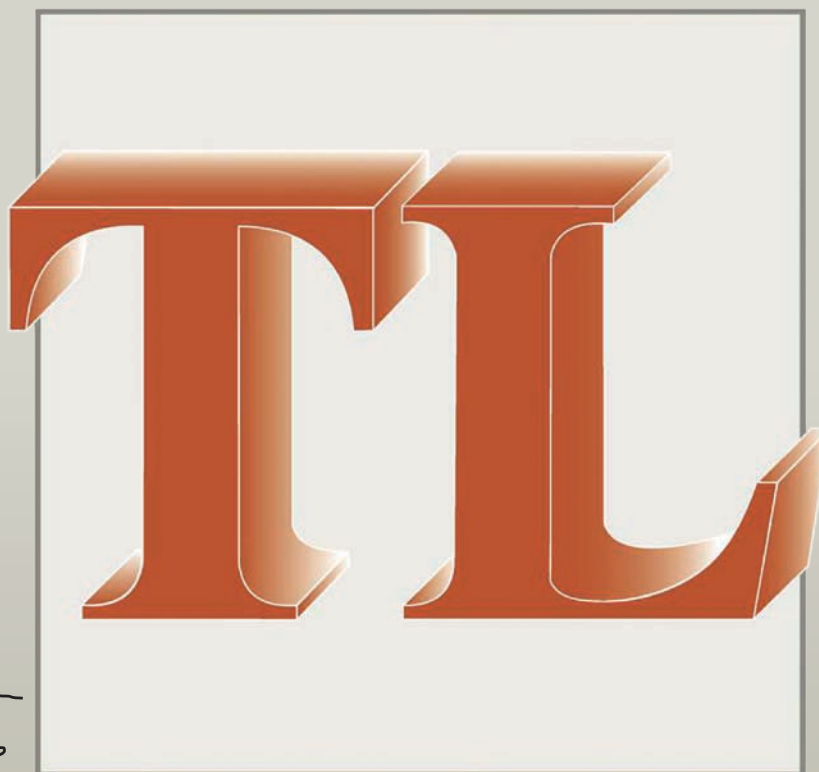
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Toxicology Letters

Official Journal of EUROTOX



EUROTOX **2021**
VIRTUAL CONGRESS
27 SEPTEMBER – 1 OCTOBER 2021

ABSTRACTS of the 56th Congress of the European Societies of Toxicology (EUROTOX 2021)
TOXICOLOGY OF THE NEXT GENERATION
virtual congress, 27th of September – 1st of October 2021

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Toxicology Letters serves as a multidisciplinary forum for research in all areas of toxicology. The prime aim is rapid publication of research letters with sufficient importance, novelty and breadth of interest. In addition to research letters, papers presenting hypotheses and commentaries addressing current issues of immediate interest to other investigators are invited. Mini-reviews in various areas of toxicology will also be published. A new feature is the provision of a forum for the discussion and interpretation of data published in the journal. Clinical, occupational and safety evaluation, legal, risk and hazard assessment, impact on man and environment studies of sufficient novelty to warrant rapid publication will be considered.

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*An International Journal for the Rapid Publication of Short Reports on all Aspects
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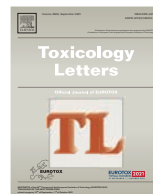
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Preface



Dear Friends and Colleagues

It is with great pleasure that we welcome you to the 2021 EUROTOX Congress. It has been a long journey to bring this Congress to fruition, but I believe that we have a fantastic mix of scientific and educational sessions, covering multiple interesting areas with something for everyone!

The programme has 6 Continuing Education courses, 28 scientific sessions and a diverse range of prize and award lectures including the Bo Holmstedt Memorial Lecture, 2 SOT Merit award lectures, the HESI lecture and, of course, our much loved EUROTOX/SOT Debate. We have incorporated 4 short oral communication sessions and there will be 10 industry sponsored events as well as a trade exhibition. There will also be more than 500 posters on display so there is no shortage of science or interest.

The Congress format will be a mix of pre-recorded and live sessions. The lectures will be pre-recorded with live question and answer sessions at the end of the prize lecture or the symposium. The debate will be live with Syril Pettit and Alan Boobis addressing the theme:

“Individualized Toxicity Is the Future of Risk Assessment”. The sessions will be available for registered participants for 8 weeks after the Congress ends.

At the start of the pandemic in 2020 the Executive Committee made the decision to postpone the 2020 Congress in Copenhagen to 2021 in the hope that we would be able to hold a face to face Congress this year. Unfortunately, the pandemic has continued, but the Executive Committee decided that we must hold a Congress in 2021 and so it had to be a virtual one. This was a disappointment to the Local Organising Committee in Denmark, but we have managed to secure the Tivoli Conference venue for 2024 so there will be a EUROTOX Congress in Copenhagen.

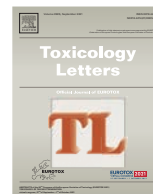
I would like to thank our sponsors and our PCO, KIT Group, for their help in bringing this congress together and importantly the Executive Committee, particularly the Core EC Group, who have taken on the organisation of EUROTOX 2021 on your behalf.

EUROTOX 2021 will be a different Congress as it will be virtual, but we have worked hard to ensure that the scientific, educational and social interactions that make EUROTOX Congresses such a success are included this year.

We hope that you enjoy the Congress and look forward to welcoming you in September.

Professor Heather Wallace
President Committee

Felix Carvalho
Chair of Scientific Programme



Continuing Education Courses (CECs)

CEC01 | Thyroid hormones, brain development and toxicity testing

CEC01-01

Thyroid Hormone Action and Disruption During Development: pregnancy, brain and rat versus human

*B.A. Demeneix

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Most brain developmental processes require thyroid hormone (TH), including proliferation, neuronal versus glial fate determination, differentiation, synaptogenesis and myelination.

The last decades have witnessed major insights into the molecular controls determining thyroid hormone availability and action in the brain. More recent findings include the presence of specific transporters across cell membranes TH transporters (THT) and the blood brain barrier, increased knowledge on the role of deiodinases activating or inactivating TH and the requirement for differential modulation of TH availability from the earliest stages of neurogenesis.

Recent epidemiological data show that maternal levels of TH during the first trimester of pregnancy in humans to be associated with IQ and brain structure in offspring^[1]. This finding echoes the importance of TH for early neurogenesis known from non-mammalian vertebrates such as birds, amphibians and fish. In these vertebrates, TH is laid down in the egg yolk. In humans, maternal supplies ensure the neuronal development until the foetal thyroid is fully functional.

A number of factors in TH signalling and metabolism between rodents and humans could underlie their different sensitivities to TH disruption. For instance, the half-life of thyroxine (T4) is often cited, although the half-life of tri-iodothyronine (T3), the biologically active form, is equivalent. Similarly, THTs are different, as is certain aspects of brain development.

In parallel to increased understanding of mechanisms controlling tissue availability of TH levels within strict physiological limits, other research has shown how vulnerable each level of TH signalling and action is to endocrine disrupting chemicals (EDCs). Experimental and epidemiological data suggest that interference with TH orchestration of human brain development could be implicated in the increased incidence of neurodevelopmental disease, such as autism spectrum disorder, as well as in significant IQ loss at a population level^[2]. Our recent results carried out in collaboration with the EDC-mix risk con-

sortium (<http://edcmixrisk.ki.se>) allow epidemiological data on prenatal exposure to different mixtures be tested on multiple models (Zebrafish, Xenopus, human brain organoids). The results underline how exposure to EDCs, including thyroid disrupting chemicals, during the prenatal period are associated with language delay in offspring.

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CEC01-02

Low thyroid hormone during pregnancy and consequences for child neurological development

*P. Taylor

Cardiff University, Thyroid Research Group, Cardiff, UK

The profound cognitive impairment displayed in children with congenital hypothyroidism or those born to mothers with severe iodine deficiency or substantial hypothyroidism due illustrates the essential role of thyroid hormones, in brain development.

The fetal thyroid gland is not fully functional until around week 18–20 of pregnancy, therefore neuronal migration and other critical brain development is largely dependent on the supply of maternal thyroid hormone. An additional challenge is that pregnancy imposes increased demands on the thyroid axis and women with previously acceptable thyroid function may fail to meet this increased demand.

This talk will summarize the associations observed between sub-optimal thyroid function (overt hypothyroidism, subclinical hypothyroidism and isolated hypothyroxinemia) during pregnancy, and offspring neurological development in terms of both IQ and behaviour. It will also explore the implications of two large clinical trials, the Controlled Antenatal Thyroid Screening study^[1] and the study by Casey et al^[2]. Follow on work from the CATS study has revealed that excess thyroid hormone replacement had no deleterious effect on IQ but did potentially have adverse implications with regard to ADHD behaviour^[3]. More recent research has also highlighted that modest changes in maternal thyroid function during pregnancy are associated in detectable changes in offspring cortical brain volumes.

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CEC01-03

Safeguarding the thyroid system – developing an *in vitro* testing battery

*S. Munn

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The European Commission's Chemicals Strategy for Sustainability (CSS) published in October 2020 aims to protect the public health and the environment from the most critical hazards posed by manufactured chemicals. The CSS is an important step towards the Commission's Zero Pollution Ambition for a toxic-free environment, as set out in the European Green Deal.

Endocrine disruption, including thyroid disruption, is considered to be one of these critical hazards and the specific actions proposed in the strategy include the development of hazard classes for EDCs under the Classification, Labelling and Packaging Regulation strengthening the information requirements under REACH and accelerating the development and uptake of methods to generate this information. Uptake relates to regulatory acceptance and acceptance translates into confidence that the method is reliable and has a predictive value, to this end validation of both aspects is an important aspect of regulatory acceptance.

In order to demonstrate that an adverse effect is the consequence of disruption of the endocrine system mechanistic methods are required to investigate the potential of a chemical to interfere with hormone production, action and clearance. The OECD's conceptual framework for the testing and assessment of endocrine disruptors has focused on the validation of methods for the detection of interference with the estrogenic, androgenic, thyroid and steroidogenesis (EATS) pathways. However, there are still important gaps in the test methods, and in particular, *in vitro* methods for the identification of thyroid disruptors are still lacking. To fill this gap, the EU's Reference Laboratory for alternatives to animal testing (EURL ECVAM) is coordinating the validation of a set of mechanistically informative alternative methods focusing on thyroid disruption in collaboration with a network of EU validation laboratories (EU NETVAL) and the developers of the methods. Candidate methods cover the different ways in which chemicals may interact with the thyroid hormone axis, including central regulation, synthesis, transport and distribution in the serum, metabolism and excretion as well as cellular uptake and intracellular (de)activation. The potential uptake of the methods within the context of the implementation of the criteria for ED identification in the EU and actions proposed under the CSS will be discussed.

CEC01-04

US and EU regulatory conundrums and practical considerations for developmental thyroid toxicity assessments

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Thyroid hormones (TH) are critical for normal human neurodevelopment, but the magnitude and pattern of changes in serum THs that disrupt TH-dependent developmental processes in brain remains

uncertain. Evidence that a chemical might target the thyroid gland directly or alter TH levels indirectly have elicited regulatory actions and further testing that can vary depending on the regulatory context (e.g. different legislation, agency or exposures). In the US, developmental thyroid system disruption (DTSD) studies (specifically the comparative thyroid toxicity study) are triggered in lieu of a developmental neurotoxicity (DNT) study. In the EU, REACH and recent 2018 ECHA/EFSA guidance favours an extended one-generation reproduction study (EOGRS), but with DTSD or DNT studies sometimes being an acceptable substitute; and comparative liver enzyme induction assays also helpful. In the EU, findings that lead to a chemical being labeled as an "endocrine disruptor" can lead to loss of registration despite adequate margins of exposure for TH changes. In contrast, US EPA focuses on risk assessment based on exposure and TH changes. These differences in regulatory framework should be considered when designing studies for submission to both the EU and US. Several OECD and EPA guideline studies include thyroid assessments in pregnant or immature rodents, however assays validated for adult TH levels cannot be assumed to have adequate dynamic range to detect decreases in TH in the fetus or young pup. In general, laboratories can achieve adequately sensitive TH quantitation levels for most ages, although issues remain for lower levels of serum TH and TSH in fetuses and postnatal day 4 pups. Recommendations for best practices for control of environmental and experimental factors that could influence variability and absolute concentrations of TH and TSH especially in pups and fetuses should be considered in designing studies. Benchmark dose analysis of T3, T4 and TSH should not be based on default 5 or 10% response level without consideration of inter-animal variability in specific TH measures. Although the concurrent control is the most important experimental control data, historical control data (HCD) from the laboratory can be useful to evaluate the laboratory's competence and quality control to enhance interpretation of study results. However, HCD is only useful in this context if studies are conducted under similar experimental conditions with means and standard deviation are available for each. Lastly, despite the assessment of serum TH in numerous study types, uncertainty remains in the magnitude of maternal/fetal/pup change required to induce DNT.

CEC01-06

Current *in vivo* guideline testing for endocrine disruption: practical issues and interpretation challenges with focus on thyroid hormones.

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One important type of endocrine disruption is by thyroid dysfunction. Early-life neurological impairments are often the primary human health concern for environmental chemicals that induce thyroid dysfunction, based on experimental and epidemiologic evidence showing a direct association between low maternal T4 levels during pregnancy and early postnatal development and neurodevelopmental outcomes in offspring.

This issue is presently very poorly dealt with in guideline studies, as these are not designed to fully assess downstream effects on the developing brain, causing neurotoxicity. This has led scientists in the field of neurotoxicology and thyroid disruption to suggest that new endpoints, which are more sensitive to perinatal thyroid disruption, should be added to these guidelines in order to better assess adverse effects on neurodevelopment. However, currently only thyroid hormone measurements are included.

In the absence of definitive biomarkers of altered neurodevelopment, general agreement exists that changes in thyroid hormone (TH) levels are appropriate starting points for risk assessment to pro-

tect against potential downstream effects on neurodevelopment. Disagreement persists regarding the magnitude of TH perturbation that could result in developmental hazard classification and labeling, which does not take into consideration dose response and exposure information.

However, currently toxicology studies according to OECD guidelines are being performed in which thyroid hormone measurements are included, and these results need to be interpreted and evaluated for its relevance for human risk assessment based on experience and scientific knowledge that is now lacking. Moreover, in June 2018 a guidance for the identification of endocrine disruptors was published by ECHA and EFSA. This guidance mentions that a decrease in T4 (total or free) in the absence of adverse histological changes should act as a trigger for further studies of F1 generation depending on the other information available. Consequently, this results in an imperative need for the different stakeholders (i.e. regulators, industry and CROs) to align how these data should be evaluated and interpreted for endocrine disrupting properties. The objective of this presentation is to provide an up-to-date overview of aspects related to TH assessments in standard toxicology regulatory testing. Implementing measurements of plasma thyroid hormone levels in OECD TG 408, 414, 421, 422 and 443 studies at our laboratory revealed important practical aspects and other knowledge that would be valuable for other laboratories. The presentation will include an overview of the current testing paradigm, practical aspects, interpretation challenges and items that need improvement.

CEC02 | Advances in conducting systematic reviews for chemical assessment: automation, uncertainty assessment and synthesis

CEC02-01

How can systematic review methods help now and also prepare for future decision making on mechanistic data?

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Systematic review methodology is a rigorous approach to answering a research question via review of existing evidence with a process that maximizes transparency and minimizes bias in identifying, selecting, critically assessing, and integrating evidence relevant to answer that question. The clear advantages of methodological rigor and transparency associated with these methods has resulted in systematic review approaches becoming the standard for literature-based reviews across many research fields including evidence-based medicine, toxicology, environmental health and, more recently, chemical risk assessment. The characterization of environmental hazards requires the integration of evidence on potential health effects associated with environmental exposures across broad evidence bases including studies in humans, experimental animals, and *in vitro* or mechanistic data. This presentation will provide an overview of the major steps in systematic review from problem formulation and protocol development, literature search and screening, data extraction, evidence appraisal, and evidence integration including uncertainty evaluation in hazard and risk assessment. Finally, the presentation will highlight opportunities and challenges these methods add to the chemical risk assessment process as the field increasingly shifts from incorporating results of alternative approaches (e.g., *in silico*, *in chemico*, and *in vitro* studies) toward reaching decisions on mechanistic data alone.

CEC02-02

Automated and Semi-Automated Approaches for Literature Searching, Screening, and Data Extraction for Systematic Reviews in Environmental Health

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Systematic review is a comprehensive, objective and transparent multistep process to identify, screen and synthesize primary scientific literature for reaching evidence-based conclusions on specific research questions. Across the field of toxicology, efforts to apply systematic review methods are gaining momentum highlighted by regulatory requirements that have been instituted globally to conduct systematic review in support of safety assessments of environmental chemicals and foods. Conducting a systematic review is a time-consuming and resource intensive process. Literature-based evaluations of environmental exposures and health outcomes can require consideration of large numbers of studies across multiple evidence streams (e.g., human, animal, *in vitro* and mechanistic evidence). The required level of effort in terms of time and resources depends on the size of the evidence base and complexity of the question, but on average, systematic reviews take more than 1,000 person hours to complete and can cost over \$100,000 USD. Similarly, for environmental health questions, the level of effort varies according to the number of questions or health outcomes being addressed, exposures of interest, and complexity of data to be extracted, and the amount of literature to evaluate. Furthermore, by the time a systematic review is published, those data can be outdated, and thus perhaps less accurate and not useful for rapid decision-making. Many of the steps of the systematic review process have the potential to utilize automated or semi-automated approaches. This lecture will introduce the concept of automation and semi-automation in the various steps of the systematic review process and highlight their utility and limitations in reducing the time and resources required to conduct a systematic review. Given the increasing volume of published studies, automation and semi-automation of labor-intensive steps in the review process, has great potential to improve the speed of systematic review and reduce the workload and resources required without comprising the rigor and transparency that is critical to the method.

CEC02-03

Synthesis, certainty (GRADE), and qualitative integration of human, animal, and mechanistic data

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Once the data of all relevant individual studies have been extracted and the studies have been appraised, the next systematic review step is the synthesis of the evidence. As the populations studied are often heterogeneous, possibly comprising human, animal, *in vitro* and *in silico* studies, a structured approach to evidence integration is required. Within populations, evidence can be summarised across studies that have similar experimental designs or that used the same species strains or similar cell types. This summary will facilitate the subsequent integration of more heterogeneous evidence within populations and finally across populations.

For the body of evidence from similar studies, summary tables are compiled that list inter alia important outcomes, effect size measurements and sample sizes per outcome. In addition, the certainty in the body of evidence is assessed per outcome. This assessment, traditionally often conducted in a weight-of-evidence (WoE) approach, can for example be conducted using the approach to grading confidence of the

GRADE (Grading of Recommendations Assessment, Development and Evaluation) working group, who started to explore the application in the context of environmental and occupational health questions.

The confidence in bodies of evidence is determined by qualitatively by integrating risk-of-bias, (in-)directness, (in-)consistency, (im-)precision, publication bias as well as large effect sizes, dose-response relationship and effect-reducing confounding. These factors can be used to either downgrade or upgrade the confidence. While most of these factors are readily applicable to toxicological questions, some require adaptation, especially the concept of (in-)directness, as relevant evidence, e.g. animal and *in vitro* studies, is often not generated in the population of interest, i.e. humans.

The confidence assessment for each population will ultimately inform the integration of evidence across populations. In addition, first approaches also make use of the concepts of (in-)directness and (in-)consistency for this final integration step. However, the integration of bodies of evidence across population remains a field of active research, especially as new approach methodologies will provide an increasing amount of evidence in the future.

CEC02-04

Quantitative evidence integration supporting toxicity value development and characterization of uncertainty

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The increased use of evidence-based methodologies, such as systematic review, provide a platform for quantitative integration and uncertainty evaluation in hazard and risk assessment. Though an area of significant interest, methods and applications remain limited. The objective of this presentation is to provide a survey of the state of the science on both qualitative and quantitative methods for integrating evidence relative to assessment of hazard potential, derivation of toxicity values, and corresponding uncertainty informed from data generated as part of the systematic review process. Emphasis will be placed on quantitative approaches, examples of which include: (1) integration of heterogeneous mechanistic data associated with assessing cancer potential via the key characteristics of carcinogens, (2) meta-regression to quantitatively integrate dose-response data from across *in vivo* animal studies of a single endpoint of interest, (3) Bayesian approaches to assessing dose response as well as applying uncertainty factors, to quantify the uncertainties in extrapolating a point of departure (derived from dose-response data) to a health-protective reference dose, and, (4) consideration of bias-adjusted analyses in addressing uncertainty. Utilities and challenges in application of these quantitative techniques provide enhanced opportunities to characterize hazard, risk, and uncertainty which ultimately provide the risk manager important information for decision making supported by evidence-based techniques.

CEC02-05

Conduct and reporting standards for systematic reviews in toxicology and risk assessment

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Systematic reviews (SRs) are complex, interdisciplinary research projects that are challenging to conduct successfully. In response, many tools and guidance documents have been developed to help research teams produce high-quality SRs. Faced with so many choices, it can be confusing for SR authors to decide which tools are optimal for their specific context and how best to use them.

In this lecture by a specialist Systematic Reviews Editor, participants will learn about conduct and reporting standards which have been developed or adapted for the specific needs of toxicology and environmental health (EH) SRs. These include the new COSTER recommendations for conduct of EH SRs^[1], the PRISMA and ROSES standards for reporting SR projects^[2,3], and the CREST_Triage tool for screening the scientific quality of EH SRs^[4].

Participants will be shown how conduct and reporting standards, in combination with critical appraisal tools, can be combined into a readily-implementable quality management workflow for EH SR projects. Such a workflow should make a significant difference to the success of a EH SR project and maximise chances of publication of any resulting manuscripts.

References

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CEC03 | Lessons learned and future directions for toxicology in water safety and security

CEC03-01

Water bodies and frame work for protection

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Water Security is to safeguard protection against water-borne pollution and water-related disasters, and for preserving ecosystems. The Lessons learned from fertilizers as stressors for water quality are that it is essential to understand the impact of pollution on water bodies in term of time lines and regional parameters. The serious environmental problems caused by the excessive release of reactive nitrogen have troubled the water resource management sector for a long time. Despite some successes in the reduction of nitrogen emissions, especially from point sources, groundwater bodies, surface waters and marine waters not only in Germany are still highly affected by nitrogen inputs. Most pollution finds its way into the surface waters via pathways that are mainly fed by agriculture such as groundwater, drainage water, and run-off.

The consequences of elevated nitrogen load from rivers into marine water at coastal line lead to an increased production of phytoplankton and the growth of short-lived macroalgae (algal bloom). A high density of phytoplankton reduces the water transparency and the reduced penetration of sunlight limits the depth to which macrophytes and sea grasses can grow. One of the most striking effects of excessive levels of nutrients is the formation of hypoxic or anoxic zones in deep water layers in which higher organisms are unable to survive. The nitrate inputs into groundwater are usually the result of leaching from the soil and for bodies of groundwater near the surface. Farming is the main cause of the nitrate inputs. In general, the nitrate pollution is spread over many areas, but often areas with a high density in livestock farming are hot spots in contamination.

Even very low input levels of reactive nitrogen can have a deleterious effect on certain species and ecosystems, because processes, such as acidification, nitrogen loading and species loss are irreversible or only reversible over long periods of time. Limit values for the protection of human health are woefully inadequate for protecting more sensitive species and ecosystems.

Water Framework Directive (WFD) sets the ambitious goal of attaining “good status” for Europe’s rivers, lakes, groundwater bodies and coastal waters already by 2015. The regulatory instrumentation are the nitrate directive, amendments on fertilizer ordinance and the revision of the National Emission Ceilings Directive.

It is urgent to get an integrated view on driving forces and sources for pollution, description of parameters for survey, impact on human and environmental health and target sites for effects. Data are needed from surveys, environmental measurements or biomarker assessments to characterize exposure. Biomarkers are particularly useful for integrating these concepts as they may include markers of exposure, susceptibility and early biological effects.

CEC03-02

Contamination pattern by pesticides in water

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Water is the dominant and most essential element of life. Specifically, clean water is essential for human health, wildlife, and a balanced environment. A great number of studies indicate the widespread occurrence of pesticides in several water bodies. Pesticides with different type such as insecticides, herbicides and fungicides were considered to be common pesticides found in the environment.

Pesticides are applied on several fields, gardens, parks, and other places, a percentage of the chemicals end up as runoff. Afterwards, this runoff moves in streams, rivers, lakes, oceans and finally in drinking water. The concentrations of pesticides in water varies greatly, both geographically and seasonally, based on land use and pesticide use patterns. Furthermore, pesticides have become universal contaminants that found in all segments of the environment as a result the creation of many hazards to the environment and human health. Pesticide contamination in fresh water has emerged an important environmental problem in the last few decades. That fact is causing concern with respect to long-term and low dose effects of pesticides on public health as well as in environment and in non-target species. Herbicides are the most frequently found type of pesticide in streams and groundwater in agricultural areas. On the other hand, in urban areas, there is a greater predominance of insecticides in streams than in agricultural areas. Also, pesticide concentrations vary yearly, based on variations in rainfall, and seasonally, based on agricultural practices. Today, agriculture has to deal with population growth, food security, health risks from pesticides, pesticide resistance, degradation of the natural environment and climate change. Furthermore, there are multiple questions that rise about the exposure to mixtures of pollutants with possible cumulative, additive or/and synergistic effects. Recently published papers on RLRS – Real Life Risk Simulation – highlighted the issue of mixtures toxicology at low dose and in long term in Risk Assessment.

CEC03-03

Biodiversity and the ecological integrity of freshwater systems

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The ecological integrity of surface freshwater systems on which humankind depends for water provision is intimately connected to their biodiversity. From a functional perspective, habitat properties influence organismal performance with consequences to population dynamics and finally, community structure of which diversity is one of the defining features. Conversely, biodiversity (whether particular species, functional groups, or diversity per se) influences a range of ecosystem services and as such mediate water properties and their variability – and therefore predictability – over time. Thus, one cannot overstate the importance of recognizing the bidirectional interaction between freshwater provision as an ecosystem service and the maintenance of freshwater biodiversity.

On another line, the assessment of conservation strategies in watersheds, and of water quality in particular, depends on monitoring. Once again, we argue that biodiversity is a most reasonable long-term indicator of freshwater integrity because (i) the selection of the ‘most sensitive indicator species’ to define water quality criteria is a meaningless quest both because species vary broadly in their sensitivities to a given stressor and because species sensitivity ranks may not be correlated across stressors; (ii) the consideration of one or few species in defining water quality criteria is an abstraction to the complexity of ecological communities in which species are embedded, so that the consequences of a stressor on a given focal species may be both direct or indirectly transmitted through other species in the food web; and (iii) different species integrate responses to abiotic and biotic stressors at varying spatial and temporal scales and therefore provide complementary sources of information.

CEC03-04

No abstract has been submitted.

CEC03-05

Dissipative use of lead – a future risk for groundwater

**T. Schupp*

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Lead (Pb) and Pb compounds have been used since ancient times, e.g. in water pipes, roofing, as pigments in paints, in ammunition, shielding material against radiation and as weighting material. Over the last decades, measures were undertaken to reduce Pb exposure and these regulatory activities have resulted in reduced Pb immissions into the environment. As a result of reduced Pb burden, blood Pb concentrations of the general population decreased. But Pb is highly toxic for humans and the remaining immissions still raises major concerns due to its presence in food. 10 µg per person per day is considered as the tolerable daily intake (TDI) based on data on developmental neurotoxicity.

To gain more insight about the intermediate and long-term consequences of Pb emissions, we established a mathematical model that considers input from fertilizer, ammunition, deposition from air, uptake of Pb by crops, and wash-out to simulate the resulting Pb concentrations in soil over extended period. Simulating current farming scenarios, a new equilibrium concentration of Pb in soil 5 mg Pb/kg represents a critical concentration in soil that should not be exceeded because this level will lead to an intake of approximately 10 µg Pb per person per day by the consumption of agricultural products.

Pb spread on soil may contribute to oral Pb exposure via drinking water and this potential additional source of Pb exposure can reduce

the margin of safety of Pb taken up by food. Pb ions in the surface water will be in equilibrium with its poorly soluble salts, with adsorbents like clay minerals, with iron and manganese oxides and with chelating ligands in soil organic material. A net water flow from the soil surface to groundwater levels will finally cause a Pb burden to groundwater.

A “Pb-soil-chromatography” peak from contaminated soil will eventually reach groundwater layers. A long time may pass before the Pb-peak in infiltrating water arrives at critical ground water layers. Areas with a previously comparatively high input of Pb into soil would call for a long-term monitoring of the groundwater even if more recent Pb deposition is deemed to be of low relevance.

In summary, we propose as efficient measures to reduce Pb input into agricultural soil to lower the Pb content of compost and to use alternatives to Pb ammunition for hunting.

Full paper “Long-term simulation of lead concentrations in agricultural soils in relation to human adverse health effects” published by Members of Beratungskommission, German Society of Toxicology
DOI: 10.1007/s00204-020-02762-x.

CEC03-06

Metagenomic Approaches for Surveillance and Testing Water

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One of the new opportunities for Water Security is to utilize the new “omic” tools to ensure human and ecosystem protection against water-borne pollution and water-related disasters. Systems based approaches allow for integrated assessments and now link methodologically both chemical and microbial evaluations. This talk will discuss basic ontologies that allow for such integrated assessments and to discuss use of global platforms that help to facilitate this integration. One Health platforms further accentuate these integrations and this is especially true with the recent Tripartite Risk Assessment agreements that link FAO, WHO and One Health Initiatives (OIE). A case study approach will be used to examine three applications: Antibiotic resistance, Microbial assessment and Chemical pollution in estuarine settings. Hallmarks for each of these assessments will be the use of common risk assessment framing, use of cyber tools available for on-line access, and linkage with decision analytic options. Multi-disciplinary risk framed approaches are shown to be essential for the use and application of these tools.

CEC03-07

Bromate in bathing water – a carcinogenic risk?

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Swimming pool water needs disinfection e.g. by ozonation, chlorination or brominated disinfectants. During disinfection processes chlorine, hypochlorites and ozone may oxidize the bromide (Br⁻) to bromine (Br₂), which subsequently may disproportionate to bromate. Therefore bromate is a typical byproduct and counts for substantial human health risks because it is classified as 1B carcinogen. Our study a) re-evaluated the carcinogenic mode of action of bromate, b) derived reliable levels of exposure by all routes under various scenarios of swimming, and finally deduced from this information cancer risk-related bromate concentrations in swimming pool water. Quality of the studies on genotoxicity and carcinogenicity were as-

essed by Klimisch criteria and SciRAP tool, respectively. Benchmark dose (BMD) modelling was performed using the modelling average mode in BMDS 3.1 and PROAST 66.40, 66.7 and 69. Different target groups (infants/toddlers, children and adults) and exposure scenarios (recreational, sportive swimmers, top athletes) were considered for oral, inhalation and dermal exposure. Exposure was calculated by frequency of swimming events and duration in water. For illustration, cancer risk-related bromate concentrations in pool water were calculated for different target groups taking into account their exposure, using the hBMDL10 and a cancer risk of 1 in 100,000.

There is convincing evidence from a multitude of studies that bromate induces oxidative DNA damage and acts as a clastogen *in vitro* and *in vivo*. Since statistical modeling of the available genotox data is compatible with both linear as well as non-linear dose-response relationships, up to now bromate should be considered as a non-threshold carcinogen. BMD modelling with model averaging for renal cancer studies resulted in a median hBMDL10 of 0.65 mg BrO₃⁻/kg bw per day.

Evaluation in different age and activity groups revealed that top athletes had the highest exposure, followed by sportively active children, sportively active adults, infants and toddlers, children and adults. The predominant route of exposure was oral (73–98%, by swallowing water, followed by the dermal route (2–27%); inhalation route was insignificant (<0.5%).

Accepting the same risk level for all population groups results in different guidance values due to the large variation in exposure. For example, for an additional risk of 1 in 100,000, the bromate concentrations would range between 0.011 for top athletes, 0.015 for sportive children and 2.1 mg/l for adults.

The full paper „A toxicological risk assessment for bromate in swimming pool water” is prepared for publication by Members of Beratungskommission, German Society of Toxicology.

CEC04 | Inflammation as a mediator of toxic responses

CEC04-01

Think beyond cytotoxicity:

Does toxic-induced immunogenic cell death (ICD) contribute to lung inflammation and serve as predictive biomarker?

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More than 20 years ago, the immunologist Polly Matzinger elaborated a revolutionary concept in immunology emphasizing the crucial role of damaged cells during the immune responses against self- and non-self-entities. In this striking danger model, endogenous signals (now termed Damage-Associated Molecular Patterns or DAMP) released by immunogenic cell death (ICD) enhance activation of inflammatory cells against microorganisms and cancer cells. Conversely, excessive ICD result in immune-related pathological scenarios such as autoimmune diseases. The applicability of Matzinger's model in fundamental and applied toxicology remains to be established. The rapid advances in ICD and DAMP release and sensing present exciting opportunities to test if adverse lung inflammatory effects induced by inhaled particles derive from their immunogenic cytotoxicity. These possible interconnected links will be extremely relevant in predictive lung toxicology.

CEC04-02**The multiple facets of skin inflammation: from direct toxic insult to specific immune responses***M. Pallardy*Université Paris-Saclay, INSERM UMR 996 Faculté Pharmacie, Châtenay-Malabry, France*

Skin inflammation is a sign of an immune response (innate or adaptive) in the body. Symptoms can include redness, heat, itching, sensitivity, and swelling. The cause or trigger of skin inflammation may be acute, such as a skin infection, chemical insult, or chronic, such as an autoimmune condition like psoriasis. Allergic mechanisms are often a cause of skin inflammation including Allergic Contact Dermatitis (ACD) and Atopic Dermatitis (AD). Skin inflammation can be the consequences of multiple agents from pathogens to chemicals.

Contact dermatitis (CD) is one of the most common inflammatory dermatological conditions and is caused by the exposure to exogenous substances that elicit an immune response resulting in inflammation in the skin. Categories of CD include ACD, photoallergic CD (PACD), irritant CD (ICD) and photoirritant CD (PICD, or phototoxic CD). ICD is caused by direct cellular toxicity leading to the inflammation and activation of the innate immune system, whereas ACD results from adaptive immunity involving innate immunity and T lymphocytes. Individuals who develop ACD acquire a specific immunity against the chemical to which they have been exposed.

ACD and ICD are both inflammation of the skin due to a contact with chemicals in the environment, but ICD is a non-specific skin response and is consequence of direct damage of the skin due to the irritating nature of the substance. Another difference between ICD and ACD is that, in ACD, symptoms do not appear after the first exposure, but rather usually within a few days after a re-exposure to the allergen.

CEC04-03**Innate immune inflammatory signaling in glial cells modulates chemical neurotoxicity***R. Tjalkens*Colorado State University, Department of Environmental and Radiological Health Sciences, Fort Collins, USA*

Risk for developing Alzheimer's and Parkinson's disease is associated with aging, genetics and exposure to environmental agents such as infectious pathogens and neurotoxic chemicals. Neuroinflammation represents a common mechanism of toxicity for each of these factors, resulting in inflammatory activation of microglia and astrocytes, the principal non-neuronal cells of the brain. Transition of these cell types from a trophic or supportive phenotype to a damaging inflammatory phenotype during neurotoxic stress promotes both aggregation of proteins such as amyloid beta and alpha-synuclein, as well as injury to neurons. Repeated exposure to infectious pathogens and other neurotoxic agents is therefore implicated in the onset and progression of prodromal and clinical neurodegenerative disease. One of the central molecular pathways regulating innate immune inflammatory signaling in glial cells is the transcription factor, nuclear factor kappa B (NF- κ B), which is activated by multiple cellular stress signals through the kinase complex, IKK2. Stimulation of NF- κ B in microglia and astrocytes activates coordinate expression of numerous inflammatory cytokines and chemokines, as well as reactive oxygen and nitrogen species and inflammatory lipid mediators. Neurotoxic chemical agents including heavy metals, pesticides and drugs of abuse can stimulate inflammatory activation of glial cells resulting in neuronal injury. Selective genetic and pharmacologic inhibition of NF- κ B in microglia and astrocytes can mitigate neuronal injury from these agents, suggesting that innate immune inflamma-

tory signaling in glial cells is critical to the progression of pathology resulting from exposure to these agents. Data from animal models of neurodegeneration are extremely informative in determining the role of inflammatory signaling in glial cells as a mediator of neuropathology relevant to chronic neurodegeneration.

CEC04-04**Evaluating cytokines in immunotoxicity testing***E. Corsini*University of Milan, Milan, Italy*

Cytokines are secreted proteins released by cells with specific effect on the interactions and communications between cells. Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action). Different cell types can secrete the same cytokine and a single cytokine can act on several different cell types (pleiotropy). In addition, cytokines may be redundant in their activity, and similar functions can be stimulated by different cytokines. They are usually produced in a cascade, as one cytokine stimulates its target cells to make additional cytokines. Finally, cytokines can act synergistically or antagonistically. They mediate all immunological and inflammatory reactions.

In the evaluation of the adverse effects on the immune system, cytokines provide important evidence to support or refute the biological plausibility of chemical-induced immunotoxicity. Therefore, their measurement represents an important tool that can be applied both to *in vivo* as well as to *in vitro* models. There are a multitude of examples on the use of cytokines as parameters in the assessment of immunotoxicity, both for immunosuppression and inappropriate immunostimulation, both in animals and human studies as well as in non-animal models. Examples will be provided in the presentation, as method available to assess cytokine production.

CEC05 | Nanotoxicology

CEC05-01**Genotoxicity of nanomaterials***J. Catalán, K. M. Siivola, K. Aimonen, G. Vales, H. Saarelainen, S. Suhonen, M. Hartikainen, H. Norppa*Finnish Institute of Occupational Health (FIOH), Helsinki, Finland*

A main safety concern related to nanomaterials is their possible genotoxicity. Genotoxicity describes the capacity of a chemical to produce genetic damage that, if it is not repaired, can lead to mutations and, eventually, to cancer. Therefore, every mutagen is considered to be potentially carcinogenic. Furthermore, mutagenicity is also involved in reproductive and developmental abnormalities.

Due to the important consequences to human health, mutagenicity is a hazard endpoint required in all product regulations (REACH, biocides, pharmaceuticals, medical devices, food additives, cosmetics, etc.). On the other hand, genotoxicity assessment at an early stage of innovation is highly advised as part of the safe-by-design strategies during product development. The mutagenicity of chemicals is usually evaluated on the basis of a battery of standard *in vitro* assays, which can be followed up by validated *in vivo* assays. However, it is presently not clear to which extent genotoxicity assays can be applied to test nanomaterials, and how well such data could be utilized in predicting the carcinogenicity of these materials. We will provide an overview on the limitations of genotoxicity assays and how much of

the available genotoxicity data on nanomaterials are relevant from the regulatory perspective. It is also unclear how different physico-chemical properties of nanomaterials can modulate their genotoxic potential. The effect of size, surface chemistry and biopersistence will be discussed.

Nanomaterials can be genotoxic through a primary mechanism, executed by the substance itself, or a secondary mechanism involving an inflammatory response. The primary mechanism can involve a direct interaction with the DNA, or an indirect effect mediated by other molecules, such as the induction of reactive oxygen species. Traditional *in vitro* genotoxicity tests are considered to detect primary genotoxicity, whereas *in vivo* assays are required to reveal secondary genotoxicity. Current genotoxicity assessment is based on identifying a substance as genotoxic or non-genotoxic, assuming that genotoxicity does not have a threshold value. Hence, no dose can be considered as 'safe'. However, recent findings in genetic toxicology are moving this paradigm forward to a more semi-quantitative approach, where a threshold mechanism of action (MoA) is assumed when genotoxicity is mediated by secondary mechanisms. Several *in vitro* co-culture systems are presently being developed and may provide a way of differentiating between primary and secondary MoAs. Finally, new approaches on genetic toxicology, aiming at reducing animal experiments while providing more mechanistical based information on the molecular changes involved in the induction of genotoxic effects, will be discussed.

CEC05-02

Nanomaterial-induced inflammation, acute phase response and risk of cardiovascular diseases

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Inhalation studies of insoluble nanomaterials show that they induce pulmonary inflammation that is predicted by total deposited surface area. Transcriptomics studies of mice show the induction of pulmonary acute phase response in parallel with the inflammatory response. Acute phase response is a well-established risk factor for cardiovascular disease. Pulmonary exposure to soluble and insoluble nanomaterials induce dose-dependent acute phase response.

The lecture will give an overview of the scientific evidence linking nanomaterial exposure to risk of cardiovascular disease, the biological mechanism of action of the acute phase response and provide examples of occupational biomonitoring studies showing correlation between particle exposure levels and systemic acute phase response. The pathway has been submitted as adverse outcome pathway AOP237 in Aopwiki.

CEC05-03

Toxicity of nanomaterials in the user phase

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Nanomaterials are increasingly used in different product groups due to improvement of characteristics of the products. The consumer is potentially exposed to nanomaterials in their final, intended use, i.e. when the nanomaterials are part of a matrix. Consumers may be exposed by inhalation, dermal exposure and oral intake. Occupational exposure may also occur in the end-of-life phase.

Based on different cases, this lecture will focus on how the toxicity of nanomaterials is affected when nanomaterials are part of a consumer matrix. Cases will include the toxicity of inhalation of dust obtained by sanding of paint and epoxy containing nanomaterials and dermal exposure due to use of nanomaterial containing sunscreen and other cosmetics. Because free nanomaterials may be liberated during the use phase, the hazard of pristine nanomaterials will also be discussed.

CEC05-04

In vitro-based high-throughput screening and toxicogenomics to support effective safety evaluation of engineered nanomaterials

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High-throughput screening (HTS) technology allows for rapid first-level safety evaluations of large numbers of engineered nanomaterials. A wide variety of cell culture-based, data-rich HTS approaches, including those that build on "omics", are in development to keep pace with the steadily increasing innovations that involve nanomaterials, and which have potential to efficiently serve for toxicity-based ranking, grouping, read across and prioritizations for deepened studies. Generation of large toxic mode of action (MoA)-informative data sets are coupled to the need for harmonized interpretation, integration, and data management approaches in order to modernize current risk assessment practices. This educational lecture will introduce recent state-of-the-art HTS testing methodologies which permit collective interpretation and visualization of diverse types of relevant data for nanomaterials, including from applying the US-EPA developed Toxicological Priority Index tool (ToxPi). Examples include combining basic toxicity indicators into scores as well as new strategies for assessment of direct and indirect (secondary) genotoxic mechanisms. Further concepts to be discussed include applying targeted or genome-wide transcriptomics data-driven predictions of toxic MoA coupled to Adverse Outcome Pathway-driven hypothesis-generation for guiding further testing needs. Finally, to be presented are current strategies towards making existing toxicity and safety-testing data "findable, accessible, interoperable and reusable" according to the FAIR principles.

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CEC06 | Toxicity assessment in drug development

CEC06-01

Challenging early target safety assessment strategies

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First target safety assessment in drug development is often based on published information on knockout mice. In some cases, the genetic deficiency may be associated with a severe, lethal phenotype. However, genetic full deficiency may not be representative of pharmacological inhibition. Related strategies on early target safety assessment are discussed based on two examples, for which the underlying previously published background information is summarized here. (i) Retinoic-acid-orphan-receptor-C (RORC) is a master regulator of Th17 cells, whose inhibition may offer an effective therapeutic principle for autoimmune diseases. However, genetic RORC deficiency in mice, while preventing autoimmunity, causes early lethality due to metastatic thymic T cell lymphomas. RORC^{-/-} mice are characterized by a lack of lymph nodes, Peyer's patches, and other lymphoid cell compartments. The thymi of RORC^{-/-} mice show specific aberrations, which occur prior to the development of T cell lymphoma. These include reduced total cell numbers, changes in CD4/CD8 representation, increased frequencies of thymocytes in the S/G2/M cell cycle phase, larger cell size, increased apoptosis in the cortex, and increased cortical/medullary ratio. RORC-deficient patients display immunological features concordant with RORC^{-/-} mice, including the absence of palpable axillary and cervical lymph nodes and reduced thymus size. (ii) Reducing the activity of sphingosine-1-phosphate (S1P) lyase has a protective effect in inflammation and may be effective in the treatment of autoimmune diseases. However, genetic S1P lyase-deficiency causes a severe, lethal phenotype associated with defects in multiple tissues. Partial restoration of S1P lyase (<10%) in human knock-in mice is sufficient to fully protect the mice from the lesions in many (not all were assessed/described for the human knock-in mice) of the tissues affected in fully S1P lyase-deficient mice.

CEC06-02

Phototoxicity of small molecules – from initial assessment to *in vivo* studies

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During this part of the CEC session 06 (Toxicity in drug development) I will go through the guidelines considered most applicable for the phototoxicity assessment of small molecules. Data needed for a phototoxicity assessment, studies available and timing of phototoxicity assessment(s) will be discussed.

This presentation will include a case that the participants may use as an example.

CEC06-03

Reversible effects of FGF21 on the female reproductive system

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Fibroblast growth factor 21 (FGF21) is a target for weight management and leads to a clear decrease in body weight in animal models. Reversible effects of FGF21 on the female reproductive system leading to infertility have been shown in transgenic mice overexpressing FGF21. However, similar effects have not been described in non-rodents. Six-week repeat-dose studies administering 3 dose levels (low, intermediate and high) of an FGF21 analogue as well as vehicle control were performed in sexually mature mice and cynomolgus monkeys. In the mouse study, reversible dose-responsive atrophic changes affecting the vagina and uterus correlating with low uterus weight and a decreased number of corpora lutea in the ovaries correlating with low ovary weight were observed at the intermediate and high dose levels. No changes were observed in the female reproductive organs in the monkeys. Both mice and monkeys showed weight loss or reduced body weight gain at the intermediate and high dose levels. Weight loss is known to affect the female reproductive system, and the effect seen in the mouse female reproductive organs may therefore both be a direct effect of FGF21 as well as a secondary effect from the observed weight loss or a combination of the two. How would you design the subsequent 16-week studies in mice and monkeys as well as the fertility study in mice supporting a 12-week clinical trial including women of child bearing potential?

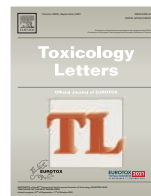
CEC06-04

PEGylated coagulation factor IX: The road to regulatory approval

*H. Offenberg

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This case relates to learnings from the recent regulatory submission of Refixia® or N9-GP, a 40 kDa glycopegylated (PEG) recombinant coagulation factor IX intended for prophylaxis, on-demand treatment of bleeding and surgical procedures in haemophilia B of all ages. Toxicity studies of limited duration were initially performed in cynomolgus monkeys, however the monkeys developed neutralising cross reacting antibodies that affected exposure and resulted in acquired haemophilia. Regulators expressed concern related to the long term effects of PEGylation and potential adverse effects related to this. The session will focus on discussion of options to investigate long term effects of N9-GP and support the approval in all indications.



Invited Sessions

EUROTOX–SOT Debate

D – Individualized toxicity is the future of risk assessment

EUROTOX Debater: Alan R. Boobis, *Imperial College London, UK*

SOT Debater: Syril D. Pettit, *HESI, Washington, DC, USA*

Each year, the EUROTOX Congress includes a debate in which leading toxicologists advocate opposing sides of an issue of significant toxicological importance. The debate continues a tradition that originated in the early 1990s. This year, the debaters will address the proposition “individualized toxicity is the future of risk assessment.” As toxicologists, we seek to define safe operating parameters for the use of a substance in a population. The opportunity to use “real-world evidence” and personal data collection devices (human biomonitoring) to gather information on toxicity from individuals and small populations allows us to envision new ways to refine these parameters as they relate to individuals. Is the future of toxicology and risk assessment headed toward a paradigm that must include approaches for patients/individuals at a public health–protection scale? If so, is it possible (or even desirable) to view risk of small groups of individuals based on discrete risk factors? Can adverse outcome assessments for groups be improved by this approach? Can recent developments in machine learning and other computational methods be applied in toxicology as they are being applied in precision medicine? Does surveillance of a patient’s response via personalized adverse effect monitoring provide a means to qualify the exposure–response relationship that can be tailored to each patient? Would better profiling be helpful to avoid lethal errors? Does an integrated exposure reduce the number of assumptions that need to be made when estimating exposure and thus help reduce the uncertainties in exposure science? Can we address the ethical and political aspects of its application?

Regardless of their personal convictions, each debater will present relevant evidence and compelling scientific arguments to persuade and appeal to the audience in order to obtain the approval or refusal of the motion. In addition to inclusion as a Featured Session at this meeting, this debate also took place (with the debaters taking the reverse positions) during the SOT Virtual Meeting, March 12–26, 2021.

Bo Holmstedt Memorial Fund Lecture

B – Context matters: next generation insights on the chemistry of DNA damage and mutation

*S.J. Sturla

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Cancer risk is impacted by exposures to chemicals from the environment, diet and drugs, together with defects or deficiencies in DNA damage tolerance pathways. Research of the past decades provides a basis for understanding how DNA oxidation, alkylation and instability, balanced by DNA damage tolerance and repair drive mutagenesis. Nevertheless, our ability to predict individual susceptibility to chemical mutagens or the etiology of individual cancers remains limited in part because there exists a mismatch between our low resolution understanding of the dynamic process of DNA damage formation and removal on a genome-wide level vs. our sophisticated single-base genome-wide picture of mutational landscapes of human cancers. Very recently, several strategies have emerged for base-resolution genome-wide mapping of DNA damage, including chemical probe- and ligation-based strategies. We have mapped DNA damage in mammalian cells, and gained new insight on how damage profiles are governed by chemical sources and cellular repair proficiency, and, finally, related chemical exposures with *in vitro* with mutation signatures. This research involves applying a combination of strategies for evaluating damage regions in the human genome based on DNA-damage-tailored chemical probes and bioanalysis methods, including single nucleotide mapping of damage in whole genomes, and *in vitro* strategies to determine the impact of enzymatic deficiencies on mutation signatures. Elucidation of DNA damage and repair from chemical exposure to DNA alkylating drugs, diet-derived reactive nitrogen species, and reactive oxygen species will be presented. Chemical structure and sequence-specific chemical probe-based strategies for tracking DNA damage overcomes previous limitations to understanding the distribution of DNA damage formation and repair, and its genome-wide impact on the accumulation of mutations. Furthermore, it provides information concerning the relationship between DNA repair enzyme function and mutation signatures that could lead to new approaches for quantifying risk on the basis of early biomarkers and characterization of causative factors of individual cancers.

SOT Merit Award Lecture 1

SOT 1 – SOT Merit award lecture 2020: Unraveling the molecular mechanisms of cannabinoid-mediated immune modulation and cannabinoid receptor 2 as a putative therapeutic target

*N.E. Kaminski

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The term *cannabinoid* encompasses a broad family of molecules, including plant (cannabis)–derived, synthetic, and endogenous compounds possessing diverse biological activities. Indeed, cannabis alone possesses over 100 structurally related cannabinoids, most of which have yet to be studied to any extent. The majority of attention during the past 50 years has focused on the primary psychotropic constituent in cannabis, Δ^9 -tetrahydrocannabinol, and very recently cannabidiol, both of which can influence immune function. A focus of the Kaminski laboratory has been on defining the profile of immune activity by cannabinoids and elucidating the molecular mechanisms mediating cannabinoid immune modulation. A significant advancement in the field came with the identification and cloning of two G-protein coupled cannabinoid receptors, CB1 and CB2, and the establishment of their expression on leukocytes. Identification of cannabinoid receptors quickly led to the discovery of endogenous cannabinoid receptor ligands, raising questions concerning their physiological role, including in immune regulation. An important backdrop to cannabinoid research has been the prevalence of cannabis use, with an estimated 37 million adult users in the US and likely increasing with recent trends toward legalization of recreational consumption. Likewise, medical use of cannabis and cannabidiol has also increased significantly, with many questions about the benefits remaining unanswered. Finally, expression of cannabinoid receptors within the immune system, coupled with the absence of CB2 within the CNS, provides a putative drug target for cannabinoid-mediated immune modulation devoid of psychotropic activity. This presentation will discuss the molecular mechanisms by which cannabinoids modulate the immune system, as well as putative health implications and CB2 as a putative therapeutic target for the treatment of inflammatory diseases. *Dr. Kaminski was the recipient of the SOT Merit Award in 2020.*

SOT Merit Award Lecture 2

SOT 2 – SOT Merit award lecture 2021: The exciting challenge of working in regulatory toxicology

*R. Henderson

Lovelace Respiratory Research Institute, Albuquerque, USA

The importance of using regulations to keep our air clean is relevant for two major reasons: to protect the health of all species and to protect the health of our planet. This lecture will address some of the challenges associated with working in this field through the lens of a prodigious career in regulatory toxicology. Dr. Henderson will discuss her early education, which included a double major in chemistry and biology as well as a double minor in German and mathematics. She will then discuss her one-year studies in Munich, Germany, on a Fulbright Scholarship and her subsequent return on a fellowship to perform a graduate study in chemistry at the University of Texas in Austin, Texas. She then will speak about her early career, when after earning a PhD in chemistry, Dr. Henderson joined the newly established Lovelace Respiratory Research Institute under Dr. Roger O. McClellan. She will present on her research, funded by the National Institute of Environmental Health Sciences, on the toxicokinetics of inhaled xenobiotics, which led to the development of novel biomarkers in bronchoalveolar lavage fluid and contributed to the modern field of inhalation toxicology. Dr. Henderson also will touch on how from this basic research, she published over 250 scientific communications, including review articles, chapters, and books, and served on numerous Editorial Boards and study sections. Dr. Henderson will then discuss her experiences as a member and later Chair of the US Environmental Protection Agency Clean Air Scientific Advisory Committee, where she pushed for reasonable regulations of air pollutants and additionally contributed to the World Health Organization's guidelines for air quality. This lecture will include a discussion of some of the challenges Dr. Henderson faced in the leadership roles she held in regulatory agencies. *Dr. Henderson is the 2021 recipient of the SOT Merit Award.*

HESI CITE Lecture

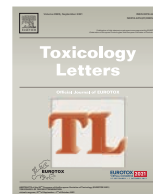
H – Linking primary mechanisms of environmentally induced neurotoxicity to human neurological disease relevance

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Neurodegenerative disease symptoms typically arise from specific neuropathology. In Parkinson's disease (PD), dopamine (DA)ergic neurons degenerate in the substantia nigra and resultant striatal DA depletion produces prototypical motor deficits. While neuropathology also occurs elsewhere, nigral DAergic neurons are especially sensitive to PD-relevant environmental insults. Diverse chemical classes of environmental risk factors have been identified, with oxidative stress, mitochondrial dysfunction, and protein aggregation repeatedly identified as key pathological responses. With respect to adverse outcome pathways (AOPs), these events would likely map to middle to late "key events". For many PD relevant neurotoxins, the molecular initiating event is unknown. To address this important gap, we study the primary neurotoxic mechanisms of action for heterocyclic aromatic amines (HAAs), organophosphates (OP), and per- and polyfluoroalkyl substances (PFAS). We broadly hypothesize that a combination of internal cellular dose and underlying DAergic neurobiology contribute to DAergic sensitivity across chemical classes. Here, we found that PD-relevant toxicants may preferentially accumulate in DAergic neurons, not through uptake by the DA transporter (DAT), but due to interactions with neuromelanin (a species-specific by-product of catecholaminergic catabolism in neurons). Blocking DAT in cellular models does not ameliorate HAA neurotoxicity, suggesting HAAs do not selectively accumulate in DAergic neurons via DAT. HAAs bind to neuromelanin models (Sepia and dopamine-associated), potentially explaining increased HAA accumulation in neuromelanin-forming cells. Moreover, neuromelanin formation modulated oxidative stress, mitochondrial dysfunction, and autophagy impairments, suggesting neuromelanin-toxicant interactions as a potentially important molecular initiating for many PD toxicants. Therefore, commonly used rodent models likely have a significant translational weakness because they are almost devoid of neuromelanin, lacking human-relevant neurobiology and likely underestimating intraneuronal dose. Our findings suggest that toxicant-neuromelanin interactions are a critical early pathogenic event and that studying PD neurotoxicity in neuromelanin-forming models is crucial for understanding the molecular initiating events of PD. In ongoing OP and PFAS models, we have identified mitochondrial import pathways and perturbations to mitochondria (respiration, oxidative damage, mitophagy) that likely mediate intraorganelle accumulation and DAergic neurotoxicity. Taken together, our findings suggest that it is critical to evaluate primary mechanisms to elucidate how toxicants selectively accumulate and exert selective toxicity. Moreover, it is critical to identify how underlying neuronal subtype biology and specific targets contribute to selective neurotoxicity to advance our understanding of human neurological disease.



Sessions

S01 | The effect of chemicals on the gut microbiota: is it the cause of all problems?

S01-01

Gut microbiota and human health through lifespan

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The microbiota refers to the trillions of symbiotic microorganisms present in the human body, the majority of which residing in the gastrointestinal tract. The gut microbiota has an organ-like fundamental role in human physiology, playing an important role in key functions such as digestion and regulation of variety of immunological and metabolic processes.

The gut microbiota emerges at birth and continues to develop and evolve throughout infancy and childhood to reach an individual-specific configuration towards adulthood. Due to the central role of intestinal microbial signals in the development and function of innate immunity, disturbed early colonization is believed to contribute to the increased risk of immune-mediated and metabolic diseases related to caesarean section birth and early antibiotic use. In adulthood, altered microbiota has been implicated in major diseases such as metabolic syndrome, cardiovascular disease, cancer, inflammatory bowel diseases, asthma, allergies, and neurodegenerative diseases. The lecture will provide an overview of the gut microbiota through lifespan, the major known modifiers of its structure and function, and discuss the importance and approaches of identifying the causal role of the gut microbiota in human health and diseases. The challenges and potential of different strategies to modulate the gut microbiota to achieve health benefits are also discussed.

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S01-02

Dietary emulsifiers, human microbiota and intestinal inflammation

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The human body is exposed to a diversity of chemical constituents from dietary and environmental origin. Recently, the use of additives in food products has become a matter of concern. It was previously demonstrated that chemical emulsifiers polysorbate and carboxymethylcellulose increase the susceptibility to chronic inflammatory diseases in genetically predisposed mice (Chassaing *et al.*, 2015). Moreover, it was shown that *in vitro* cultured human gut microbiota that got exposed to these chemical emulsifiers were enriched in flagellated microbial species and were able to trigger an inflammatory phenotype in germfree mice upon transfer of these pre-exposed microbiota (Chassaing *et al.*, 2017). It is not clear whether the microbial modulation and pro-inflammatory effects from chemical emulsifiers can be extended towards natural emulsifiers and clean-label emulsifiers from biotechnological origin. Here, we performed short- and long-term incubations of a chemical (polysorbate), natural (soy lecithin) and biotechnological (rhamnolipid) emulsifier with gut microbiota from 10 different human study participants and we investigated the impact on microbiome composition, functionality and subsequent cross-talk with host epithelial cells. We demonstrate that emulsifiers – independent from their origin – elicit microbiota-modulating effects in a concentration dependent manner with rhamnolipids triggering the most severe disruptions in both microbiome composition as fermentation activity. The observed effects were characterized by a large interindividual variability (Miclotte *et al.* 2020). On the long term, rhamnolipids had the most pronounced effects with decreasing abundance of *Faecalibacterium* and *Agathobacter*, accompanied by a significant decrease in butyrate levels. Interestingly, exposure of co-cultures of epithelial, goblet and immune cells to intestinal water, derived from emulsifier-gut microbiota incubations revealed a pro-inflammatory tone from emulsifiers with a strongest response for rhamnolipids. Overall, our results confirm previous observations that dietary emulsifiers can increase the risk for posing stress towards the gut epithelium in a microbiota-dependent manner and that formerly observed effects triggered by chemical emulsifiers are equivalent or probably even exceeded by natural and biotechnological alternatives. While the current research approached

the microbial exposure to emulsifiers from an acute exposure scenario at higher concentrations, it is not clear whether similar effects are also present at lower concentrations. Yet, the chronic nature of emulsifier exposure that is characteristic of westernized diets should trigger more in-depth investigations of the putative health risks posed by this class of food additives.

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S01-03

Low calorie sweeteners and their impact on the gut microbiota

*I. Rowland

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The human colonic microbiota is a large and complex microbial community. Over 1000 bacterial species have been identified with about 160 being found in the gut of any individual. The size and diversity of the microbiota is reflected in extensive metabolic activities.

Observational studies comparing the faecal microbiotas of healthy subjects with those of patients, strongly suggest an association of gut microbiota composition and the aetiology and/or development of a range of gastrointestinal diseases and also a link with obesity and diabetes. However, the precise organisms involved have not been fully elucidated. The interactions of low calorie sweeteners (LCS) and gut microbiota have been the subject of numerous studies in laboratory animals and to a lesser extent in human subjects. LCS are a structurally diverse group of compounds that have very different metabolic fates following consumption. Most (e.g. acesulfame K, saccharin, and sucralose) are not metabolized by gut bacteria. Stevia is a notable exception as its glycosidic forms are hydrolysed by the microbiota, releasing steviol, which is then absorbed intact without further bacterial metabolism.

LCS are consumed at very low levels and hence they are unlikely to have a direct, clinically meaningful impact on the gut microbiota. Nevertheless, some studies on saccharin (mostly in laboratory animals) have shown effects on microbiota composition or metabolism, although only at very high doses above normal human consumption. Studies with other LCS show either inconsistent, or no effects on the microbiota, probably as a consequence of design issues and lack of adequate controls.

There are relatively few human studies on LCS and gut microbiota and the effects seen are inconsistent and difficult to interpret due to a lack of dietary control or assessment and lack of control groups. Overall, the current evidence indicates that LCS have minimal impact on gut microbiota. Well controlled, human studies are needed to explore further the interaction of LCS with gut microbiota especially on microbial gene function.

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S01-04

The role of diet on stability, resilience and modulation of the human gut microbiota

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The gut microbiome of the human population shows a high degree of inter individual variability and dynamic diversity throughout life. This variability is linked to different host and environmental co-varying factors, being medication and diet the main contributors. Yet, they only explain a small percentage of the microbiome variation suggesting that other factors such as microbiome-host physiology interactions and individual microbiome temporal dynamics need to be considered.

The microbiome of healthy adult individuals tends to reach a structural and functional baseline stable state. Resilience is defined as the amount of an external perturbation that the gut microbiome can stand before changing towards a different equilibrium state. If a threshold of no return is achieved, a microbial dysbiosis state characterized by a strong imbalance in the microbiome structure can be induced, and this can occur over a few days or being permanently acquired during life time. The rapidly increasing generation of human longitudinal data, helps to understand the causality and directionality of microbiome associations with perturbations, among which the longest perturbation is the diet.

Diet has shaped gut microbiome profiles through human evolution. Short-term interventions may transiently alter the microbiome community structure, but long-term diet changes are required to shift to a new steady-state. Traditional plant-based diet with high carbohydrates and low meat and dairy consumption are found to be drivers of gut microbiomes dominated by high levels of the enterotype *Prevotella*. On the other hand, the enterotype *Bacteroides* positively associated with animal protein and saturated fats, is being the gut predominant microbiome among urban-industrial societies. The progressive loss of microbial diversity and functionality over generations observed in these societies along with the emerging increase of chronic non-transmissible diseases, have been related to the decline in the consumption of dietary fiber and loss of dietary diversity.

Targeted microbiome modulation strategies are intended to push the gut microbiome towards a eubiosis state through interventions that include newly developed probiotics, prebiotics and targeted diets. But these interventions have been shown to elicit differential gut microbiome responses among individuals. However, today multi omics data obtained from large scale human studies are successfully being used to predict responses to dietary interventions. These high-throughput powerful technologies can be combined to help understanding the dynamics of transcriptional regulation and facilitate the use of machine-learning algorithms to identify microbial gene or taxon-based signatures as disease biomarkers. Further use of experimental models to validate the generated data are needed to elucidate underlying mechanisms and understand the interrelationships between diet and structure and function of the human gut microbiome.

S02 | *In vitro* organotypic models for predicting the toxicity of chemicals or drugs

S02-01

Predictive (diseased) 3D lung models to assess effects of aerosolized nanomaterials and nanodrugs

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The lung with its huge internal surface provides a non-invasive entry portal for any kind of aerosol, e.g. containing micro- and nanoscale particles, or drugs. Inhaled materials can enter the lung, and depending on their size and properties can penetrate into different regions of the lung, and subsequently interact with epithelial tissue. While larger aerosols are deposited in the conducting airways, smaller ones reach the peripheral gas exchange region and may even pass the air-blood tissue barrier to enter the systemic circulation.

Many human lung models are described to investigate structural and functional aspects of the different lung compartments when exposed to aerosols. Such *in vitro* systems range from simple monocultures to highly sophisticated 3D models that include a combination of relevant cell types, e.g. epithelial cells, endothelial cells, immune cells, fibroblasts. It has been recognized that advanced 3D models bridge the obvious gap between simple monolayer cultures and *in vivo* models/humans, combining the advantages for increased throughput capabilities and predictive power. In addition, air-liquid interface (ALI) exposure is more realistic to mimic *in vivo* conditions in the lung than the suspension exposure. The advantage of the ALI exposure approach is that the particles/drugs mass deposition on the lung cell surface can be fully controlled by on-line monitoring, allowing a dose-deposition / effect correlation.

There is an urgent need for novel technologies to accelerate the hazard assessment of chemicals/materials in an occupational setting and the selection of safe particles and novel drug targets that can be applied *via* inhalation. This presentation will give an overview of the advantages of ALI exposure systems compared to conventional submerged conditions as well as 3D (diseased) lung models, and will further critically discuss the limitations of such approaches.

This work has received funding from the European Union's H2020 research and innovation programme under grant agreement No. 760813 (PATROLS).

S02-02

Advanced *in vitro* models for nephrotoxicity testing: as complex as possible, but simple in use

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Adverse effects caused by exposure to foreign compounds, including drugs, often involve the kidney because of its role in maintaining body homeostasis. Early prediction of those effects, such as drug-drug interactions and renal toxicity, is imperative for the development of new and safe drugs by the pharmaceutical industry. Current *in vitro* assays do not accurately allow such prediction, predomi-

nantly due to inadequate preservation of the organs' microenvironment. The kidney epithelium is highly polarized, and the maintenance of this polarity is critical for optimal functioning and responsiveness to environmental signals influencing cell proliferation, migration and differentiation. This presentation will provide an overview of advances in 3D cultures of human renal cells and organoids in microfluidics, and in particular kidney tubules, thereby improving physiological performance of the tissue. These kidney-on-a-chip platforms have great potential for drug screenings and provide novel alternative strategies for prediction of renal drug disposition and safety assessment in a human-specific context.

S02-03

Human 3D brain model to study developmental neurotoxicity

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The increase of neurodevelopmental disorders continues at an alarming rate^[1], bringing concerns about the implication of toxicants to these disorders. However, the significance of exposure to xenobiotics at developmental stages is not fully understood. The current existing guidelines (OECD TG 426 and US EPA 712-C-98-239) are based on animal experiments, where observations are made to detect gross neurological and behavioral abnormalities, including the assessment of physical development (OECD 2007, EPA 1998). This entails the use of a large number of animals (about 1,000 rats), for a long period of time (between 3 months and 2 years), producing a very high cost (\$1.4 million per substance). For these reasons, DNT assessments are not done routinely generating a lack of toxicological information in the vast majority of chemicals. OECD and EFSA are developing a new framework^[2,3] to evaluate DNT compounds based on a suggested DNT *in vitro* testing battery to be able to replace current animal guidelines. These methods cover important key events of brain development such as proliferation, migration, neurite outgrowth between others. However, despite myelin has been considered one important key event by OECD experts, this assay has not been incorporated yet on the testing battery due to the difficulty to obtain myelin *in vitro*. We have developed a 3D human brain model derived from iPSC (also called BrainSpheres). The model consists of aggregates that are very consistent in shape and size (350 µm in diameter), presenting different cell types such as neurons, astrocytes, and oligodendrocytes^[4,5]. The model reaches a higher maturation level compared to classical human *in vitro* cell cultures, as suggested by the presence of compact myelin around axons and strong spontaneous electrical activity^[6]. In the latest year, we have developed a quantitative myelination assay in which we have used reference compounds to internally validate the efficacy of the assay. Myelin was assessed by quantifying co-localization of myelin basic protein and neurofilament proteins by confocal microscopy, and PLP1 protein by immunofluorescence and western blots. Results demonstrated that the positive reference compound (cuprizone) and two of the three potential myelin disruptors (Methylmercury chloride, Bisphenol A, Tris(1,3-dichloro-2-propyl)phosphate) decreased myelination, while ibuprofen (negative control) had no effect. In this presentation, we define a methodology that allows to quantify myelin disruption with several and provide reference compounds for chemical-induced myelin disruption.

S02-04**Mini-Gut organoids for therapeutic testing**

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Organoid cultures have originally been developed as model systems to understand biological events, including development, tissue regeneration and oncogenesis. It is clear now that they have many other applications, including a strong potential for testing drug efficacy and potential toxicity. Miniaturization of organoid cultures now allows to perform many tests and to follow many readouts.

We have set up and miniaturized organoid cultures of human colon, using surgical resections or biopsies to isolate stem cells. Organoids from healthy individuals and from patients (Inflammatory Bowel Disease: IBD patients) were grown and demonstrated different phenotypes. The effects of drugs currently used in clinics and of newly developed targets were followed on the disease-associated phenotypes. Results demonstrated that such mini-gut cultures can be used to test drugs that favor the regrowth of functional epithelium. Other approaches have tested the effects of anti-cancer drugs on cancer organoid cultures.

Taken together, data are now available to suggest the use of organoid cultures not only for toxicology testing, but also for therapeutic testing, directly in diseased tissues. This could be an opening to personalized medicine.

S03 | Artificial intelligence and machine learning in chemical risk assessment

S03-01**Systematic reviews and chemical risk assessment: current challenges and the need for AI in overcoming them***P. A. Whaley^{1,2}¹ Johns Hopkins University Bloomberg School of Public Health, Evidence-based Toxicology Collaboration, Baltimore, USA;² Lancaster University, Lancaster Environment Centre, Lancaster, UK

Chemical risk assessment (CRA) is a data-intensive process. While recent innovations in the application of systematic review (SR) methods to CRA are considered to hold much promise in terms of improving the transparency and validity of the outcomes of individual risk assessments, it cannot be denied that the rigour of SR is unlikely to make the evidence analysis process proceed any faster. Meanwhile, the volume of data needing to be analysed increases daily: more risk-relevant studies are being published than ever before, while the demand for evidence-based approaches to regulating chemical substances means the number of analyses we need to conduct is rapidly increasing as well. In order to respond to this situation, in which we need more, better, and faster analyses of increasing volumes of information, we need to consider how artificial intelligence (AI) methods can accelerate the systematic analysis of risk-relevant research.

This presentation explores a number of emerging themes in this space. It charts a course from how evidence has traditionally been systematically reviewed in the past, where people have manually retrieved and synthesised small bodies of evidence in response to highly-focused research questions, to what evidence synthesis will look like in the future, when computational approaches will massively increase our ability to access, organise and process risk-relevant data^[1]. This includes the training of machines to automatically “read” and extract data from scientific studies on our behalf, and how “knowledge

graphs” will result in AIs replacing humans in the analysis of risk-relevant information^[2,3]. The implications, both philosophical and practical, will be flagged, before discussion closes on the role scientists will have in a society where machines do the thinking on our behalf.

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S03-02**Use of chemical informatics, quantum chemistry modelling and artificial intelligence algorithms to predict molecular initiating events***T. E. H. Allen¹, A. J. Wedlake², M. N. Grayson³, A. M. Middleton⁴, M. Folia⁴, M. Baltazar⁴, P. Piechota⁴, E. Gelžinytė², J. M. Goodman², P. J. Russell⁴, P. Kukic⁴, S. Gutsell⁴¹ University of Cambridge, MRC Toxicology Unit, Cambridge, UK;² University of Cambridge, Centre for Molecular Informatics, Yusuf Hamied Department of Chemistry, Cambridge, UK;³ University of Bath, Department of Chemistry, Bath, UK;⁴ Unilever, Safety and Environmental Assurance Centre, Sharnbrook, UK

Molecular Initiating Events (MIEs) provide good targets for *in silico* modelling using a variety of approaches, as they are well defined chemical-biological interactions^[1]. A wide variety of algorithms have been used to predict these important key events, and these models are seeing use in a variety of hazard identification and molecular screening safety decisions. However, using these algorithms in a chemical risk assessment remains a challenge. Next-generation risk assessment (NGRA) generally involves the comparison of quantitative exposure and hazard values considered as probability distributions^[2]. Can computational approaches provide the mechanistic insight and uncertainty estimation required for such decisions?

We have deployed several different algorithms in this area. This includes transparent structural alert models constructed using a maximal common substructure search, random forest models developed using physicochemical descriptors and neural networks in Python 3 using TensorFlow^[3,4]. All three computational approaches consistently provide models with over 90% accuracy against test set data and combining these models allows us to provide the best possible prediction – increasing model performance when compared to any individual model.

Quantum chemistry approaches have also been investigated for the prediction of chemical bond forming MIEs, specifically to predict the results of the Ames assay^[5]. DNA can directly react with some electrophilic chemicals, modifying its structure and causing damage that can lead to genotoxic adverse outcomes. By computationally modelling the transition state of these reactions and calculating the activation energy we have been able to establish why some molecules can react with DNA, and return a positive Ames test, and why some chemically similar molecules cannot.

To further extend the use of some of our developed MIE models to NGRA, qualitative activity predictions and uncertainty estimates are required. Bayesian learning neural networks use probability distributions to produce an output prediction with a mean and standard deviation. This uncertainty accounts for both how close the new molecule is to the existing data and how much variation exists within the training set. These networks produce quantitative activity estimates with errors within one log unit, even on external validation

data. We have also started to apply our algorithms to the prediction of key events further down the adverse outcome pathway, allowing them to provide more mechanistic understanding of toxicity. Chemical risk assessment requires information on molecular potency, uncertainty and toxicity mechanism, and decision-makers and regulators need to have confidence in the tools being used. The models we have developed provide additional understanding and confidence, vital for their future use in risk assessment.

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S03-03

Machine learning *in silico* models in chemical hazard identification

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In silico models can be developed by use of Machine learning (ML), a form of artificial intelligence (AI), where knowledge is automatically learned from training data. Many ML modeling techniques exist. Once a model has been developed and validated for a given endpoint, it can be applied to make predictions with high computational speed for new substances similar to the training data. A large number of free and commercial *in silico* models for many human health-related endpoints are available, and many new models are being developed, especially for adverse outcome pathway (AOP) molecular initiating events (MIEs) and other key events (KEs). Together with *in vitro* and *in chemico* assays, *in silico* approaches are part of so-called new approach methodologies (NAMs). Besides being used for screening, prioritisation, classification and labelling, benign-by-design chemistry etc., *in silico* NAMs are expected to be increasingly used in e.g. integrated approaches to testing and assessment (IATA) and next generation risk assessment (NGRA) to support regulatory decision making. To enhance regulatory acceptance of *in silico* models, the OECD defined principles for their validation >15 years ago. The EU has for more than a decade promoted the development and use of non-animal approaches including *in silico* models under different chemical regulations including REACH, and in the U.S. the aim to significantly reduce animal testing by 2025 and eliminate it completely by 2035 has been announced. This talk will go briefly through the above outlined topics and give some examples of our own *in silico* systems like the Danish (Q)SAR Database and Models free websites as well as examples of our *in silico* screening and assessment projects for the Danish EPA.

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S03-04

The virtual physiological human: bringing the community together and the field forward

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The use of computer modeling and simulation in medicine, also called *in silico* medicine, is taking big strides towards fulfilling its promise: contributing to a faster, safer and more personalised healthcare. Developments in *in silico* medicine are very relevant to the field of (*in silico*) toxicology - and vice versa - as we face similar technical, societal and regulatory challenges. This presentation discusses the latest (non-technical) advances in the area of *in silico* medicine. It demonstrates how building a strong community and stakeholder interaction allows to bring the whole field forward.

In silico technologies are increasingly adopted in all aspects of healthcare, from prevention over diagnostics to treatment prediction and clinical follow-up. This progress is owed to a collaborative effort of a wide variety of stakeholders, from researchers to clinicians, from industry to regulators, and from policy makers to funders. In recent years, FDA and ASME published guidelines and standards on reporting, verification and validation of computer models used in the context of medical devices. In Europe, regulators are working together with academia and industry to establish guidelines for the evaluation of *in silico* technologies (beyond the classical well-regulated pharmacometrics) in drug design and development. Organizations such as the Virtual Physiological Human institute (VPHi, the international scientific society for *in silico* medicine) and the Avicenna Alliance (the alliance of VPHi and industry) are driving initiatives that will further accelerate the adoption of *in silico* medicine. One such initiative is the work on 'Good Simulation Practice' which will be a quality standard defining how to assess and approve *in silico* tools before they can be used to produce regulatory evidence on the safety/efficacy of a new medical product. Another initiative is the Community Challenge for the adoption of consensus protocols for the characterization of biological tissue properties (C4bio), which will lead to a 'certificates of birth' for the generated data meeting regulatory requirements. Key to all these initiatives is the involvement of multiple stakeholders, which ensures a wide basis for acceptance and uptake of the developed standards and guidelines and which is the only way to remove the remaining barriers for the full adoption of *in silico* medicine.

S03a | EAPCCT symposium on COVID-19 and the toxicity of therapeutic drugs and vaccines

S03a-01

Specific treatments in the critically ill COVID-19 patients: benefits and toxicities

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Introduction: Since 18 months, the world has been fighting against a terrible pandemic due to severe acute respiratory syndrome coronavirus-2, responsible for the coronavirus disease-2019 (COVID-19). Rapidly, in addition to supportive care mainly based on oxygen administration and mechanical ventilation when requested, several

anti-COVID-19 therapies including drugs with demonstrated and drugs with only hypothetical benefits have been used. Many unapproved therapies including cheaper drugs available as over-the-counter have been marketed. We aimed to review the benefits and toxicities of the main specific drugs used in the critically ill COVID-19 patients.

Method: A narrative review based on published articles in PubMed or as preprints.

Results: Anti-COVID-19 pharmacotherapy mainly consisted in antiviral and immunomodulatory drugs given either in an early stage to strengthen the anti-COVID-19 immunity or in a late stage to limit the extent of the deleterious anti-COVID-19 immune response responsible for the so-called cytokine storm. Lopinavir/ritonavir combination and remdesivir have been initially used despite significant liver and renal toxicities but rapidly abandoned due to the absence of limited effectiveness, respectively. The use of hydroxychloroquine and its combination to azithromycin has been also stopped due to the absence of established efficacy in the randomized placebo-controlled studies and the risk of cardiac dysrhythmias in severe COVID-19 patients with organ dysfunction. To date, corticosteroids and anti-interleukin-6 receptor Fab fragments are extensively used due to their demonstrated contribution to limit mortality and severity of COVID-19; however, their use has resulted in marked increase in bacterial and mycological infections, especially in mechanically ventilated patients. Various other drugs such as colchicine and ivermectin are prescribed and the extent of their toxicity in COVID-19 patients is still worthy assessing. Finally, dietary supplements and vitamins including vitamin D and high-dose vitamin C have also been used. Despite an expected excellent tolerance and an interesting rationale to support their anti-COVID-19 activities, their preventive or therapeutic effectiveness has not been confirmed in randomized trials.

Conclusions: The role of clinical toxicologists and poison control centers remains essential to identify drug-related toxicities. Evaluation of anti-COVID-19 drug safety should rely on single or case series and reported side effects in clinical trials. Unapproved treatments should systematically require attention of health authorities.

S03a-02

Understanding vaccine-induced immune thrombocytopenia and thrombosis

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Vaccine-induced immune thrombocytopenia and thrombosis (VITT) is a rare but serious complication seen post COVID-19 vaccination, most notably in the UK after the Astra Zeneca vaccines, occurring in 1 per 50,000 vaccines in the under 50s and about 1 per 100,000 in the over 50s.

VITT causes thromboses in both the arterial and venous beds, generally targeting unusual sites, with cerebral venous sinus thromboses accounting for 50% of the thromboses, and splanchnic vein thrombosis accounting for another 30%. Thrombosis is associated with thrombocytopenia, often a low fibrinogen and very high D-dimers. The syndrome has many similarities to heparin-induced thrombocytopenia and thrombosis and indeed the same antibody to Platelet Factor 4 is found in VITT and is considered to be the pathogenic cause. This is an immune response and therefore no identifying risk factors have been yet found other than younger age.

The presentation and evolving management will be discussed.

S03a-03

Impact of the COVID crisis on European Poison Centres

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On behalf of EAPCCT COVID-19 and PCCs activities WG

The pandemic has also crystallized the role of clinical toxicology and identified a new role for clinical toxicologists. Poison Control Centers (PCC) and clinical toxicologists have been committed on several fronts: prevention, including campaigns, collaboration with Government Agencies, toxicovigilance, etc. Our activity in addition to pre-hospital triage of intoxicated patients helped to stabilize the flooded emergency system. An ad hoc EAPCCT Covid-19 Working Group was established so as to evaluate the impact of Covid-19 on European PCCs activities. A pilot study was performed in 4 European PCCs [Copenhagen, Pavia, Utrecht, Zurich] to evaluate critical points on methods, data collection and resources. The study was then extended to all European PCCs to investigate the effect of the Covid-19 pandemic during the first wave on the activities of European PCC. All 65 European PCCs listed in the WHO directory were asked to supply epidemiological data on poisonings (e.g., number of calls, patients, type of caller (medical professional or public), type of exposure (accidental, intentional (all), intentional suicide attempt), and age groups). Investigated exposures were disinfectants, household cleaning products (according to the European Chemicals Agency (ECHA) classification) and drugs, including antivirals. Data was analyzed during a 4-month period (March-June 2020) and compared with the previous two years. Furthermore, all members of EAPCCT were asked to participate in a survey on organizational changes during the COVID-19 pandemic. Results: The study included data from 36 PCCs from 21 countries (55% of EU PCCs). 60 % of Head of PCCs from 24 countries submitted pandemic related organizational data. Twenty per cent of PCC saw an increase in length of shifts while 42% saw an increase of total number of shifts. 25% indicated an increase of time spent on duties other than PCC activities, such as activities in the emergency departments (35%), intensive care units (12%), and nursing wards (9%). Over 50% of PCCs lacked protocols to manage PCC staffing upon massive sick leave. In contrast safe working protocols were in place in nearly every PCC. No centers reported receiving special funds for activities arising from the pandemic. Conclusion: European PCCs activities vary in different countries. A substantial improvement can be made in many areas such as data harmonization, establishing a European database of poisonings, and an improved network and cooperation of European PCC, and timely toxicovigilance especially in times of global emergencies.

S03a-04**Rapid Development of the FDA ACMT COVID-19 ToxIC (FACT) Pharmacovigilance Pilot Project to Monitor Adverse Events Reported in Association with COVID-19 Therapeutics**

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Background: When the COVID-19 pandemic emerged, many treatments were administered either off-label, under U.S. Food and Drug Administration (FDA) Emergency Use Authorizations, or through compassionate use programs. The rapid spread and high mortality of COVID-19 emphasized a need to develop a surveillance system to identify adverse drug events (ADEs) specifically related to emerging COVID-19 therapeutics. The FDA contracted with the American College of Medical Toxicology's (ACMT) Toxicology Investigators Consortium (ToxIC) to establish a novel multi-center active surveillance network to capture ADEs associated with COVID-19 therapeutics.

Methods: In October 2020, ToxIC recruited medical toxicology site investigators from 15 medical centers across the United States to establish a pilot toxico-surveillance program to proactively identify and report ADEs associated with COVID-19 therapeutics to a new ToxIC Sub-registry. This FDA ACMT COVID-19 ToxIC (FACT) Pharmacovigilance Project focused on providing timely data about adverse drug events associated with exposures to medications or substances used for the treatment or prevention of COVID-19 in inpatient and outpatient settings. Site-based research assistants worked with the principal investigators at all 15 sites to proactively identify cases of interest via site-specific mechanisms. These included reports from medical toxicology consultations, the patient's treatment team, the pharmacy, and by chart review. A HIPAA-compliant web-accessible REDCap data collection instrument was developed in collaboration with the FDA to facilitate case submission and provide for real time reporting. Over the initial 6 months of data collection, case identification criteria evolved to specifically target serious (e.g., death or hospitalization) and unlabeled adverse drug events.

Results: Between 11/23/20 and 6/30/21, 610 cases were submitted across all 15 sites. The majority of the cases involved ADEs with remdesivir and monoclonal antibodies. Through close collaboration with the FDA and continued assessment of emerging literature, investigators were guided to identify important ADE signals. For example, we developed a data collection form specific for remdesivir-associated bradycardia (heart rate below 60 after therapy initiation), which ToxIC shared with the sites, leading to the identification of 166 cases.

Conclusion: In partnership with the FDA, ToxIC rapidly developed a multi-center pharmacovigilance pilot project to help identify and analyze ADEs associated with COVID-19 therapeutics. Early identification of potential safety signals and creation of enhanced data collection instruments allowed timely investigation of the potential causality and clinical significance of adverse drug events reported with COVID-19 therapeutics. This project demonstrates that in the face of a national public health emergency, ToxIC can adapt to collect real-time data for public health analysis.

S04 | Personalized nano-immunotoxicology for the workplace**S04-01****Does immunotoxicity of nanomaterials depend on the individual pre-existing conditions? A need for a personalised testing strategy**

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Interaction of nanomaterials with the immune system mainly involves cells and mechanisms pertaining to innate immunity, the ancient defensive system that includes inflammatory reactions the involvement of phagocytes and other innate effector cells, and non-specific effector molecules such as complement.

The variability of nano-effects on innate immunity/inflammation in different *in vitro* and *in vivo* models is mainly attributed to the different characteristics of the nanomaterials, a concept that has promoted an extensive effort towards "safety by design", i.e., the design of nanomaterials with physical-chemical features unable to trigger unwanted reactions. This concept however does not appropriately consider the huge diversity of human innate immune reactivity. The recent attention on "innate memory", the mechanism by which innate immune responses change depending on previous exposures, opens the way to a revisitation of nanoimmunosafety that considers the human immune variability in addition to the nanomaterial characteristics.

In this view, it is notable that even nanomaterials that do not have a direct capacity to trigger inflammatory effects, such as gold nanoparticles, were found able to induce or modulate innate memory in some individuals (assessed *in vitro* on blood monocytes), thereby changing their response to a subsequent infectious challenge.

Based on these notions, a personalised profiling of the effects of nanomaterials of the innate memory is recommended, which would help identifying people at risk of developing inadequate immune responses as consequence of exposure to nanomaterials.

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S04-02***In vitro* immuno-nanotoxicological methods that take pre-existing conditions into account**

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In the workplace environment, where nanoparticles (NPs) may reach higher concentrations, the co-existence of allergens may result in new combinations of NPs with allergens. Normal as well as pre-disposed individuals, suffering from pre-existing allergic sensitization, may thus be affected by inhalation of aerosols carrying these new combinations having different immunological consequences. Recently, epithelial barrier integrity has been recognized to exert major impacts on the general health status of humans (epithelial barrier hypothesis, <https://doi.org/10.1038/s41577-021-00538-7>), responsible for increase in allergy, autoimmunity and other chronic condi-

tions. Moreover, for therapeutic approaches allergens are conjugated with different types of particulate matter, which serve as adjuvants, to boost the immune reaction or, in the particular case of allergen-specific immunotherapy, to drive immunomodulation from a type 2 to a more tolerogenic response. Here, the variety of materials can range from classical alum-based to more sophisticated nanovaccine carriers. Decreasing the size-range into the nano-scale goes in line with specific surface-related effects, known as protein corona formation, having impact not just on the NP itself, but even more importantly on the bound allergen. We have recently established a suite of *in vitro* assays that elucidate effects at a structural and molecular level with a particular focus on responses specific for sensitized individuals, to sort out alterations in the allergic effector function. Data enrichment from *in vitro* experimentation may be accomplished by use of suitable *in silico* modeling tools. Therefore, protein corona prediction was tested and evaluated for its potential to complement the experimental workflow. Ultimately, combined *in vitro* and *in silico* data including comprehensive metadata was uploaded to the Nano-Commons Knowledge Base to aid data FAIRness, i.e. Findability, Accessibility, Interoperability, and Reusability. In conclusion, such integrated workflows can help in early-stage decision-making processes in therapeutic development, and hence, promote safe-by design approaches in nanomedical innovation, as well as – in combination with *in silico*-based exposure-relevant dose-finding – serve for risk monitoring at specifically loaded areas, e.g., workplaces, taking into account pre-existing conditions such as allergic sensitization.

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S04-03

Understanding the biological impact of industrial engineered nanomaterials upon the alveolar epithelial barrier *in vitro* using occupational and *in vivo* relevant concentrations

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Human exposure to engineered nanomaterials (ENMs) is inevitable. With the constant increase in their production, and subsequent use within a plethora of human-based applications, deducing relevant and realistic exposure levels to test ENM-hazard is of paramount importance. Within the occupational setting, it is well accepted that inhalation exposure is of primary concern towards human health. However, known exposure concentrations within the occupational setting are currently limited. With clear understanding that ENMs penetrate to the lower lung, the alveolar region is therefore of significant interest. With the need to assess the hazard potential of a wide range of different ENMs, recent focus has been given towards the use and implementation of different *in vitro* models representing the key cellular components of the alveolar region. Furthermore, consideration of not only how such *in vitro* systems can be constructed to mimic the anatomy of the lower lung, but also to encompass specific physiological attributes (e.g. fluid-flow dynamics). Deduction of which is optimal, not only towards ENM hazard-screening, but to further predict the response observed *in vivo* is essential. Thus, by combining both approaches (occupational exposure and *in vitro* hazard assessment), a true understanding of the potential (inhalation) risk of ENM in the occupational environment can be elucidated. The purpose of this presentation therefore will be to introduce approach-

es taken to firstly understand occupational exposure levels within ENM production facilities. Then secondly, how these exposure levels were incorporated through *in silico* means to represent *in vivo* relevant concentrations, in addition to *in vivo in vitro* extrapolated concentrations from past studies for additional ENM types, which were then all subsequently assessed for their biological impact using *in vitro* models of the human lower lung.

S04-04

Impact of nanomaterials on haemodynamic parameters in normal and disease conditions

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Nanotechnologies like liposomes are developed as new therapeutic and diagnostic agents and requires knowledge of their compatibility with the blood components. The aim of this talk is to promote guidelines to study haemocompatibility of nanodrugs in normal and in pathological conditions. The six most important haemodynamic parameters are erythrocytes integrity, platelets aggregation, coagulation cascade, complement system, white blood cell recruitment and endothelial cell alteration. Haemostasis includes all physiological phenomena which prevent and lead to stop bleeding; maintaining vascular integrity. A dysfunction of haemostasis can slow down or even completely stop blood circulation. It is therefore primordial to know what nanodrugs can have an effect on coagulation. Assessment on normal and pathological models is of major importance to proof efficacy of nanodrugs as, for example, absence of potential multidrug reactions. Various techniques assessing the impact of nanodrugs on these six parameters were investigated. Based on the results obtained, we suggest guidelines for testing nanodrug haemocompatibility. This is required by international agencies and responds to a request of scientific community due to a lack of recommendations.

S05 | The use of minipigs in juvenile studies in an evolving regulatory landscape

S05-01

Use of juvenile minipigs in testing drugs and foodstuffs to demonstrate safety for human children – practical issues

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Regulations for testing of medicines and nutritional substances products prior to human use are in a state of constant evolution in order to keep pace with technological developments, and with a present huge focus in paediatrics use. Within the field of pharmaceuticals, the newly published ICH Guideline S11^[1] concerned with nonclinical safety testing of paediatric medicines sets the parameters for future testing of medicines for use in human children. Within the nutritional field, the European Food Safety Authority (EFSA) has published guidance on risk assessment of substances present in food intended for infants below the age of 16 weeks^[2]. Over the last many years, there has been an increased interest in, and acceptance of, the use of minipigs as a non-rodent species in toxicology and safety assessment and both mentioned guidelines reference juvenile minipigs. We assess the reasons for interest in the minipig and present examples of their use in toxicology and safety assessment, in particular from the point of view of practical use of these animals in satisfying the regulatory requirement.

In this presentation the background is set out and context will be given to the other presentations in this session.

References

- [1] ICH harmonised Guideline S11 – Nonclinical safety Testing in Support of Development of Paediatric Medicines, Step 2 document, September 2018
- [2] Guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age. EFSA Journal 2017; 15(5) : 4849

S05-02

The Minipig – A Rising Star for Nonclinical Safety Testing in Support of Development of Paediatric Medicines?

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Toxicity studies in juvenile animals can be required to support the development of paediatric medicines. The need for and the design of such juvenile animal studies (JAS) is regulated by the ICH guideline S11. Rats were traditionally used in JAS, mainly for practical, budgetary, and animal welfare reasons. When JAS in non-rodents were required, dogs or monkeys were often employed because these are species generally used to support adult drug development, which typically precedes paediatric development.

Minipigs have multiple advantages over dogs and monkeys, and even compared to rabbits, which make them a particularly attractive non-rodent species for JAS. Advantages include a shorter development period until maturity, an advantageous litter size and remarkable technical feasibilities, combined with numerous anatomic, physiologic and developmental similarities to humans. A good understanding of species-specific characteristics relevant to JAS is needed to prevent shortcomings.

The increasing number of pharmaceuticals developed for children-specific indications could significantly raise the use of the minipig in JAS. For such indications, a comprehensive non-clinical safety data package can include repeated-dose toxicity studies performed in both juvenile rodents and juvenile minipigs, with exposure during all relevant stages of development up to maturity. This approach can also improve the assessment of those organ systems that develop at very different pace in animal species and humans.

Overall, the minipig is still not used to its maximum potential in non-clinical safety testing and certainly not in support of the development of paediatric medicines.

S05-03

The juvenile Göttingen Minipig: role of organ development in view of food and drug safety in neonates, infants and toddlers

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In order to assess safety of pharmaceuticals and risk of nutritional products when used in the paediatric population, guidelines have been issued by ICH and EFSA, respectively. Studies in juvenile animals can be required, especially for the youngest age groups, and the (mini)pig is considered to be a relevant model in both guidelines. Indeed, the piglet shows several anatomical and physiological similarities with human neonates, infants and toddlers. Especially the (timing of) development of the gastrointestinal tract, but also the skin, immune, cardiovascular and urogenital system, are generally considered to resemble more the ones in the human paediatric population than other nonrodent species. The current presentation will focus on the development of the gastrointestinal tract in the juvenile Göttingen Minipig in view of absorption and metabolism of drugs

and nutritional products, but also the development of other organ systems will be briefly discussed. The relevance of these developmental milestones will be illustrated with real-life examples.

S05-04

Early life nutrition and later life cardiometabolic health in Göttingen Minipigs

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The Developmental Origins of Health and Disease (DOHaD) hypothesis states that the neonatal (dietary) environment shapes the subsequent development of the adult phenotype including possible disorders. In this study the later life cardiometabolic effects of high and low glycemic infant nutrition were compared in Göttingen Minipigs. This minipig was chosen as a representative large animal model for human infant nutritional physiology due to gastrointestinal similarities and their propensity to obesity and cardiometabolic disease.

Minipigs were weaned at 3 weeks of age and fed high or low glycemic diets until the age of 3 months. Then, the piglets were fed standard and/or obesogenic diets until reaching 6 or 12 months of age. Cardiometabolic health was quantified in permanently venous and arterial catheterized minipigs. Fasting plasma insulin and metabolite concentrations as well as blood cell profile were measured; blood pressure was recorded by intra-arterial measurement and atherosclerosis by aorta fatty streaks; insulin secretion and glucose tolerance were assessed by the hyperglycemic clamp and insulin sensitivity was estimated by the hyperinsulinemic euglycemic clamp.

The results indicate that early life high glycemic nutrition predisposes to later life elevation in fasting plasma glucose, a reduction in free fatty acids and a tendency towards elevation in fasting plasma insulin concentrations. Number of red blood cells tends to be increased. Insulin secretion, glucose tolerance and systolic blood pressure are increased. Insulin sensitivity and atherosclerosis are unaffected. In conclusion, this study shows that early life glycemic nutrition has a programming effect on cardiometabolic health in later life.

S06 | Human induced pluripotent stem cell (iPSC)-based test systems for future mechanism-based chemical safety testing.

S06-01

Human iPSC-based 3D-Neurosphere Assays for the detection of Developmental Neurotoxicity

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There is an unmet need to convert the traditional animal-based developmental toxicity tests to *in vitro* assays using human cells to detect and predict chemical hazards. However, the highly complex structure of the human brain and the limited availability of human neuronal cell lines, and primary human CNS tissue makes *in vitro* modelling difficult. Human induced pluripotent stem cells (hiPSCs), due to their pluripotent nature as any cell type of the human body can be differentiated from them, could change the field by providing unlimited cell sources for *in vitro* studies. Besides, the intensive de-

development of 3D cell culture technologies which could mimic the complexity and homeostasis of tissues opened new possibilities in *in vitro* testing. Combining the two technology we developed a hiPSC-based 3D neuro-spheroid *in vitro* toxicology assay that can be used to test developmental neurotoxicity. Our results revealed that the 3D neuro-spheroids contain different subtypes of neurons (GABAergic, glutamatergic, cholinergic), astrocytes and oligodendrocytes. By exposing them to different classes of toxicants and analysing them at various time points, representing distinct developmental stages and maturity, concentration-responses were investigated. The results demonstrated that different classes of toxicants resulted in distinct cell susceptibility profiles in each developmental stage, indicating that hiPSC-based *in vitro* models might be used effectively to evaluate developmental neurotoxicity. By incorporating reporter iPSC lines where major toxicity pathways are labelled by homologues recombination approach the response of the differentiated cells can be investigated based on the reporter gene expression which can further enhance to predict the toxic effect.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 681002 (EU-ToxRisk), No. 721975 (IN3, MSC-ITN), No. 814978 (TUBE) and No. 250619 (ADAIR).

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S06-02

Creation of functional pluripotent stem cell-derived hepatocyte-like cell and more complex liver models

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With the advent of pluripotent stem cell (PSC) technology, one aim is to create differentiated progeny that mimics primary tissue cells. As many drugs and chemicals can cause liver damage, modeling this toxicity is of great interest in the field of chemical toxicology and toxicity prediction. Over the last several years, we therefore developed methods that improve liver cell differentiation from PSCs to levels that approximate primary liver cells. Specifically, we used genome and metabolic engineering approaches to create hepatocyte-like cells (HLCs) from PSCs, that display (at least some) drug metabolism characteristics similar to levels seen in primary hepatocytes (PHHs), and the ability to identify DILI causing drugs with the same precision as PHHs. However, unlike PHHs, drug metabolism characteristics of PSC-HLCs are stable for several weeks in 2D culture. By combining these PSC-HLCs with PSC derived hepatic stellate cells (HSCs), PSC-derived endothelial cells (ECs), and PSC-derived macrophages (Mfs) in a fully defined functionalized polyethylene glycol based hydrogel, we created liver mimics, containing AFP- /CYP3A4⁺ mature hepatocytes, and all three non-parenchymal cell types in a long-term stable manner. Such liver mimics have further improved CYP activity, and comprise HSCs that can be activated by e.g., TGFβ and macrophages that can be activated by e.g., LPS. Most importantly, the response of the 4-cell liver mimics outperforms any of the monocultures (in 2D or in 3D) in detecting liver damage caused by for instance oleic acid, to model NASH. Studies are ongoing to determine the ability of the liver mimics to assess DILI as well as iDILI responses.

S06-03

Dissecting lineage specific oxidative stress response dynamics using high content imaging of the hiPSC HMOX1 fluorescent reporter line

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Cellular stress responses are critical for the repair of injured cells during diverse types of tissue damage, under pathological conditions as well as adverse responses to drug exposures. Targeting cellular stress response activation can contribute to cellular fitness and resilience to stress conditions. We generated an eGFP tagged HMOX1 expressing human induced pluripotent stem cell (hiPSC) line, to visualize the dynamics of oxidative stress on a single cell level. Both endogenous and scar free tagging was achieved using CRISPR/Cas9 technology, resulting in the maintenance of a representative cellular stress response *in vitro*. HiPSCs are an excellent model for *in vitro* safety screening since they rapidly divide, have undisturbed metabolic activity and can be differentiated into a whole range of cell types. This overcomes many of the limitations found in current *in vitro* models such as availability, inter-donor variability and stability. In order to dissect lineage specific oxidative stress response dynamics upon chemical exposure we differentiated the HMOX1-GFP hiPSC reporter into different lineages including hepatocytes, cardiomyocytes, neuronal cells and kidney cells. The various differentiated reporter cells were exposed to different oxidative stressors, CDDO-Me and diethyl maleate, followed by live cell imaging of the reporter activation using automated high content confocal imaging (HCI). We found large cell type dependent differences in amplitude of the oxidative stress response as well as the dynamic profile as a whole. Targeted sequencing was used to validate the differentiation status of the different lineages and specific induction of oxidative stress responses. These results provide insights into organ specific sensitivity and dynamics towards chemically induced oxidative stress response signalling. We anticipate that our hiPSC cellular stress response reporters in combination with HCI may play a key role for refined understanding of the dynamic stress response signalling in diverse target tissue under adverse drug reactions.

This work was part of the EU-ToxRisk project and received funding of the European Union's Horizon 2020 research and innovation program under grant agreement No 681002.

S06-04

Nephrotoxic liability assessment using hiPSC-derived renal glomerular and proximal tubular epithelial cells

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Within human populations a significant amount of genetic diversity exists, including in genes that can contribute to effects on pharmacokinetics and toxicity of drugs. Currently used toxicity models, including animal models or human cell lines coming from a single donor, may not capture these effects. Human induced pluripotent stem cells (iPSCs) offer exciting possibilities to overcome some of these limitations in the (near) future; however, currently available renal iPSC-derived models are often considered immature and less differentiated than primary cells.

Recently, we reported the differentiation of human iPSC into renal proximal tubular-like cells (PTL)^[1] and into renal podocyte-like cells^[2,3]. Both differentiation protocols are fast (14 and 10 days, respectively) and result in relatively stable target cells that maintain specific differentiation markers, including megalin (PTL) or synaptopodin and WT1 (podocytes), for more than 7 days in culture.

To evaluate their usefulness for toxicology testing, we challenged both models with nephrotoxic compounds and analysed differential gene expressions using TempO-Seq transcriptomics. PTL were treated with the aminoglycoside gentamycin, known to induce proximal tubular damage, and gene expression levels were compared to human primary proximal tubular cells and the human renal proximal tubular cell lines RPTEC/TERT1 and HK2. Podocytes were treated with doxorubicin and pamidronate, both known to induce glomerular injury.

Both iPSC-derived renal models, could show relevant genes and toxicity pathways activation. Furthermore, PTL cells that exhibit megalin facilitated endocytosis, showed a more similar response to gentamycin with primary cells compared to RPTEC/TERT1 cells that are not expressing megalin.

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S07 | Setting the European Environment and Health Research Agenda, 2020–2030: the HERA project

S07-01

Introducing the HERA Core Agenda for the European Environment, Climate & Health Research

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The overall aim of the HERA project is to set the priorities for an environment and health research agenda in the EU by adopting a holistic, systematic and inclusive approach the face of global environmental changes. Research is expected to support decision-making at all levels and to help attain the ultimate goals of protecting and improving ecosystem quality and human health." (HERA (heraresearch.eu)). An agenda has been prepared based on the 24 HERA partners participation sharing their deep own competencies and undertaking extensive reviews of current knowledge, policies and activities through surveys targeting research communities and stakeholder groups.

The EU Research Agenda developed by the HERA project identifies six major research goals on the environment, climate and health to be addressed and numerous specific recommendations for research. The agenda presented to the Commission emphasizes research that form a scientific base for policy and management decisions related to environment, climate and health. The six overarching research

goals (RGs) are: *i*: Climate change and biodiversity loss – reduce effects on health and the environment; *ii*: Cities and communities – promote healthy lives in sustainable and inclusive societies; *iii*: Chemicals and physical stressors – prevent and eliminate harmful chemical exposures to health; *iv*: Improve health impact assessment of environmental factors and promote implementation research; *v*: Develop infrastructures, technologies and human resources for sustainable research on environment, climate change and health and *vi*: Promote research on transformational change in environment, climate change and health. A total of 30 research goals are specified in the HERA Core Agenda. Each of these needs are motivated by both their scientific and policy relevance, the latter commonly related to the Green Deal initiated strategy or action plans other EU relevant EU plans as well as the UN Sustainable Developments Goals. Numerous research needs are presented and tentative call areas have been formulated under each one of the 30 research goals.

The presentation will introduce the process to identify the proposed research goals but will primarily focus on goals regarding Climate change and biodiversity loss and on Chemical and physical stressors. The global perspective in relation to the research needs and the infrastructural needs are covered separately in the introduction to the Round table "Setting the European Environment and Health Research Agenda, 2020-2030: the HERA project.

S07-02

Major environmental stressors and their effect on health: a global perspective

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Pollution knows no boundaries, thus requiring global commitment. A global perspective on major environmental stressors and their effect on health can address global environmental stressors. It can also focus on major global agreements and policies related to common interests of their parties that are being formed to mitigate environmental stressors. Major categories of environmental stressors are associated with environmental pollution, land use change, degradation of ecosystems and loss of biodiversity and climate change. Global environmental pollution of long-range transport of pollution is related to air, ocean and water pollution, toxic chemicals and material flows. Causes and sources of pollution vary, and their impact on human and ecosystem health occurs through various pathways – not all of which are well known or assessed. In the European context, tackling global pollution includes international and EU policies related to: the "EU Action call for zero pollution", UNECE multilateral environmental agreements, black carbon; polluter pays principle; Agenda 2030 for Sustainable Development; various UN resolutions and treaties; sound management of chemicals and waste; the Basel, Rotterdam, Stockholm and Minamata Conventions on waste and electronic equipment, hazardous chemicals, persistent organic pollutants and mercury; plastics; biodiversity; and restricting the export of certain products and wastes. In addition, to achieve progress in addressing global pollution, development of policy interventions must be based on scientific understanding of multi-sectoral interactions. Policies and international coordination must be advanced in the context of other global processes and trends such as climate change, exploitation of natural resources including biodiversity loss, land degradation, and international trade. Not only is it necessary to advance knowledge on pathways and magnitude of global pollution as well as on quantitative assessment of short- and long-term related health impact, in addition research is needed to quantitatively assess the magnitude, dynamics and trends of global pollution flows and their impact on human and ecosystem health. Research should tackle

causes and consequences of global pollution in the context of governance and regulatory dimensions related to specific sectors. Research related to the impact of environmental drivers (e.g. climate change) on global pollution is needed in order to tackle negative consequences to humans and planetary health, in general. Finally, special attention should be given to vulnerable populations, gender and equity.

S07-03

Infrastructure needs in the field of environment and health

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Research infrastructures are key entities enabling high-level research and fostering innovations in any research area. Effectiveness and sustainability of environment, climate and health research also relies on availability of an adequate infrastructural framework providing an access to necessary capacities, innovative technologies, and expert human resources. The gaps identified in current infrastructural landscape in Europe include, for instance, large population-based cohorts as a valuable resource supporting research in environmental health. Coordinated infrastructures in cohort studies are needed covering parent-child, adult, and ageing cohorts as well as occupational studies to capture impacts of general environment and occupation exposures throughout the lifetime. There is also a need for laboratory capacities for targeted and non-targeted analyses of complex chemical mixtures in environmental matrices and human tissues and for analyses of effect biomarkers. Innovative experimental and modelling tools should enable an assessment of links between the environmental exposures and toxicity outcomes. Additional data for informed exposure and impact assessment can be collected through satellites, ground-based and wearable devices, social media data or citizen-sciences projects. A comprehensive monitoring system connecting human health and environmental indicators in time and space is essential. Research is needed primarily on data integration and analysis to connect indicators of global environmental changes with those of human health outcomes. Improved capacities to predict the impacts of climate and environment on human health are needed as well as capabilities to develop and communicate intervention strategies to mitigate these impacts. This would require big data infrastructures and effective governance structures for integration of data across sectors, disciplines, and multiple stakeholders. Such effort should reinforce the development of new technologies and data sciences as a transdisciplinary activity promoting research and innovations on machine learning and artificial intelligence. Numerous research infrastructures have been developed in Europe during the last decades in both the environment and health domains, however, the infrastructural capacities bridging the closely interlinked environmental domains to human health are largely missing. To tackle environmental challenges, we need to work in a more integrated manner towards more holistic understanding of the Earth's system and human health and wellbeing.

S08 | Back-translation from clinical outcomes, how did investigative toxicology, modelling and simulation actually perform?

S08-01

Mathematical modelling combined with new *in-vitro* technologies to enable quantitative translation between preclinical and clinical safety

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When target-related safety signals are detected in preclinical settings, mathematical modelling can help predict clinical risk at efficacious dose-schedules to avoid safety failures in clinical trials.

Mechanistic models can predict, in particular, adverse effects mediated by on-target mechanisms at early stages of drug development prior to the initiation of clinical trials. Of high relevance to the development of quantitative systems toxicology (QST) models is the increasing integration of novel *in vitro* techniques, such as organoids and microphysiological systems (MPS), into the pharmaceutical testing strategy. These advanced *in-vitro* technologies provide a variety of dynamic, high-resolution measurements of multiscale nature to help the development of QST models. In turn, QST can enhance pharmaceutical applications of MPS by quantitatively translating MPS toxicity into clinical risk. Their combination results in a highly powerful approach to enhance our understanding of biological processes and mechanisms of toxicity.

We will discuss the development of QST models as a back and forth process between preclinical and clinical toxicology to quantitatively assess haematological safety of a range of oncotherapeutics with different mechanism of action including compounds inhibiting DNA damage repair mechanisms and cell cycle proteins. In particular, we will present data on the response of hematopoietic progenitors to drug exposure in a bone marrow MPS. We will describe how the MPS progenitors' response can be integrated into novel QST modelling solutions to predict the haematological profile of patients exposed to relevant doses and schedules. Importantly, we will demonstrate that this approach enables the comparative safety assessment of dose schedules in early stages of the drug development pipeline to support decision making and help design clinical trials.

S08-02

A case study of a retrospective review of risk factors for DILI based on *in vitro* and *in vivo* data

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BAY 1128688 is a selective inhibitor of the aldo-keto reductase family 1 member C3 (AKR1C3). Inhibition of AKR1C3 has been proposed as drug target for endometriosis. During preclinical evaluation of this drug candidate, exposure levels, dose-response relationship, target organs of toxicity, and reversibility of effects were investigated in toxicological studies. The preclinical safety assessment, performed according to ICH guidelines, included repeat-dose toxicity studies in rats and cynomolgus monkeys dosed for 4 and 13 weeks. No hepatotoxicity was observed up to the highest tested dose of 70 mg/kg in female rats and 40 mg/kg in female monkeys over the 13 weeks of administration. These doses, identified as NOAELs, corresponded to an approximately 21-fold (rat) and 16-fold (monkey) higher systemic exposure (by the area under the unbound concentration-time curve at steady state over 24 hours, AUC_{[0-24]_{ss,u}) than the anticipated hu-}

man exposure at the maximum intended clinical dose of 60 mg twice daily (BID). Some adaptive changes, such as transiently increased serum bilirubin in monkeys without concomitant increase of transaminases, increased liver weight in both species and microscopic findings in the liver (prominent bile ducts, hepatocellular hypertrophy) in rats were observed. These changes were, however, reversible and occurred at multitudes above maximum expected human exposure.

In addition to the *in vivo* toxicological studies, *in vitro* assays were performed to investigate the potential for a DILI liability. In these assays, BAY 1128688 was found to be an inhibitor of transporters commonly involved in uptake and efflux processes of human liver cells, especially in bilirubin and bile salt transport (bile salt export pump [BSEP] and MRP 2 [multidrug resistance-related protein]). The investigated bile salt transporters showed low IC_{50} values in the range of 1–10 μ M, but still with a safety margin compared to the c_{max} (unbound) predicted for the Phase I trial.

Based on the overall preclinical assessment, no prohibitive findings for starting the clinical development were identified. Monitoring of bile acid levels in blood was included as parameter for the clinical trials. In clinical trials with multiple dose administration of up to 4 weeks in healthy volunteers, isolated dose-dependent, modest increases in serum total bilirubin were observed after single or multiple doses, but no concomitant increase in transaminases. In a subsequent trial with adult premenopausal women with symptomatic endometriosis (Phase IIa) hepatotoxicity was detected after more than 8 weeks of treatment indicated by a ALT increase ranging from 2x ULN to 76x ULN observed in 10 subjects (8.3%). The trial was prematurely terminated. The presentation will discuss hypotheses why the preclinical DILI assessment failed to predict the clinical findings and whether additional investigations could be done to reduce the probabilities of such failures in the future.

S08-03

Moving from detection of cardiovascular liabilities to quantitative mechanistic translational understanding: challenges and opportunities

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Cardiovascular safety finding encompass a range of perturbations covering ECG changes, haemodynamics and cardiac pathology. These changes can occur independently or concomitantly, either directly or indirectly related to PK parameters. Within cardiovascular safety, molecular understanding is key to developing quantitative translational insights and ultimately to predicting quantitative outcomes in patients. The concepts and techniques used depend on whether the molecular mechanism is known or unknown. We will discuss both scenarios using real examples to highlight the difficulties, challenges and impact associated with developing quantitative mechanistic understanding within drug discovery.

Molecular understanding of different cardiovascular effects varies, currently the key ion channels responsible for changes in QT, QRS and PR intervals are well established. In addition to some mechanisms for haemodynamic perturbations are known, for example inhibition of VEGFR2 and blood pressure increases. Integrated mathematical models have been developed that apply this mechanistic knowledge to predict the changes in ECG parameters or blood pressure based on *in vitro* inhibition at key molecular targets, predicted PK and determined transduction factors. This approach relies on both mechanistic insight and a single cellular perturbation underlying the physiological response.

However, cardiovascular effects are often multifactorial, and the mechanisms are largely unknown, presenting bigger challenges.

Technological developments in terms of 'omics' technologies, off-target profiling and data mining/bioinformatics have the potential to begin to fill this void. These approaches allow hypotheses to be developed that require further investigations. Application examples include off-target profiling utilising multiple kinase and omics (proteomics, transcriptomics and metabolomics) technology platforms. These approaches are enabling molecular understanding of cardiac pathology. If confirmed such approaches could be used as the basis for further quantitative mechanistic understanding incorporating systems pharmacology modelling. Success will facilitate quantitative predictive outcomes in patients and informed drug design.

S08-04

eTRANSAFE's Rosetta stone – a new approach to overcome safety translational hurdles

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Despite numerous attempts at establishing the meaningfulness of preclinical animal data for human clinical safety assessment, there are no systematic and automated tools able to perform this task on a large scale and on demand. Beyond issues related to the lack of available harmonised and curated preclinical data in large quantity, the incongruity between preclinical and clinical vocabularies is mostly responsible for this situation. Hence, the two main requirements to produce such a system are i) establishment of the required preclinical data collection and sharing process, since most of them are in private archives; ii) a vocabulary translation system able to alleviate the mismatch between the terminologies used for describing effects in preclinical studies (mainly SEND and HPATH, (histopathological ontology, <https://github.com/Novartis/hpath>) and the terminologies used for clinical studies (mainly MedDRA).

eTRANSAFE, a research project funded within the Innovative Medicines Initiative (IMI), aims to overcome the described hurdles. In this project, 11 pharmaceutical companies share *in vivo* toxicity data together with clinical data in repositories set-up by honest brokers. Academic partners in the project develop 'semantic services', which combine the terminologies of the preclinical with the clinical world – the so-called Rosetta stone project (Pognan *et al.* 2021: Pharmaceuticals 2021, 14, 237. <https://doi.org/10.3390/ph14030237>).

This mapping strategy is based on the use of a large, semantically rich ontology that functions as an intermediate (the Rosetta stone) between the preclinical and clinical terminologies of interest in eTRANSAFE. We selected the Systematized Nomenclature of Medicine, Clinical Terms (SNOMED CT) as the intermediate ontology. Mappings from the eTRANSAFE vocabularies to SNOMED CT were manually established and tools were developed to support an iterative verification process. To limit the manual curation work, mapping efforts were directed towards terms that are actually used in eTRANSAFE data sources. Also, to inform the mapping process, we made use of existing terminological resources, such as the Unified Medical Language System (UMLS), which already contain mappings between part of our terminologies and SNOMED CT.

The semantic services perform basic semantic operations on the terminologies in eTRANSAFE, e.g., term normalization or mapping of a preclinical concept to a clinical concept. The mapping function takes advantage of the granularity and ontological structure of SNOMED CT to infer mappings when concepts from one terminology cannot be mapped directly to concepts from another terminology. The semantic services are the building blocks for more elaborate software components of the eTRANSAFE platform, such as a query tool

or the construction of concordance tables. The Rosetta stone approach works both ways, allowing also reverse translation from clinical to preclinical terminologies.

S09 | Increasing confidence in non-animal approaches for regulatory decision-making

S09-01

Acceptance of *in silico* methods for regulatory purposes

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This presentation will discuss how confidence and acceptance of *in silico* methods for safety assessment can be enhanced through the development of *in silico* toxicology protocols. The use of *in silico* methods as part of the ICH M7 pharmaceutical impurities guideline will be reviewed in addition to how position papers have supported the use of computational models for regulatory submissions. These papers detail methodologies and procedures for performing assessments combining experimental data and *in silico* results, conducting an expert review of the information as well as documenting the outcome. Leveraging the experience of developing such protocols for ICH M7 assessments, a more expansive project was initiated to develop *in silico* protocols for major toxicological endpoints, such as skin sensitization. A large consortium was established to develop several protocols for various toxicological endpoints and publish associated position papers. This presentation will discuss the *in silico* toxicology protocol framework and outline progress to date on the development and implementation of several endpoint protocols.

S09-02

Strengthening a grouping/read-across case using omics-derived molecular mechanistic evidence from an invertebrate model

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Grouping and read-across is frequently used in chemical risk assessment to fill data gaps for human health and environmental endpoints without using vertebrate animals. Traditionally, substances are grouped based on structural and/or physico-chemical parameters. However, the quality of this approach could be improved by adding further relevant experimental evidence to support the grouping hypothesis. The objective of this case study was to evaluate the capability of multi-omics mechanistic data to substantiate (or not) the formation of chemical categories derived from conventional structural approaches.

The study investigated the molecular responses of *Daphnia magna* to seven disperse azo dyes. The target substance was disperse yellow 3 (DY3), and six potential source substances were considered. First, all these substances were grouped using structure, physicochemical properties and (Q)SAR profiling – to generate a grouping hypothesis against which the multi-omics findings would be compared. Next, a series of acute toxicity studies were conducted with *Daphnia* generating samples for multi-omics analyses (with low, medium and high

dose per dye). Metabolomics analyses were conducted using direct infusion mass spectrometry (DIMS) and transcriptomics using a newly-designed BioSpyder Tempo-Seq 1991-gene platform. The data streams were processed individually and then fused to allow an integrated analysis of the metabolic and transcriptional perturbations to discover the molecular response-based grouping of the dyes. In addition, the gene expression data were analysed using Ingenuity Pathway Analysis to identify perturbed molecular pathways that could provide a mechanistic rationale for the grouping.

Traditional approaches for grouping identified DY3 as being in one group, though two other dyes (Sudan Red G and Sudan 1) were also classed as 'phenols'. Hierarchical cluster analysis of the multi-omics data streams was used to group the azo dyes based on the similarities of their molecular responses. This revealed that the two Sudan dyes showed a higher toxicological similarity to DY3 than the 4 other azo dyes, and Sudan 1 and DY3 were most similar. Ingenuity Pathway Analysis also identified Sudan 1 as the most similar to the target substance, DY3.

In conclusion, this study establishes a workflow that enables molecular mechanistic data to be considered alongside conventional physicochemical approaches, to derive more robust chemical groups for read-across. For these seven azo dyes, while conventional approaches provided some indication of chemical grouping with DY3, it was only by incorporating molecular mechanistic data that we were able to define a single source substance (Sudan 1) to read across the *Daphnia* chronic reproductive toxicity endpoint. This case study thereby demonstrates the application of a New Approach Methodology, in a non-vertebrate model, to increase the scientific rigour of grouping and read-across.

S09-03

A new path for pesticide assessment: using the AOP framework as a tool in risk assessment

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The European pesticide regulation being very data-hungry, triggers vast animal data covering standard general repeat dose toxicity as well as specific studies addressing e.g. endocrine disruption and developmental neurotoxicity. Also, the regulation calls for integration of *in vitro* and human observational evidence in the risk assessment when available and relevant. The AOP framework provides a useful tool to "glue" these different types of evidence streams together. This talk will discuss cases illustrating the use of AOPs in different contexts exemplified within the area of developmental neurotoxicity, neurotoxicity and endocrine activity. Also, perspectives will be presented on future opportunities, but also blockers that need to be overcome to increase the confidence in non-animal approaches in pesticide risk assessment.

S10 | Computational modeling of AOP networks to assist risk assessment of chemicals

S10-01

Quantitative Bayesian Network Analyses of Mitochondrial Toxicity

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Adverse Outcome Pathways (AOPs) are increasingly used as a framework to support use of *in vitro* data in hazard assessment of chemicals which describes a series of events leading from a molecular initiating event to an adverse outcome. Chemical risk and safety assessment requires quantitative relationships from exposure to effect timing and magnitude: quantitative AOPs (qAOPs) use mathematical models to describe the relationship between each key event based on *in vitro* data, and are able to provide dose-time-response predictions. We have shown that Bayesian networks can be used in that case, and are suitable for refined hazard assessment. We first briefly review the method and its main applications so far.

We present an analysis of data obtained in three cell lines, LUHMES (neuronal cells), HepG2 (liver cells) and RPTEC/TERT1 (kidney cells). Using similar experimental protocols, these cell lines were used to construct and calibrate a qAOP of mitochondrial toxicity for two substances, rotenone and deguelin. The objectives were to determine whether the relationships could be modelled with the same equations for both substances, and to test chemical-independence with a dataset obtained for eight other chemicals in neuronal cells. Although the same modelling approach was used, and the data was obtained in similar conditions, repeating the calibration approach for rotenone and deguelin in three cell lines highlights various practical difficulties in this case-study. Chemical independence of the qAOP constructed is not clear, nor is tissue independence. This raises questions about the possibility to use the same qAOP for various substances and various tissues. Recommendations regarding experimental design of *in vitro* experiments targeted at calibrating qAOPs for a single chemical in a single tissue are proposed.

This project has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 681002 as well as from the Innovative Medicines Initiative 2 Joint Undertaking (IMI2/JU) under grant agreement No 777365.

S10-02

Logic modelling of toxicology pathways

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Traditional toxicogenomics studies aim at finding genes that are relevant in drug induced organ injury. However, the advancements in experimental techniques allow us to study the drug effects on multiple levels at the same time, including gene expression, (phospho-) proteomics and metabolomics. In the recent years, network based methods emerged that not only identify the key responders to the drug perturbation, but also capture the mechanistic relationship

among the components and help to cope with the complexity of these datasets.

In this work we present multiple network based methods, including COSMOS (Causal Oriented Search of Multi-Omics Space), a causal reasoning based method that integrates metabolomics, transcriptomics and phosphoproteomics datasets using prior knowledge networks about metabolic, gene regulatory and signaling interactions. As a case study we analysed human colon and small intestine data after treatment with Fluorouracil.

S10-03

Data-driven computational modeling of the DNA damage response and liver toxicity

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Cellular homeostasis is essential for maintaining a healthy and normal-functioning liver. Homeostatic disruption caused by chemical compounds can cause temporary or chronic liver injury and even complete liver failure. There is a vast array of proteins involved in cellular homeostasis that controls the response to diverse types of stress. One of these proteins is the transcription factor p53, primarily known for its function to maintain genomic stability, control transient and permanent cell cycle arrest and apoptosis. Activated p53 regulates the expression of many proteins such as Mdm2, p21 and Btg2, which play a role in shutting down the p53 response (Mdm2) and in regulation of cell cycle arrest (p21, Btg2). We aim to improve our understanding of the mechanism by which p53 signaling determines cell fate decisions and its relevance for potential liver toxicity. We therefore developed an Ordinary Differential Equation (ODE) model that couples the expression dynamics of p53 and these three downstream targets, Mdm2, p21 and Btg2, to cell cycle progression. We based our model on data acquired with p53, Mdm2, p21 and Btg2 HepG2 BAC-GFP reporter cell lines with which we measured chemical-induced protein expression dynamics using live-cell imaging. Cells were exposed to various concentrations of cisplatin to induce DNA damage and we subsequently performed time-lapse imaging for 65 hours to measure GFP intensities per cell, reflecting the amount of GFP-labelled protein present in that cell. Moreover, we monitored cell cycle progression in single cells using fluorescent ubiquitination-based cell cycle indicator (FUCCI) technology in HepG2 cells exposed to the same concentrations of cisplatin. With our data-driven ODE model, we were able to mechanistically describe the interactions between proteins involved in the DNA damage response with p53 as central regulator. Moreover, our model predicted that p21 primarily affects the G2/M transition, rather than the G1/S transition. We anticipate that our models can in the future contribute to the establishment of quantitative adverse outcome pathways for chemical safety assessment.

S10-04

Spatial-temporal multiscale-multilevel modeling of APAP damage and its consequence on ammonia detoxification in a virtual liver lobule: steps towards a virtual liver

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In vivo experiments are expensive, time consuming, and underlie strict ethic rules. Their results only partially apply to human. Modern

experimental methods composed of imaging at high resolution, in 3D, or in living tissues provide information that increasingly permit development and parameterization of multi-level computational models, that allow for virtual *in vitro* and *in vivo* experiments. In such models, hypotheses on molecular, cell-level or tissue-level mechanisms can be implemented and their consequence been tested *in silico*. The results can be used to guide experimental decisions and designs (Drasdo, J. Hepat. 2014). Prospectively, this can permit feeding computational models with patient-specific information at each of the above levels and studying the prospective impact of therapeutic interventions. As a step towards a virtual liver lobule, we will report the stepwise development of a multilevel model of APAP (acetaminophen, paracetamol)-induced damage, regeneration and the detoxification of ammonia during regeneration. Hyperammonemia (too high ammonia blood concentration) is a severe complication after drug induced liver damage, for example resulting from overdosing APAP, and can lead to encephalopathy and death of the patient. We will present a spatial-temporal micro-architectural agent-based model (STM) of drug-induced liver damage and regeneration in a liver lobule, the smallest functional micro-anatomical unit of liver. The intracellular ammonia-detoxification model builds upon earlier work in which a compartment model guided identification of a previously unrecognized ammonia sink mechanism missing in the 40-years old consensus reaction scheme and led to a potential therapy approach in hyperammonemia (Schliess, Hepatology, 2014; Ghallab, J. Hepat. 2016). We demonstrate that the spatial-temporal model of a liver lobule constructed from histological images (Hammad, Arch. Toxicol. 2014; Friebe, Bioinformatics, 2015) hence directly representing architectural alterations, in which the detoxification reactions are executed in each individual hepatocyte, is able to identify in how far changes of ammonia detoxification shall be attributed to alterations of lobule micro-architecture or alterations in the intracellular reactions. We demonstrate this by simulating ammonia detoxification in a healthy liver lobule, after APAP-induced pericentral damage, and in a fibrotic liver lobule. The STM may be a prime candidate to simulate *in vitro* to *in vivo* drug toxicity as we sketch by corresponding simulations for APAP toxicity *in vitro* and *in vivo*. Most recent model advancements permit establishment of a pipeline of individual cell resolved virtual simulations of *in vitro* settings up to *in vivo* processes, that integrate realistic cell biomechanics (Van Liedekerke, Biomech and model in mechanobiol, 2020) with intracellular signaling/metabolism and cell-cell signaling hence generating virtual twins of experimentally studied systems of interest.

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S11 | Emerging tools for the investigation and prediction of liver toxicity

S11-01

ULA 3D spheroids as a tool for studying normal and diseased liver function and for prediction of drug pharmacokinetics and hepatotoxicity

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Much effort is devoted to the generation of phenotypically appropriate *in vitro* systems for studying hepatic drug metabolism and clearance, drug hepatotoxicity and liver disease. Of specific importance among liver diseases is NASH which is becoming the leading hepatic disease in the world. The lecture will be focused on a 3D spheroid liver system based on monocultures or co-cultures of PHH and NPCs having a phenotype similar to human liver *in vivo*, where toxicity, clearance and metabolism of drugs can be predicted. Furthermore, in this system it is possible to i) elucidate mechanisms behind chronic drug hepatotoxicity, ii) mimic liver diseases like steatosis and fibrosis and iii) study genetic, mechanistic and environmental factors of importance for NASH and its pharmacological treatment. By choosing different genetically defined donors and siRNA based gene silencing, the contribution of different polymorphic genes and gene products of importance for developing the diseases can be studied.

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S11-02**Functional imaging of hepatotoxicity: Interruption of paracellular bile acid cycling after acetaminophen overdose ameliorates hepatotoxicity***J.G. Hengstler¹, A. Ghallab²¹ Leibniz Research Centre for Working Environment and Human Factors, Dept. of Toxicology, Dortmund, Germany;² South Valley University, Department of Forensic Medicine and Toxicology, Qena, Egypt

Background & Aims: Acetaminophen (APAP) overdose remains a frequent cause of acute liver failure, which in patients is generally accompanied by increased levels of serum bile acids (BA), including glycodeoxycholic acid, a marker of poor prognosis. However, the pathophysiological role of BA remained elusive. Here, we investigated the role of BA in APAP-induced liver injury.

Methods: We performed intravital imaging to investigate BA transport in mice, quantified endogenous BA concentrations in the serum of mice and patients with APAP overdose, analyzed liver tissue by MS and MALDI-MSI, assessed the integrity of the blood-bile barrier by immunostaining of tight junction proteins and intravital imaging of fluorescent markers, identified the intracellular cytotoxic concentrations of BA, and performed interventions to block BA uptake from blood into hepatocytes using the Ntcp inhibitor, Myrcludex B and the Oatp knockout mouse model.

Results: Prior to the onset of cell death, APAP overdose causes a breach of the blood-bile barrier resulting in the leakage of BA from the bile canaliculi into the sinusoidal blood, which is then followed by the uptake of BA into hepatocytes via the basolateral membrane, its secretion into bile canaliculi and repeated cycling. This, what we termed 'futile cycling' of BA, led to increased concentrations of intracellular BA that were high enough to cause hepatocyte death. Importantly, however, the interruption of this cycling by pharmacological Ntcp blockage and Oatp knockout strongly reduced APAP-induced hepatotoxicity.

Conclusions: APAP overdose induces a breach of the blood-bile barrier which leads to futile BA cycling that causes hepatocyte death. Prevention of paracellular BA cycling may represent a therapeutic option after APAP intoxication.

Lay Summary: Only one drug, N-acetylcysteine (NAC), is approved for treatment of APAP overdose and is effective only when given within ~8 hours after ingestion. We identified a mechanism by which APAP overdose causes an increase of bile acid concentrations in hepatocytes that exceeds toxic thresholds. Blocking this mechanism prevented APAP-induced hepatotoxicity in mice and evidence from patients suggests that this therapy may be effective for longer periods after ingestion than NAC.

S11-03**Dynamic imaging of stress response pathway activation for quantitative systems liver toxicity approaches***B. van de Water

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Drug safety testing is a multi-scale process by design, often beginning in cell-based models before progressing to clinical and nonclinical studies. Quantitative systems toxicology models capture the multi-scale nature of drug toxicity from injury at the cellular level, to adverse outcome in tissues and organs. At inception of injury, cellular stress response pathways allow cells to adapt or with sustained activation lead to cell injury and signal progression to adverse cellular and tissue outcomes. Drug-induced liver injury (DILI) remain impor-

tant concern in drug development and an area where QST modeling can improve translational safety predictions. This presentation focuses on modeling drug-induced cellular stress response pathways in liver models across different scales of complexity. First, high content single live cell confocal imaging of GFP-tagged protein captures the dynamics of stress-responsive transcription factors, e.g. Nrf2, Xbp1, p53 and NF- κ B, and corresponding downstream target proteins, allowing detailed quantification of cellular stress-responsive and inflammatory pathways after drug treatments. The detailed temporal- and concentration-dependent dynamics of stress pathways are used to develop computational models of the stress responses linking drug treatment to liver and kidney toxicity. Secondly, *in vitro* stress responses are linked to *in vivo* stress response pathways by building gene co-expression networks for liver. Activation levels of the various stress gene networks by the above cellular stress responsive transcription factors are assessed systematically and quantitatively for both DILI. Recently, we have linked the stress response networks to the association of the various network perturbations that occur concurrent with adverse pathological outcomes in liver providing mechanistic insights into pathogenesis. Ultimately, mapping cellular stress response network activation and the preservation of networks across scale of complexity from cells to tissues and across species will enable quantitative translational risk assessment for new drugs entering clinical trials.

S11-04

No abstract has been submitted.

S12 | Application of high throughput transcriptomics in mechanism-based chemical safety assessment**S12-01****Utilisation of high throughput transcriptomics for unraveling mechanisms of chemical-induced nephrotoxicity***P. Jennings, L. Capinha, G. Carta

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The prediction of chemical-induced nephrotoxicity and the mechanisms thereof, is an important part in chemical hazard identification. Here we utilised two classes of compounds to compare and contrast transcriptomic profiles related to chemical induced perturbations. The human cell line RPTEC/TERT1 cell line was exposed to different concentrations of a variety of halogenated alkenes and mitochondrial electron transport chain inhibitors. Transcriptomic alterations were assessed using the Tempo-Seq (Biospyder) assay. While both these chemical classes had certain transcriptomic pathways in common, some were strikingly different. The results are presented and the implications discussed.

S12-02**Early prediction of late adverse outcome using bench-mark dose modelling of high throughput transcriptomics data***S.S. Auerbach

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Dose-related changes in gene expression are increasingly being used to characterize the response to chemicals in both *in vitro* and *in vivo* systems. The quantitative accuracy by which biological potency de-

rived from genomic dose response approaches forecasts the toxicological potency of a test article has been characterized extensively in *in vivo* systems and is currently under investigation in *in vitro* systems. However, characterization of the quantitative aspects of toxicity amounts to an incomplete representation of a chemical's toxicological properties with the other major component being the qualitative outcomes which can range from minimal to extreme severity and can impact risk assessment. To address this question, we have undertaken a data mining exercise to identify molecular biomarkers from short-term *in vivo* studies that forecast liver and kidney pathological manifestations in longer term rat studies. Further we explore the behavior of these biomarkers in *in vitro* systems, specifically as it relates to their qualitative translatability and their quantitative relationship to *in vivo* effect levels.

S12-03

Transcriptomic profiling of the inter-individual variability of chemical-induced cellular stress response activation in primary human hepatocytes

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Drug-induced liver injury (DILI) remains a major concern for the clinic and pharmaceutical companies, and therefore there is a need to improve its prediction at an early phase during drug development. One of the early key events of DILI is the activation of adaptive stress responses, a cellular mechanism to overcome stress. Given the diversity of DILI outcomes, it is key to map the inter-individual variability in activation of these stress responses. Accurately capturing this variance, could aid in the improvement of drug toxicity screening strategies. Therefore, we profiled the transcriptome of a panel of 50 cryo-preserved primary human hepatocytes derived from different individuals exposed for 8 or 24 h to a broad concentration range of tunicamycin for unfolded protein response (UPR), diethyl maleate for oxidative stress response, cisplatin for DNA damage response and TNF α for NF- κ B signaling. Transcriptomic profiles were related to LDH leakage as a measure for cytotoxicity. The variance in the concentration-dependent stress response activation among individuals could be captured, where the average of point-of-departures (PoD) for UPR-related genes resulted in a maximum difference of 866-fold between different hepatocytes. For each stress response, hepatocytes were classified based on a sensitivity score using maximum fold change and PoD for pathway-related genes. Correlation of the sensitivity scores and their background such as disease status was identified. The most sensitive and insensitive hepatocytes for UPR or oxidative stress response were further characterized using various hepatotoxicants. Given that often a small panel of PHHs is used during drug toxicity screening, the influence of the panel size on the estimation of the variance in stress response activation was evaluated. Using a population mixed-effect framework, the distribution of the PoDs and maximum fold change were modelled allowing to simulate smaller or larger PHH panel sizes. Here, small panel sizes systematically under-estimated the variance by 2-fold indicating that either PHH panel size should be increased or safety factors could be used to obtain improved predictions of the variability and DILI susceptibilities at an early phase of drug development.

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S12-04

Genomics-based platforms in combination with machine learning algorithms enabling well informed and reliable risk assessments for different toxicological endpoints

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The use of animal-based testing approaches within the field of toxicology is under increasing scrutiny, and efforts are being made to drive a paradigm shift to replace such methods with animal-free and resource-effective New Approach Methodologies. This shift is accelerated by innovative methods which leverage on the latest scientific and technological advances and integrate these into the assay development process. In this context, robotics and high-throughput screening has accelerated the generation of high informational content (i.e. "big" datasets), which in combination with state-of-the-art machine learning algorithms have been used to develop transcriptomic-based human-relevant *in vitro* test systems capable of providing mechanism-based and well-informed hazard and potency predictions.

The purpose of this presentation is to illustrate the potential for next generation transcriptomics-based *in vitro* methods to improve hazard assessments and provide reliable quantitative potency information for a broad diversity of chemicals. The GARD technology platform and the endpoint of skin sensitization will be used as a proof-of-concept to demonstrate how such methods can bring novel elements to the field of regulatory toxicology and contribute to highly accurate predictions. The GARD assay integrates state-of-the-art components, such as transcriptomic-based biomarker signatures, machine learning-assisted classification models and a streamlined analysis pipeline facilitated by cloud-based software.

This presentation will describe the assay development, including the selection of an appropriate cell system, the unbiased exploratory driven analysis utilized during biomarker discovery, and discuss the mechanistic relevance of the selected biomarkers. Results from a formal validation study will be presented, demonstrating an added value in terms of performance and broad applicability to existing strategies for hazard and risk assessment. However, despite the demonstrated advantages, the use of advanced technological components also pose specific hurdles towards regulatory acceptance. These will be discussed, and our view on how such hurdles may be overcome will be shared with the audience. Moreover, novel results from dose-response analysis of the transcriptomic data will be demonstrated, and a tentative approach describing how this information can be utilized for quantitative assessment of relative sensitizing potency on a continuous scale will be described. Finally, a proof-of-concept study on respiratory sensitization will be provided to illustrate how the conceptual framework can be further extended to other toxicological endpoints beyond skin sensitization.

In summary, this talk will demonstrate the potential for *in vitro* assays based on toxicogenomics, here exemplified by the GARD assay, to fill data gaps and provide well-informed and highly accurate hazard and potency predictions, fit for purpose for regulatory decision-making.

S13 | Modes of action in non-genotoxic carcinogenesis

S13-01

Developing an integrated approach for the testing and assessment of chemical non-genotoxic carcinogens for global regulatory purposes

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While regulatory requirements for carcinogenicity testing of chemicals vary according to product sector and regulatory jurisdiction, the standard approach starts with a battery of genotoxicity tests (which include mutagenicity assays). If any of the *in vivo* genotoxicity tests are positive, a lifetime rodent cancer bioassay may be requested, but under most chemical regulations (except plant protection, biocides, pharmaceuticals) this is rare. The decision to conduct further testing based on genotoxicity test outcomes creates a regulatory gap for the identification of non-genotoxic carcinogens (NGTxC). In 2016 the Organisation of Economic Cooperation and Development (OECD) established an expert group to address the development of an integrated approach to the testing and assessment (IATA) of NGTxC, with the objective of addressing this gap. Through that work, a definition of NGTxC in a regulatory context was agreed. Using the adverse outcome pathway (AOP) concept, various cancer models were developed, and overarching mechanisms and modes of action were identified. After further refining and structuring with respect to the common hallmarks of cancer and knowing that NGTxC act through a large variety of specific mechanisms, with cell proliferation commonly being a unifying element, it became evident that a panel of tests covering multiple biological traits will be needed to populate the IATA. Consequently, in addition to literature and database investigation, the OECD opened a call for relevant assays in 2018 to receive suggestions. Here we report on the definition, on the development of the overarching NGTxC IATA, the development of ranking parameters to evaluate the assays, selected evaluations, and a preliminary regulatory framework. Ultimately, the intent is to select best scoring assays for each IATA module for further validation towards OECD test guideline development, and for their integration in a NGTxC IATA to better identify carcinogens and reduce public health hazards.

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S13-02

Cellular and newly proposed models to study the transforming ability of pollutants for translational toxicology

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Human carcinogenesis is a complex process, which requires the alteration of several biological traits and the simultaneous modulation of multiple gene pathways. Although it is well recognized that chemical carcinogens may disrupt cell and tissue homeostasis playing a fundamental role in human carcinogenesis, it is still difficult to un-

derstand their mode and mechanisms of action. Hence, there is an urgent need to improve and complement current testing methods for risk assessment of carcinogens with novel technologies based on rational implementation of what we learn from the fast-paced advances in cancer biology and evolution. For example, there is increasing evidence that the exposure to xenobiotics triggers an immune response leading to inflammation, which represents one of the main drivers in the onset and progression of several human tumors. To explore the role of carcinogens in the initiation and progression of cancer, we developed the transformics assay by combining the cell transformation assay (CTA) with the microarray transcriptomics. CTA has been long regarded as a promising tool to study the process of carcinogenesis *in vitro*, even if it cannot be used as a stand-alone test for the classification of carcinogens. However, CTA provides an easily detectable endpoint of oncotransformation, which can be used to anchor the exposure to the acquisition of the malignant phenotype. We applied the transcriptomics approach to the BALB/c 3T3 CTA in order to identify the molecular steps leading to *in vitro* malignant transformation following the exposure to chemicals. The analysis of modulated genes, gene pathways and associated biological processes gave evidence for the involvement of the innate immune response, including the upregulation of genes coding for inflammatory chemokines, sustaining the process leading to the epithelial mesenchymal transition, which marks the steps to progression in human tumors. The chemical concentration is a key factor in the turning point towards malignancy. In conclusion, the transformics innovative approach has the potential to be a valuable additional asset in the identification of the role of carcinogens in the multistep process of carcinogenesis, and in the evaluation of thresholds for non-genotoxic carcinogens in translational toxicology.

S13-03

Non-coding RNAs mechanisms enforcing oncogenic programs and allowing establishment of metastatic niches

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The acquisition of genetic lesions is the defining initiating events of tumorigenesis, however, there is an increasing realisation that in a number of different cancer types organ-damage can pre-dispose individuals to the development of tumours. For example, individuals that have fatty liver disease or cirrhosis of the liver from alcohol consumption are more likely to develop hepatocellular carcinoma. These conditions are both associated with fibrosis as a result of altered and increased production of extracellular matrix components. We have been investigating how these gene-expression programs are established and controlled by both coding and non-coding RNA molecules. Importantly, we have been examining how the translation landscape is changed to achieve the enhanced requirement of endoplasmic reticulum coupled protein production in these conditions. These studies are starting to shed light on the role of fibrotic deposition by extracellular matrix production, and the parallels to the metastatic niches, to tumour initiation and maintenance.

S14 | New approaches using *in vitro* assays and 3D models can improve prediction of immune reactions to xenobiotics

S14-01

Immune response to chemicals and drugs: understanding is key for prediction

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Allergic reactions to drugs and chemicals are mediated by an adaptive immune response involving T and B lymphocyte responding to the xenobiotic bound to a protein or not. During thymic selection, T cells that have not yet encountered their cognate antigen are considered naive T cells. Due to the artificial nature of drug/chemical-T cell epitopes, it is not clear whether thymic selection of drug/chemical-specific T cells is a common phenomenon or remains limited to few donors or simply does not exist, suggesting T-cell receptor (TCR) cross-reactivity with other antigens. Several models have been published in the literature suggesting that activation of the adaptive immune response in the case of chemical or drug allergy can follow different rules depending on multiple factors. In the hapten model, drugs/chemicals or haptens bind to self-proteins to form a complex of a sufficient size to trigger an immune response. This structure is then processed by antigen presenting cells (APCs) and the resulting haptenated peptides are presented through MHC class or class II-dependent pathways to TCRs as *de novo* antigens. In the pharmacologic interaction with immune receptors (p-i) model, the drug binds non-covalently to either the TCR (p-i TCR) or MHC protein (p-i HLA) or to both in a peptide-independent manner to directly activate T cells. In this model, several experimental evidence support that some drugs can trigger T-cell activation without requiring intracellular antigen processing. This interaction leads to rapid T-cell-mediated reaction, which has features of hypersensitivity, and/or alloimmune and/or autoimmune reactions. Finally, a new concept emerged recently: the altered peptide model where a molecule can bind non-covalently to the MHC-binding cleft directly or in the endoplasmic reticulum (ER) altering the specificity of peptide binding and resulting in the presentation of novel peptide ligands.

It is clear that the mechanisms of immunisation and/or stimulation of adaptive immunity to chemicals and drugs is complex but it is an absolute prerequisite for allergy to occur. Understanding these mechanisms is necessary to develop adequate cellular and molecular models to identify these possible mechanisms. An AOP for allergic dermatitis has been published and is extensively used. Developing an AOP for other types of allergy is the next step for developing new approaches and tests.

S14-02

Development of new approaches to predict drug-induced hypersensitivity with an increased understanding of reaction mechanisms

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T-cell-mediated drug hypersensitivity reactions represent a major clinical problem as they are currently impossible to predict. Susceptibility is dependent on: (1) exposure to the drug antigen in a relevant form (2) the availability of a T-cell repertoire for the drug antigen and

(3) the availability of HLA molecules for drug antigen binding. However most individuals who express all three factors do not develop hypersensitivity when exposed to drugs. This is because immune regulatory pathways also contribute to susceptibility through controlling the strength, nature and duration of the immune response. In this presentation I will review how immune regulatory pathways impact on the development of a drug-specific T-cell response and how a knowledge of drug immune cell exposure and immune regulation has assisted the development of *in vitro* assays to prime naive T-cells to drugs. I will also detail how a step-wise screening approach is being developed for drug immunogenicity assessment using lymphocytes from HLA-typed healthy donors.

S14-03

The challenges of predicting biological products immunogenicity using T-cell assays

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Owing to the risk that immunogenicity of new biopharmaceuticals (BPs) stops their clinical development, an important request from pharmaceutical companies is to anticipate these issues at early stages of drug development. Generally, drug selection is driven by pre-clinical studies performed in animal models. However, animal models are not considered as good predictive models of immunogenicity in humans, the humanized proteins being recognized as non-self in animals. As CD4 T-cells are key players of the initiation of the immune responses, including anti-drug antibody (ADA) response, predictive tools of immunogenicity aim at deciphering the ability of BPs to stimulate T cells in humans. The main predictive approaches evaluate different steps of the process leading to T cell stimulation. *In silico* prediction predicts the capacity of binding to frequent HLA class II molecules, of peptides comprised into the BP sequence. The MHC associated peptide proteomics approach identifies the peptides displayed at the surface of antigen presenting cells loaded with the BPs. Finally, T cell assays using cells collected from healthy donors evaluate whether BPs prime a new T cell response *in vitro* and hence evaluate whether T cells specific for the BPs are found in the naïve T cell repertoire. Prediction tools of immunogenicity do not therefore directly predict immunogenicity but reveal a potential of response, which is one of the main factors contributing to immunogenicity. Many formats of T cell assays exist but all the approaches have in common to detect BP-specific T cells in donors, who have never been exposed to BPs. Because of the low frequency of specific T cells in the naïve repertoire, we have developed the T cell amplification assay, which relies on weekly rounds of stimulation with BPs to enrich the cell culture in specific T cells. In this assay, antibodies known to be immunogenic in multiple treated patients generate a higher T cell response than poorly immunogenic antibodies, while immunogenic human cytokines, hormones or growth factors such (erythropoietin, relaxin, beta-interferon) also lead to *in vitro* T cell stimulation, revealing the low tolerance induced to this fully human proteins. Multiple T cell epitopes have been also identified in therapeutic antibodies and some other BPs using the T cell amplification assay. In all antibodies, the non-germline amino acid sequences, mainly found in the CDRs, combined with their capacity to be presented by HLA class II molecules appear to be the main driver of immunogenicity. In contrast, T-cell epitopes identified in human cytokines, hormones or growth factors are not mutated as the T cell response to these BPs results from an escape of T-cells from thymic selection. Together, the recent outcomes about T-cell response to BPs highlight the advances done in immunogenicity assessment at early preclinical stage but also differences of mechanism of immunogenicity across the different types of BPs.

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S14-04

Challenges and opportunities of 3D-skin models: the way forward for assessing chemical sensitizers?

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Contact with the skin is inevitable or desirable for daily life products. Whereas the majority of these products are harmless, a number can become metabolized and/or activate the immunological defense via innate and adaptive mechanisms resulting in sensitization. Repeated exposure with the same sensitizing substance may result in allergic contact dermatitis. Therefore strict safety (hazard) assessment of actives and ingredients in products and drugs applied to the skin is essential to prevent sensitization. This means determining i) whether the chemical is a potential sensitizer and if so ii) what is the safe concentration for human exposure to prevent sensitization occurring. A clear overview will be presented of the organotypic *in vitro* skin models (reconstructed human epidermis, reconstructed human skin, immune competent skin models incorporating Langerhans Cells and T-cells, skin-on-chip) that are currently commercially available or which are being used in a laboratory research setting for hazard assessment of potential sensitizers. This will include models being used for investigating the mechanisms (sensitization key events 1–4) related to allergic contact dermatitis. The limitations of the models, their current applications and their future potential in replacing animals in allergy related science will be discussed.

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S15 | Impact of climate change on food safety

S15-01

The impact of climate change on mycotoxin and related fungi risks in Europe: current scenario and future perspectives

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As determined by the Intergovernmental Panel on Climate Change, warming of the climate system is unequivocal and has been associated with rising sea levels, diminished amounts of ice and snow and increasing oceanic and atmospheric temperatures. Such climate changes have a significant impact on stages and rates of toxigenic fungi development and can modify host-resistance and host-pathogen interactions, influencing deeply also the conditions for mycotoxin production that vary for each individual pathogen. Moreover, the new combinations mycotoxins/host plants/geographical areas are arising to the attention of the scientific community and require new diagnostic tools and deeper knowledge of both biology and genetics

of toxigenic fungi. In this review, it is underlined that an extension of the aflatoxin contamination risk in maize in South and Central-Europe is highly likely in the next 30 years, due to favorable climatic conditions to the growth of *Aspergillus flavus*. Moreover, the myco-toxigenic *Fusarium* species profile on maize and minor cereals such as wheat in Europe is in continuous change in Northern, Central and Southern-Europe with, in particular, a worrisome growing contamination of *F. graminearum* in the Central and Northern Europe on both maize and minor cereals, *F. proliferatum* on wheat in Southern-Europe, and *F. verticillioides* in Northern Europe on minor cereals. The current scenario of main toxigenic fungi occurrence in cereals in Europe will be provided, together with the main risk that we will face in the future perspectives.

S15-02

Global change impacts on harmful algal bloom toxicity

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Global environmental change involves shifts in a wide range of environmental factors including elevated CO₂ levels, warming and changes in nutrient availabilities. Many cellular processes rely on the availabilities of nutrients, while temperature is a major driver for metabolic rates, and CO₂ availability governs photosynthesis and carbon acquisition in primary producers. Consequently, global change is expected to affect algal growth and toxin synthesis, which both ultimately determine harmful algal bloom toxicity. In this talk, I will highlight key findings from meta-analyses on the impacts of global change factors on harmful algal growth and toxin production. Results demonstrated that elevated CO₂ levels promoted growth of harmful algae, and thus may support their proliferation. Similarly, warming caused enhanced growth rates, though only for species isolated in temperate regions^[1]. The impacts of elevated CO₂ levels and warming on toxin synthesis strongly varied and did not show a consistent response across species. Algal toxins can be classified as nitrogen rich when they consist of amino acids, or rely on amino acids as precursor, or as carbon based, for instance when they rely on polyketide synthesis pathways. Shifts in the cellular balance of carbon and nitrogen may therefore alter toxin synthesis. Indeed, recent findings confirm strong dependency of nitrogen for nitrogen rich toxins, which generally decrease under nitrogen limitation, but increase under phosphorus limitation when nitrogen is in relative excess^[2]. Carbon rich toxins tend to increase under both nitrogen and phosphorus limitation, where carbon is in relative excess^[3]. Overall, our findings demonstrate that algal toxin synthesis show stoichiometrically predictable patterns. Moreover, while elevated pCO₂ and warming seem to benefit the proliferation of harmful algae, the overall impacts on bloom toxicity will strongly depend on concomitant changes in nutrient availabilities.

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S15-03

Ocean warming and the risk of ciguatera poisoning

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Ciguatera Poisoning (CP) is the most common, non-bacterial illness associated with consumption of seafood (certain fish and gastropods) contaminated with neurotoxins (ciguatoxins and/or maitotoxins) produced by the marine microalgae in the genus *Gambierdiscus* and *Fukuyoa*. CP causes acute gastrointestinal, neurologic, and cardiac symptoms and long-term sequelae, particularly due to chronic exposure to the toxins. Thus, CP has substantial human health, social, and economic impacts in the affected areas. CP is endemic in pantropical regions, were approximately 50,000 cases documented annually, although the real impact is not known given to underreporting (90%). Since the beginning of the 21st century, CP cases have been detected in more temperate zones with no previous history of this disease. Some cases seem to be due to an increased international trade of tropical sea-products. In addition, climate and anthropogenically-driven environmental changes may be favouring a potential expansion of CP. In particular, ocean warming, projected on 0.4–1.4°C by mid twenty-first century, is expected to affect the biology, cell proliferation and biogeographic range distribution of the microalgae involved in CP. However, there are still many unknown aspects that require in-depth and multidisciplinary research, specially related to the vectors of toxin transfer. This presentation will summarize the most recent knowledge about the potential risk of CP in the near future.

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S15-04

Tetrodotoxins in seafood from European waters

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Tetrodotoxin (TTX) and its analogues are a well-known toxin because of severe and lethal incidents in people consuming pufferfish (fugu) and occasionally other seafood^[1]. Several years ago it was detected in shellfish from European waters, first in Greece^[2], then the UK^[3] and subsequently the Netherlands^[4]. In Greece this was based on follow-up of false-positive test results in the mouse bioassay, in the two other countries after targeted LC-MS analysis of mussels and

oysters. The producing organism of the toxin is still unclear, as well as the exact conditions leading to the formation and contamination of shellfish. It is also unclear whether this is a new phenomenon or has been undetected thus far, potentially due to no clear poisoning cases at the levels present in the shellfish or an extension of the harvesting period, now covering also the summer months.

Surprisingly, no proper risk assessments, including health based guidance values, had been published for this well-known group of toxins, although in Japanese papers a Minimum Lethal Dose of 2 mg is mentioned. Other papers mention hospitalization of consumers after an estimated intake of 0.2 mg TTX. EFSA was therefore asked by the European Commission to perform a risk assessment^[5]. Based on a novel study in mice^[6], EFSA derived an acute reference dose (ARfD) of 0.25 µg/kg bw. This was based on a NOAEL of 25 µg/kg bw and an uncertainty factor of 100. Reported human cases did not allow their use for deriving a reference point for the assessment but indicate that the ARfD is not conservative. EFSA also calculated that a large portion size of 400 gram shellfish should not contain more than 44 µg/kg of TTX or analogues to exclude effects in consumers. This level is occasionally exceeded, as shown in monitoring programs.

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S16 | Human microengineered organs-on-chips: advancing regulatory science through innovation

S16-01

Organs-on-chips for safety testing and disease modeling

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Over the last decade, the ascendance of translational science within the Pharmaceutical Industry has resulted in an increased implementation of *in vitro* models within drug projects to advance preclinical safety and efficacy. Of particular interest are models developed using organ-on-a-chip technology. Briefly, Organ-Chip models combine engineering and biology to recreate the delicate microenvironment found *in vivo*. Typically, this is achieved through microfluidics which provides a shear stress to the cells, and where appropriate, mechanical stretch to emulate the forces cells experience during peristalsis or breathing. Organ-Chips are also designed to create the tissue-

tissue interface where epithelial cells of an organ are placed in co-culture with organ-specific endothelial cells. The hypothesis is that by recreating *in vivo* conditions outside the body, the data produced will have a greater translational confidence into the clinical phases of development. This presentation will begin by briefly outlining Organ-Chip technology. It will then cover some use case examples for both assessment of drug safety and some newer examples of where Organ-Chips can be used for efficacy.

S16-02

Human body on a chip – are we there yet?

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Single- and multi-organ-on-a-chip systems are becoming a sustainable part of the *in vitro* assay landscape for preclinical drug testing. This triggered first attempts to emulate the next level of human biology – miniaturized human bodies on a chip. Autologous organoids of various organs have been differentiated from a single individual donor using induced pluripotent stem cell technologies. These can be used to create miniaturized on-chip models of entire human bodies. Such human body-on-a-chip devices bear the potential of testing new drugs in personalized on-chip trials prior to clinical trials in patients. Although ambitious, the development of such human body-on-a-chip systems has been recognized as a promising route toward a paradigm shift in the drug development. Progress, challenges and visions are discussed in the presentation.

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S16-03

Incorporating organ-on-chip into integrated approaches to testing and assessment

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Researchers continue to generate new approach methodologies (NAMs), including methods with increasingly sophisticated technology. When such methods recreate a more accurate physiology, their integration into the regulatory decision-making is of major value.

Microphysiological systems such as organ-on-chip (OoC) have been praised for their increased biological relevance. Nevertheless, they are still not used in regulatory decision-making despite the fact of being already utilised in other areas (such as in research and Pharma). One can argue if this is due to their lack of standardisation or to the assumption that OoC data are used in isolation. What if the data generated is part of an integrated process and is weighted for its relevance and the associated uncertainties? Is there enough understanding, confidence and standardization among these systems that would allow using them in an Integrated Approach to Testing and Assessment (IATA)?

IATA is a flexible tool for hazard identification, characterisation and/or safety assessment of a chemical or group of chemicals developed at the OECD to evolve the current regulatory toxicity testing and approaches. Such chemical evaluation results from the integration and translation of the data derived from multiple methods and sources. Besides integrating, this approach also weights all relevant evidence, frames its uncertainty and guides the targeted generation of new data, where required. IATA also puts *in vivo* data in context with mechanistic studies and at the same time avoids duplication of studies. This not only increases the relevance of the assessment, but

it also allows the trustworthy introduction of NAMs. At the Joint Research Centre (JRC) level, EURL ECVAM has been actively involved in several efforts towards the further development and implementation of IATAs. Examples range from the drafting of the overview of concepts and available guidance on IATAs and their components to the participation in the OECD IATA case studies and in the development of specific IATAs for non-genotoxic carcinogenicity, developmental neurotoxicity (DNT) and acute fish toxicity.

OoC generated mechanistic data in regulatory decision-making. The presentation will also discuss questions such as, the level of OoC standardisation and the flexibility needed to embrace the rapid evolution of this technology as well as the formality motivated by expectations for validation and standardization.

S16-04

An industry perspective: importance of organs-on-chips for advancing drug discovery and development

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Using human-relevant, translational *in vitro* models has been widely considered to reduce attrition during drug discovery and development. Over the past decade a considerable hype emerged regarding the transformative potential of microphysiological systems for pharmaceutical research; yet – while it is agreed that there are several obvious areas where such models could bring value – currently, mostly proof-of-concept studies are available and widespread application is still lacking. Thus, while acknowledging the opportunity and value such human relevant cell systems could provide, the adoption by pharma companies is moderate at best. Realizing the full potential of these models will need more clear use-cases demonstrating clinical translation, improvements on technical ease of use and greater collaboration between stakeholders. Furthermore, it is proposed that refining existing platforms for specific contexts of use where significant gaps exist in drug development will help broader application, rather than unrealistic claims that microphysiological systems can right away replace the complete drug discovery engine at once. Key advantages of such tissue systems over traditional pre-clinical models, e.g. the ability to mimic human-specific biology such as immunology or defined contexts of rare diseases should be further exploited to establish more use cases that demonstrate true added value. Modeling & analytics can help with back- and forward translation using real world data. Furthermore, the ability to generate patient-derived tissue models will allow personalization of treatments and support precision medicine approaches in clinical trials.

S17 | Designing toxicology studies to support development of cell-based therapies

S17-01

Regulatory Considerations for Cell Based Therapies

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The talk will cover the nonclinical regulatory expectations for clinical trial authorisation (CTA) applications and Marketing Authorisation Applications (MAA). the talk will focus on European expectations, but reference will be made to the USA and rest of the world.

S17-02**Non-clinical study design consideration in the development of cellular therapeutics and cellular and devices combinations***M. Johnson*Northern Biomedical Research, Inc,
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Non-clinical development of cell therapies and combination products include a cellular and device component has been ongoing for more than three decades. Development of these therapies has accelerated in the recent years with development of more complex combination therapeutics to treat unmet needs. As these have developed, regulatory agencies worldwide have adapted new review processes and a wide range of guidance documents to aid researchs in designing non-clinical safety, biodistribution, and efficacy assessments. This presentation will provide an overview of the regulatory guidelines, some key considerations in the non-clinical development and testing of cellular therapies and detail several case studies.

S17-03**Preclinical assessment of a pluripotent cell therapy for treatment of Parkinson's Disease***A. Kirkeby^{1,2}¹ *Lund University, Dept. Experimental Medical Sciences,
Lund, Sweden;*² *University of Copenhagen, Dept. Neuroscience,
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Parkinson's Disease involves the loss of dopamine neurons in the midbrain, giving rise to the main motoric symptoms of the disease. Previous work has shown that replacement of the lost dopamine neurons through transplantation of new neurons from human fetal ventral midbrain can, when successful, restore dopamine levels in the brain and lead to marked clinical improvement. In this project, we have developed a novel ATMP cell product (STEM-PD), consisting of human dopaminergic neuron progenitor cells derived from human embryonic stem cells. STEM-PD has been manufactured under GMP and undergone extensive preclinical safety and efficacy testing. These studies have provided evidence that the cell product is efficacious in restoring motoric function in animal models of Parkinson's Disease, while showing long-term persistence of grafts with no toxicological concern or biodistribution to other tissues.

S17-04**How cell therapies derived from pluripotent stem cells are evaluated for tumorigenicity *in vivo* and *in vitro****D.B. Toft*Novo Nordisk A/S, Stem Cell R&D, Måløv, Denmark*

One of the main concerns developing cell therapies derived from human embryonic stem cells is the tumorigenic potential, that could arise from residual undifferentiated pluripotent or transformed cells in the Drug Product. Given the complex nature of cell therapies a thorough risk assessment, based on donor history, stem cell bank specifications, release assay sensitivity, and the robustness of the differentiation protocol, is required. This talk will present options for evaluating tumorigenicity *in vitro* and/or *in vivo* and the alignment of these methods with relevant guidelines from the European, US and Japanese Health Authorities. Even though potential tumorigenicity of a cell therapy in development should be evaluated on a case by case basis in close dialogue with regional and local regulatory agencies, some level of harmonisation across regions would be of great benefit for entities involved in the global development of new cell therapies.

S18 | Mechanistic toxicology as the basis for modelling and prediction of organ-specific toxicity**S18-01****Applying the adverse outcome pathways network for understanding and predicting neurotoxicity***A. Bal-Price*European Commission, Joint Research Centre (JRC), Ispra, Italy*

Recent developments have prompted the transition of empirically based testing of late stage toxicity in animals to more efficient and predictive mechanistically based approaches. To support this paradigm shift in regulatory toxicology testing and risk assessment the Adverse Outcome Pathway (AOP) concept has been proposed which relies on understanding causal relationship between molecular initiation event and a sequential series of measurable key events at cellular, tissue, organ and organism level that ultimately result in adverse outcome.

New testing approach, based on a battery of *in vitro* assays anchored to common key events (CKEs) identified in the existing neurodevelopmental and adult neurotoxicity AOP network including alterations in BDNF levels, neurite outgrowth and synaptogenesis will be presented. These CKEs are relevant to impairment of learning and memory in children, the most frequent adverse outcome identified in the existing DNT AOPs. During this presentation the alteration of CKEs triggered by exposure to POPs mixtures at the concentrations found in human blood will be discussed. The obtained data suggest that low concentrations of POPs become neurotoxic in mixture, especially after longer exposure (14 days) and alterations in neurite outgrowth and synaptogenesis are the most sensitive *in vitro* test methods. The applied *in vitro* assays (anchored to CKEs of AOP network) facilitate mechanistic understanding of the obtained data and the applied AOP-concept driven testing represents a valuable conceptual framework to evaluate developmental neurotoxicity induced by single chemicals or in mixture with potential to cause learning and memory impairment in children.

Furthermore, AOP-informed Integrated Approaches to Testing and Assessment (IATA) will be proposed for different regulatory purposes including screening and prioritization, hazard identification/characterization and risk assessment. For generation of new data IATA framework based on a set of non-testing and *in vitro* assays anchored to key neurodevelopmental processes and AOP KEs used in a flexible combination (fit-for-purpose) as well as challenge for information integration, coming from these multiples sources will be discussed. Such IATA would facilitate an application of mechanistic knowledge built in relevant AOPs into neurotoxicity evaluation and prediction, increasing scientific confidence in decision-making process.

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S18-02**Application of the adverse outcome pathway conceptual framework for translation of mechanistic data into regulatory decisions: adverse outcome pathways for kidney injury as case study***A. Mally*University of Würzburg, Department of Toxicology, Würzburg, Germany*

There is general consensus that comprehensive understanding of how chemicals and drugs cause adverse effects is key to the development of human relevant, animal-sparing solutions for safety testing. Adverse Outcome Pathways (AOPs) represent a formal description of the mechanistic linkage between a molecular initiating event, a series of intermediate steps and key events (KEs) at different levels of biological organization, and an adverse outcome. In line with recent OECD activities on the use of AOPs in developing Integrated Approaches to Testing and Assessment, identification of KEs and systematic mapping of AOPs may provide a mechanistic framework for the development and implementation of mechanism-based *in vitro* endpoints capable of predicting quantitative points of departure for safety assessment that may form part of an integrated testing strategy to reduce the need for repeated dose toxicity studies. As a case study, mechanistic understanding of kidney injury initiated by (1) inhibition of mitochondrial DNA polymerase γ (mtDNA Poly) and (2) receptor mediated endocytosis and lysosomal overload were systematically captured in a formal description of AOPs in line with the OECD AOP development programme. Using model nephrotoxic drugs and *in vitro* assays covering KEs in each AOP, quantitative response-response relationships between KEs were established and successfully used to predict *in vitro* toxicity. Integration of *in vitro* KE data with toxicokinetic models comprising a proximal tubule cell compartment to transform *in vitro* effect concentrations to a human equivalent dose allowed prediction of *in vivo* effective doses. These were compared to human clinical data to assess the performance of the *in vitro* approach to risk assessment.

S18-03**Novel means of enabling high-throughput toxicogenomics and adverse outcome pathways for prediction of lung toxicity**

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Over the past few years several putative Adverse Outcome Pathways (AOPs) for lung pathologies have been developed through data mining and case study approaches. Recently, an AOP network for lung injury was proposed, which highlighted the intermediate node key events (KEs) that can be used to inform systematic development and application of alternative methods for prediction of lung toxicity induced by nanomaterials. Advanced toxicogenomic approaches and data from cell-based high-throughput transcriptomics (HTTr) assays are suggested to greatly accelerate the development of AOPs, to generate the data required for validating KEs, and to support first tier hypothesis-generation during the early stages of various risk assessment procedures. However, translation of the complex data into useful information for uptake and application in decision making remains a challenge. This talk will summarize a few of the ongoing collaborative

initiatives on the effective refinement and anchoring of toxicogenomic results and tools to AOPs. Examples include application of bioinformatics approaches integrating and linking biological pathway databases to enable verification and quantification of toxicogenomics-defined KEs, AOPs and AOP networks in independent experiments, and the establishment of AOP-informed *in vitro*-based predictive models of lung toxicity. Insight into new concepts of AOP-linked toxicogenomics-driven hypothesis-generation early in risk assessment procedures to support testing prioritization, will be provided. In addition, novel means of translating and linking FAIR (Findable, Accessible, Interoperable and Reusable) data, results and approaches to tools and parameters relevant for regulatory risk assessment practices, will be discussed.

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S18-04**Development and application of an adverse outcome pathway of cholestatic liver injury***M. Vinken*Vrije Universiteit Brussel, Department of In Vitro Toxicology, Brussels, Belgium*

Adverse outcome pathways (AOPs) are pragmatic tools in toxicology and human risk assessment with broad potential. AOPs are designed to provide a clear-cut mechanistic representation of toxicological effects that span over different layers of biological organization. Because of its unique location and function in the organism, the liver is a frequent target for systemic toxicity. Not surprisingly, several AOPs related to liver toxicity have been introduced over the past few years, yet only one focuses on accumulation of bile acids. Nevertheless, this so-called cholestatic hepatotoxicity underlies a majority of drug-

induced liver injury cases and has also been associated with various other types of chemicals, including food additives, cosmetics and biocides. The present presentation will give an overview of the development of this AOP on cholestatic liver injury. Furthermore, testing of the robustness and applicability domain will be presented, and its recent optimization and modification into an AOP network will be discussed.

S19 | Can we panelize seizure?

S19-01

Seizure liability in drug discovery and development: current state of play

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Drug-induced convulsions or seizures are a serious, potentially life-threatening adverse drug reaction that can result in failure of drugs to be licensed for use, withdrawn from the market or labelling implications. Compounds associated with this liability span a wide variety of pharmacological classes and therapy areas, including many not targeted at the central nervous system. Assessment of seizure risk traditionally occurs late in the drug discovery process using low-throughput, resource intensive *in vivo* behavioral models. For example, one known CNS active compound was associated in preclinical species with convulsions confirmed as being seizures. The overall risk assessment did allow to progress into Phase I clinical trials with appropriate safety monitoring (incl., EEG) but several adverse events (syncope, clinically observable seizure, asymptomatic EEG spikes) were noted at exposures below where seizure was seen preclinically, halting the clinical trial. In a second example from a neuroscience project, candidate compounds induced convulsions in rodent and non-rodent species, at low exposure levels. All compounds inducing convulsions *in vivo* showed a strong inhibitory activity on GABA-A channels, whereas compounds with mild or no inhibitory effect did not induce seizures supporting the hypothesis of an off-target mechanism. In EEG experiments, convulsions were always preceded by premonitory clinical signs and abnormal EEG, confirming their CNS origin. The lead compounds in this project were discontinued. These examples illustrate how current approaches do not allow sufficient time to mitigate risk by influencing chemical design. Early identification using cheaper, higher throughput and predictive assays with lower animals and compound requirements would be preferable. Over the last decade, several assays that are compatible with the design-make-test-analyze cycle of drug discovery have become available. *In silico*, *in vitro* and *in vivo* assays may be combined in a rational step-wise cascade to allow more effective i) hazard detection and elimination in hit identification and lead optimization, ii) risk assessment on drug candidates taking into account the safety margin, therapeutic indication and patient population and iii) risk mitigation and management in clinical development with appropriate monitoring and rescue therapies.

S19-02

Exploring the utility of an ion channel panel for detecting seizure liability

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There is clear evidence for the involvement of ion channels in seizure (Easter *et al.*, 2009). Genetic studies have pointed to a role for voltage-gated sodium and potassium channels and the ligand-gated ion channels; GABA-A and nicotinic acetylcholine receptors. Pharmacologically, a number of ion channel modulators are known to be seizurogenic, e.g. Chlorpromazine.

Attrition due to cardiac adverse events has benefitted from the routine screening against the cardiac potassium, sodium and calcium channels, and optimisation of medicinal chemistry away from these liabilities. These assays are automated and high throughput, translate well in humans, and predictive *in silico* models have been built (Bridgland-Taylor *et al.*, 2006; Redfern *et al.*, 2003). Significantly, this strategy has reduced the number of drug withdrawals due to QT prolongation – linked with a fatal ventricular arrhythmia called Torsades de Pointes.

Currently, the detection of seizure liability is reliant upon observations in pre-clinical rodent and non-rodent studies (Easter *et al.*, 2009). Until recently, the only *in vitro* laboratory test available was hippocampal brain slice (Easter *et al.*, 2007) or zebrafish larval locomotor assays (Winter *et al.*, 2008). Both labour intensive and of limited throughput.

Should we therefore be looking to screen compounds in early drug discovery against a panel of seizure-related ion channels, and what would this panel look like? The challenge is to identify which channels to screen and which is the best cellular reagent to use; recombinant cell lines expressing an individual ion channel, or a more physiologically relevant substrate such as stem-cell derived neurones. Since mutations can be both gain- and loss-of-function, the assay needs to be a functional measure of ion channel activity; to measure both agonist and antagonist activity.

An exhaustive list of ion channels involved in seizure would represent over 100 targets (Kullman *et al.*, 2010). This compares with a cardiac ion channel panel of seven (Crumb *et al.*, 2016). Given this level of complexity, we have reduced this list of ion channels to a more manageable number. This ion channel panel can be deployed in two ways. Firstly, mechanistic investigation of seizurogenic compounds; and secondly, for the testing of compounds early in the discovery pathway, to identify ion channel liabilities in seizure-related targets. This strategy, performed with high-throughput and rapid turnaround times, will allow these liabilities to be designed-out during lead optimisation.

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S19-03

Development of seizure prediction methods using *in vitro* microelectrode array (MEA)

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In vitro microelectrode array (MEA) assessment using human induced pluripotent stem cell (iPSC)-derived neurons holds promise as a method of seizure and toxicity evaluation. However, there are still issues surrounding the analysis methods used to predict seizure and toxicity liability as well as drug mechanisms of action. In the present study, we developed an artificial intelligence (AI) using deep learning based on raster plots of neural network activity, and a multivariate analytical method that identifies a parameter set. These AI prediction model and multivariate analytical method are able to identify seizure liability concentration-dependence, rank the level of seizure liability based on the seizure liability probability, and identify the mechanism of action of compounds. The next challenge for the *in vitro* assessment system is extrapolation *in vivo*. We also report the correlation with *in vivo* data and the evaluation method using human brain organoids.

S20 | Modernizing Cancer Risk Assessment: Beyond the Bioassay

S20-01

Current challenges in a paradigm shift for cancer risk assessment

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The lifetime bioassay in rodents has become the benchmark for assessing the carcinogenicity of chemicals. The methodology was developed when there was little understanding of the biology of cancer or of species-specific responses. It is now apparent that chemicals can cause cancer by a number of different mechanisms, some of which reflect direct mutation of DNA, whilst others reflect a continuum of repeated toxicological perturbation, such as cytotoxicity, endocrine activity and growth stimulation. In addition, it is now apparent that rodents are susceptible to carcinogenicity by a number of modes of action that are not relevant to humans, due to fundamental differences in biology. An example would be renal carcinogenicity in male rats due to interaction with alpha 2u-globulin, a protein for which there is no orthologue in humans. Chemicals that are potentially carcinogenic through DNA mutation can be detected by means other than a lifetime bioassay and of those that are carcinogenic by other modes of action, the results of a cancer bioassay are often not indicative of a hazard, let alone a risk, to humans. The current paradigm requires the use of a lot of animals, leading to results which are often uninterpretable, necessitating further experimental studies. There must be a better way of assessing the potential carcinogenicity of chemicals in the light of current knowledge. This session will describe some of the approaches and activities ongoing in this area.

S20-02

Current transcriptional benchmark dose approaches that are available to safely assess carcinogenicity risk

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The two-year rodent bioassay has been the gold standard for predicting human cancer risk for over 50 years, since toxicology has historically been an observational or 'top-down' science. The bioassay protocol has remained largely unchanged, while its predictive utility of human carcinogenicity risk has been challenged. Our current knowledge of carcinogenesis, including molecular initiating and early key events, and our tools to measure and predict this essential information, has expanded considerably. The use of advanced technologies to probe systems biology and short-term mechanistic assays incorporating early biomarkers, aided by substantial progress in quantitative *in vitro*-to-*in vivo* extrapolation and *in silico* models, now allow toxicology to be a 'bottom-up' or predictive science. This talk will use a variety of case studies to demonstrate how early key events in carcinogenesis can be measured, with their dose-responses, temporality, modes of action and potency predictive of the adverse outcome, based on transcriptional benchmark dose (BMD_T) estimates for key genes and pathways. Short-term studies in rats and mice will demonstrate how BMD_T have been modeled from microarray, RNAseq, and miRNA data streams across multiple carcinogenic modes of action, including nuclear receptor activation and cytotoxicity and in target tissues such as the liver and urinary bladder. Further, unlike traditional apical responses to short-term exposures, BMD_T could distinguish tumorigenic and non-tumorigenic class members, at times in the absence of measurable apical responses. Modern integrated approaches are more efficient, feasible, and human-relevant for toxicity testing and carcinogenicity risk assessment.

S20-03

Strategies for a weight of evidence-based carcinogenicity assessment of human pharmaceuticals

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Within the framework of the International Council of Harmonisation on the Technical Requirements for Pharmaceuticals for Human Use (ICH), in 2012 a process, called S1, was started to study the possibility of an additional approach to predict the carcinogenic potential of human pharmaceuticals within conduction a 2-year rat study. Sponsors were encouraged to submit Carcinogenicity Assessment Documents (CADs) to Drug Regulatory Authorities for all investigational pharmaceuticals with ongoing or planned 2-yr rat carcinogenicity studies. The CAD should address a weight-of-evidence (WoE) approach of the carcinogenic risk of the investigational drug and provide a rationale for why the conduct of long-term studies would not add value to that assessment.

Drug Regulatory Authorities, which are member of ICH and have mutual confidentially agreements, have independently reviewed the submitted documents and evaluate the degree of concordance with Sponsors. During this prospective evaluation period the studies are just expected to go on, as the data are collected solely for real world experience. Submitted CADs were finely compared to the real outcome of the 2-yr carcinogenicity studies. Main objective has been the assessment of accuracy of the predictions. This symposium will inform about the present details of this retrospective analyses. Eventually 48 CADs have been collected, with more than 20 agreed cases with the outcome of "no added value". The presentation will discuss the strengths and weaknesses of the CADs, and the reasons why the

DRA's decided to deviate from the initial categorization by the sponsor. The Expert Working Group has developed a document as an addendum to ICH-Guideline S1B, which is currently under public consultation.

In the criteria presented as important in the WoE approach available pharmacology and toxicology data are crucial. Rodent carcinogenicity appears to be driven by pharmacological properties in a positive as well as in a negative sense. Furthermore, chronic (6-months) toxicity data are important to predict the outcome of a carcinogenicity study. An important category for discussion are those pharmaceuticals representing a new pharmacological class, where no outcomes for congeners are being known.

When using this additional WoE approach a substantial reduction in the number of 2-yr rat studies might be reached to accommodate the 3R-approach.

S20-04

Predicting non-genotoxic carcinogenic potential of agrochemicals: a mechanistic approach

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To protect man and the environment from health risks, chemical substances are subject to hazard and risk assessments before being marketed, including assessment of carcinogenic potential. Non-genotoxic carcinogens (NGTXC) do not directly interact with DNA and therefore cannot be detected based on genetic toxicity assays. Consequently, the risk of NGTXC may remain largely undetected unless lifetime carcinogenicity studies are performed. A transition toward a mechanism-based weight-of-evidence approach is considered a requirement to enhance the prediction of carcinogenic potential for environmental (agro)chemicals. The resulting approach should make optimal use of innovative (computational) tools and abide to the 3R principles. An extensive analysis of 411 unique agrochemicals that have been evaluated for carcinogenicity by the European Chemicals Agency (ECHA) and the United States Environmental Protection Agency (US EPA) revealed that about one-third of these substances (74) could be categorized as NGTXC in rodents with an average of approximately two tumor types per substance, observed in a variety of organs. However, for 116 out of the observed total of 340 cases of tumor formation, no mode of action (MOA) could be identified after

the initial review. These so-called 'unknowns' have been studied in more detail, with the aim to expand or consolidate the list of MOAs relevant to non-genotoxic carcinogenesis. In total, these 116 occurrences of tumor formation were observed in 16 different organ systems. Well-defined criteria have been applied to rule out tumour observations due to exposure to (too) high doses of the substance of interest. For the remaining substances, available reports and literature have been reviewed for (indirect) indications on the MOAs involved, with a focus on common denominators across substances. This review also has taken into account secondary tumours and relevant non-neoplastic findings. The results are a valuable contribution to the development of a weight-of-evidence approach for the prediction of carcinogenic potential of agrochemicals without a 2-year rodent carcinogenicity assay.

S21 | Drug – exposome interactions

S21-01

The exposome: drugs, toxicants, and metabolites

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Derived from the term exposure, the exposome is an omic-scale characterization of the nongenetic drivers of health and disease. With the genome, it defines the phenome of an individual. The measurement of complex environmental factors that exert pressure on our health has not kept pace with genomics and has not provided a similar level of resolution, this is especially true with regards to pharmacological agents. Emerging technologies make it possible to obtain detailed information on drugs, toxicants, pollutants, nutrients in a systematic manner. In collaboration with investigators at Emory University, a platform for enzymatic generation of xenobiotic metabolites for use with high-resolution mass spectrometry (HRMS) for chemical identification was developed and validated (Liu *et al.*, Nature Communications, 2021). Generated xenobiotic metabolites were used to confirm identities of respective metabolites in mouse and human samples based upon accurate mass, retention time and co-occurrence with related xenobiotic metabolites. Human liver S9 enzyme prepared in a 96-well plate format allowed analysis of multiple xenobiotic reactions in a single plate. Human liver S9 enzymes mediated Phase I and II biotransformation reactions to generate xenobiotic metabolites or other downstream adducts from reactive intermediates. High-resolution mass spectrometry (HRMS) analysis of enzyme-generated xenobiotic metabolites provided authentic metabolites for matching accurate mass *m/z*, retention time (RT), and MS2 to detected metabolites in humans. The use of HRMS to assess endogenous and exogenous molecules can facilitate our understanding of drug interactions, improve the detection of adverse effects of drugs and toxicants, and provide data on biological responses to exposures. This model can provide data at the individual level for precision medicine, group level for clinical trials, and population level for public health.

S21-02**Impact of dietary xenoestrogens and other food contaminants on drug metabolism and action****B. Warth**University of Vienna, Faculty of Chemistry, Department of Food Chemistry and Toxicology, Vienna, Austria*

Many environmental and food-related chemicals have the potential to interact with drugs and may influence therapeutic efficacy and side effects. To date, this concept of 'drug-exposome interactions' or 'pharmaco-exposomics' has not been systematically investigated. However, adverse effects have been observed between these xenobiotics and a number of drugs, including chemotherapeutics for breast or colon cancer therapy. This contribution will give an overview of literature examples and highlight how technological advances in mass spectrometry-based metabolomics/exposomics now enable a broad and systemic investigation of these interactions at the systems biology level. A specific example includes the interplay between the two dietary xenoestrogens genistein and zearalenone and the palbociclib/letrozole combination breast cancer therapy. A global metabolomic approach was applied to unravel metabolic pathway modifications in a cell model. Thereby, the combinatory effect of palbociclib/letrozole and xenoestrogen exposure on cellular metabolism was investigated. The drugs specifically attenuated amino acids and central carbon metabolites while higher abundances were observed for fatty acids and most nucleic acid-related metabolites. Importantly, co-exposure to the two model xenoestrogens appeared to counteract the effects of the drug treatment. This behaviour was confirmed in proliferation experiments and functional assays. Moreover, it will be highlighted how drug metabolism may be modulated by co-exposure to xenobiotics via food or lifestyle habits. This includes glucuronidation, a major phase II conjugation pathway in mammals. Its important role in the biotransformation and detoxification of xenobiotics, including dietary mycotoxins or smoking carcinogens, and the potential impact on drug treatment will be discussed.

S21-03**The central european longitudinal studies of parents and children (CELSPAC) from an exposome perspective****J. Klánová**IMasaryk University, RECETOX, Brno, Czech Republic*

A holistic concept of exposome shifts a focus from the individual determinants of health to the interdisciplinary assessment of a wide range of direct and indirect exposures mediated through the environmental and socioeconomic interactions and the lifestyle choices. A systematic characterisation of the exposome represents a challenge of simultaneous evaluation of thousands of synthetic and natural chemicals with a wide range of physicochemical properties, concentrations, and biological effects. Suitable analytical approaches are being developed using innovative untargeted high-resolution mass spectrometry methods.

This opens a door for a completely new direction of the population-based research enabling joint assessment of the chemical exposures and their downstream biological effects. Launching such methods in the high-throughput facilities would enable a new generation of large-scale studies focused on better understanding of the impact of exposures on the onset and development of diseases. Measures of psychosocial and perceived factors must be incorporated together with biological and physical factors.

To address these challenges, the CELSPAC population study platform was established around the Czech branch of the ELSPAC (European Longitudinal Study of Parents and Children) project initiated by the European branch of WHO in the late 1980s and focused on eco-

nomic, environmental, social and psychological factors affecting the health and development of children. The generation of children born in South Moravia in the late 1990s was followed since the pregnancy of their mothers until their adulthood together with both of their parents. A resulting database created a unique information source describing the development of the first generation of children growing up in the rapidly changing conditions of the post-communistic societies in Central Europe after 1989.

Since 2015, this study has served as a base for development of new protocols to follow up the ELSPAC birth cohort (CELSPAC: Young Adults) and assess the role of environmental exposures, and to recruit the next generation birth cohort (CELSPAC: The New Generation). An integration of the HAPIEE ageing cohort into the CELSPAC platform gave a birth to the unique cluster of the mother-child, productive age, and ageing cohorts enabling joint assessment of the complex population exposures and their potential adverse health impacts. This ambition was further supported by the construction of a biobanking facility for high-quality management of valuable human tissue samples.

This ambition was further supported by the construction of a biobanking facility for high-quality management of valuable human tissue samples. The accredited trace laboratory was complemented by high-resolution techniques for suspect screening and non-target analysis of emerging chemicals as well as protein, metabolic, meta-genomic and epigenetic markers. New capacities in biostatistics and bioinformatics were developed to handle and interpret the large volumes of multidimensional data and extract novel information on the most important factors affecting human health and development of chronic conditions.

S21-04**Biotransformation- and DNA Repair-driven interactions and precision responses to chemotherapy****S.J. Sturla**ETH Zurich, Department of Health Sciences and Technology, Laboratory of Toxicology, Zurich, Switzerland*

Drugs that induce DNA damage are amongst the most commonly used and effective strategies for treating cancer, whereby replication and transcription are stalled. However, problems related with adverse toxicities and drug resistance are common and indicate a need to better understand the basis of toxic selectivity of DNA damage and to create new strategies for stratifying responsive cancer patients. Certain patients respond poorly to therapy, therefore, the capacity to identify cancers that may be prone to resistance and responsive to alternative therapeutic strategies is expected to reduce the incidence of relapses and improve long-term survival. These strategies can exploit underlying molecular characteristics of the targeted cancer, including those associated with drug metabolism and DNA repair. The presentation will focus on the identification of chemical biomarkers, based on mass spectrometry profiling of DNA adducts, as sentinels of responsiveness to DNA-alkylating agents used in conventional and experimental therapies. Results show a relationship between genetic characteristics of patient-derived xenograft models of leukemia and chemical indicators of the sensitivity of individual cancers to alkylating pro-drugs that may function more effectively than conventional drugs in resistant cancers. Additionally, we demonstrate strategies for combination effects related to food-drug and drug-drug interactions that can further improve pro-drug activity. Focusing on models of healthy and cancerous colon cells, and xenograft models of leukemia, we have identified a profile of candidate diagnostic biomarkers. We further characterized the influence of cellular processes, including drug metabolism and DNA repair on drug-induced cytotoxicity, and DNA adduct profiles. Finally, we have determined how combined chemical exposures impact drug function

by stimulating metabolism or inhibiting DNA repair. The outcomes of the research improve our understanding of DNA alkylating drug function and provide practical tools for promoting the safety and efficacy of alkylating anticancer agents.

S22 | Computational models to reliably predict chemical mixture toxicity

S22-01

SMF-1, SMF-2 and SMF-3 DMT1 orthologues regulate and are regulated differentially by manganese levels in *C. elegans*

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Manganese (Mn) is an essential metal that can exert toxic effects at high concentrations, eventually leading to Parkinsonism. A major transporter of Mn in mammals is the divalent-metal transporter (DMT1). We characterize here DMT1-like proteins in the nematode *C. elegans*, which regulate and are regulated by Mn and iron (Fe) content. We identified three new DMT1-like genes in *C. elegans*: smf-1, smf-2 and smf-3. All three can functionally substitute for loss of their yeast orthologues in *S. cerevisiae*. In the worm, deletion of smf-1 or smf-3 led to an increased Mn tolerance, while loss of smf-2 led to increased Mn sensitivity. smf mRNA levels measured by QRT-PCR were up-regulated upon low Mn and down-regulated upon high Mn exposures. Translational GFP-fusions revealed that SMF-1 and SMF-3 strongly localize to partially overlapping apical regions of the gut epithelium, suggesting a differential role for SMF-1 and SMF-3 in Mn nutritional intake. Conversely, SMF-2 was detected in the marginal pharyngeal epithelium, possibly involved in metal-sensing. Analysis of metal content upon Mn exposure in smf mutants revealed that SMF-3 is required for normal Mn uptake, while smf-1 was dispensable. Higher smf-2 mRNA levels correlated with higher Fe content, supporting a role for SMF-2 in Fe uptake. In smf-1 and smf-3 but not in smf-2 mutants, increased Mn exposure led to decreased Fe levels, suggesting that both metals compete for transport by SMF-2. Finally, SMF-3 was post-translationally and reversibly down-regulated following Mn-exposure. In sum, we unraveled a complex interplay of transcriptional and post-translational regulations of 3 DMT1-like transporters in two adjacent tissues, which regulate metal-content in *C. elegans*.

S22-02

Systems toxicology models for the development of AOP networks induced by exposure to complex mixtures

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The scope of the present study concerns the development of systems biology models in order to determine the toxicity pathways and the dose-response relationship associated with the adverse outcome pathways associated with endocrine disruptors. It is important to

determine the quantitative relationships that key events relate to each other, utilizing transcriptomics and metabolomics analysis data from a combination of *in vitro* and *in vivo* data. More specifically, the results of transcriptional and metabolic analysis after a range of environmentally related doses of combination exposure to endocrine disruptors to be applied to *in vivo* (zebrafish) and *in vitro* models (A459, HepaRG, HL1-hT1, 3D immortalized hepatocytes, as well as co-cultures with Mz-ChA-1 bile, stellate LX-2 and THP1 macrophage, hMADS, SGBS, MIN6, TC1-9), but also in combination with metabolic analysis in human samples, will be combined to identify networks pathways associated with metabolic disorders. Systems biology-based AOP models will be developed by integrating the cross-omics data generated in relationship to internal exposure estimates and the relevant health outcomes. AOP models will be quantified by linking to the internal dose estimated by PBBK models to bridge external and internal exposure. More in detail, the internal dose will be used for the systems biology qAOP model incorporating different biological scales (organs, tissue, molecular level) for creating robust qAOP. The AOPs models will be parameterized using cross-omics data from experiments *in vitro* and in non-vertebrate *vivo*, as well as from the analysis of human cohort data. Establishment of causality when assessing the effects of environmental factors inducing deviation from homeostasis that eventually results in adverse neural health outcome phenotypes requires establishment of biological plausibility and mechanistic confirmation. *In vitro* assays assessing complex toxicological endpoints of relevance to disease endpoints (cellular migration and plasticity; cell proliferation, etc.) in addition to targeted functional assays will be used (causality checking phase) for that. The methodology outlined herein, addresses one of the major systems toxicology challenges, which is the link of observed network perturbations of *in vitro* systems to phenotypes, a feat that requires understanding pathways and networks that give rise to adverse responses. This is of importance for using systems toxicology data to inform health risk assessment leading to better-informed policy making. Considering the multitudes of exposure and the fact that most toxicants act via more than one discrete pathway, there will be a need to combine linear pathways into network models. This is of particular importance, considering that most cases of regulatory decision making will require more than one AOP within an interconnected network to fully account for the biologic processes that may influence the final outcome.

S22-03

Computational modelling: A new paradigm for chemical mixtures risk assessment?

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Data available on chemical mixtures point out that the combined effects of individual components at levels below no-observed effect levels may cause toxicity. Unlike single chemicals, experimental datasets of mixtures toxicity are scarce and the potential infinity of possible combinations of chemicals that are plausible represents an important limitation for mixture risk assessment. Advances in new technologies have increased the efficiency of toxicity testing and permit the assessment of large numbers of chemicals. This has led to an ongoing paradigm shift in toxicity testing based on increased use of high-throughput *in vitro* screening and *in silico* methods for the hazard identification and characterisation of chemicals. This approach not only reduces the number of animal studies but also allows for filling hazard information gaps for data poor chemicals. It also enables moving towards a better mechanistic understanding of the toxicity pathway of chemicals, both in isolation and in mixtures, that

lead to adverse outcomes *in vivo*. This approach can be complemented with a parallel vision for exposure leveraging on high-throughput exposomics and computational methods for exposure assessment.

New Approach Methodologies (NAM) include relevant *in vitro* and *in chemico* methods, -omics technologies and *in silico* tools. The latter are capable to computationally modelling the relationships between toxic response and substance properties based on chemical structure and physicochemical properties. Such relationships can be reliably used to predict toxicity, or to prioritize chemicals, in a short time, with reduced cost and without using experimental animals. High-throughput *in vitro* screening has the potential to identify concentrations that trigger biological perturbations. These *in vitro* bioactive concentrations can then be used to predict a human equivalent dose, as potential point of departure for risk assessment, using *in vitro* to *in vivo* extrapolation (IVIVE). High-throughput toxicokinetic (HTTK) methods can also be used to provide chemical-specific predictions of toxicokinetics for chemicals where there are no other data, and to derive IVIVE. In sum, these modelling tools can be applied to high throughput *in vitro* toxicity screening data of environmentally relevant chemicals for a quantitative assessment of human adverse health effects. Although computational approaches have so far been used for modelling toxicity of single chemicals, current developments expand the computational toxicology paradigm to chemical mixtures. The current conceptual model of toxicity assessment is gradually moving towards systems biology approaches which integrate, under a weight of evidence framework, multiple data streams from diverse experimental systems into a unified risk assessment paradigm. These novel approaches not only provide improved predictions of toxicity, but also enable mechanistic interpretations of the molecular mechanisms underlying toxicity.

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S22-04

PBPK modelling: Bridging animal-free toxicology tools and conventional *in vivo* testing for cumulative risk assessment after long-term-low-dose exposure to chemical mixtures

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Environmental chemicals are currently regulated almost exclusively on a single-chemical exposure basis. In reality people are exposed to chemical mixtures on a daily basis via many different exposure scenarios. The global scientific community has supported efforts to de-

velop the toxicological evaluation and regulation of chemical mixtures for a considerable number of years. The study of the effects on human and animal health, as well as the environment, from exposure to chemical mixtures requires appropriate fit-for-purpose approaches including New Approach Methodologies (NAM) which endeavour to replace animal studies. For the foreseeable future these approaches will continue to depend on some *in vivo* testing although they might provide data for the development of animal-free toxicology testing tools.

The Real-Life Risk Simulation (RLRS) approach aims to study long-term-low-dose exposure to chemical mixtures and includes replicating such exposure scenarios in rodents. However, the current accepted paradigm of animal testing, does not provide sufficient information chemical mixtures risk assessment. The *in vivo* data currently generated could be combined with modelling approaches to: optimise experimental protocols (such as appropriate dose selection for the studied mixtures), study possible interactions (as with comparison of modelling predictions and real experimental *in vivo* data), and to move towards predictions of toxicity. Having these in mind we consider PBPK modelling as a key future element of the RLRS approach.

In this presentation mixtures toxicology assessment approaches undertaken to date will be summarised, data gaps highlighted, and the potential for PBPK modelling to provide a bridge to the development of animal-free toxicology tools and conventional *in vivo* testing for cumulative risk assessment following long-term-low-dose exposure to chemical mixtures will be discussed.

S23 | The value of micro-physiological systems for drug safety assessment – a series of case studies

S23-01

Microphysiological systems evolution – introducing the immune compartment

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Advanced immunotherapies have been a game changer for cancer treatment, continually extending disease-free survival and with the ability to eradicate cancers. However, it is emerging that it is challenging, based on preclinical studies, even using non-human primates, to adequately predict adverse events and de-risk immunobiological drugs. This is due to the very nature of these drugs because they engage the specific, complex, contextual and poorly cross-species conserved immune system. Thus, as our new class of immunotherapies harness the full force of the immune system, and show tremendous potency, highly predictive fully human models are urgently needed. Toward these ends, we are continuously developing a broad range of human primary cell-based models to test safety and specificity of immunotherapeutics to better predict and identify potential adverse reactions facilitating transition into informed and safe testing in human. This presentation will focus on addressing the challenges of enabling such models of vital organs with immune cell adaptive and innate compartments, needed for animal-free non-clinical safety assessment of immune-engaging therapies.

S23-02**A microfluidic two-organ chip to investigate species specific differences of thyroid-liver crosstalk in human and rats**

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Rodents, which are widely used for regulatory toxicity testing, are particularly sensitive to perturbations of the thyroid homeostasis. Distinguishing between species-specific aspects of the mechanism of thyroid disruption is a major challenge especially when determining whether the effects are human relevant. In addition, the delineation between direct effects on the thyroid gland function and indirect or secondary responses on the hypothalamus-pituitary-thyroid function is key for the regulatory acceptance of a risk assessment. However, there is currently no distinctive assay available to evaluate direct vs. indirect thyroid hormone dysregulations and their relevance to humans.

Multi-organ-chips represent a promising approach to reproduce physiological and toxicological interactions of target organs which generally require animal studies or clinical trials. However, adoption of MPS by the pharmaceutical or agrochemical industry is still slow, mainly driven by a lack of qualified assays to predict the safety of drugs and chemicals.

Here we present ongoing work towards developing and characterizing *in vitro* liver-thyroid models for both the rat and the human. A commercially available multi-organ-chip platform enables co-cultivation and thereby organ crosstalk of three-dimensional (3D) thyroid and liver organ models. The structural and functional integrity of both organoids was reproducibly demonstrated over at least 14 days. Treatment with known inducers of liver biotransformation enhanced T4 glucuronidation (thyroid hormone elimination) as reported from *in vivo* studies. Additionally, chemicals known to directly perturb thyroid hormone formation resulted in a decrease of *de novo* synthesized thyroid hormones.

Thus, we show for the first time a functional model of the hepatic-thyroid axis as single *in vitro* assay for two different species. These two chip-based models represent a major step towards an improved assessment of potential species similarities/differences of certain toxicity findings observed in rats with significant contributions to the 3R principles.

S23-03**Adopting organ-chips as internal decision making-tools: a quantitative evaluation of human liver-chip predictive validity**

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Organ-Chips are viewed as a breakthrough technology for drug discovery and development. Their use has seen rapid growth in recent years. Arguably, however, adoption has been based on a high degree of biological plausibility but relatively few pharmacological “demonstration proofs,” where organ-chips have yielded human-congruent results for a handful of exemplar drugs. However, no one has yet shown how Organ-Chips perform versus simpler *in vitro* models at classifying the known human pharmacology of a broader “calibration set” of drugs; necessary information for understanding how Organ-Chips may further support R&D decisions. We have therefore performed a quantitative evaluation of the performance of Emulate hu-

man Liver-Chips in detecting drug induced liver injury (DILI) versus recent results from *in vitro* human liver spheroids. We selected 25 drugs that spheroids had classified as DILI false negatives, false positives, true negatives, or true positives. Drug selection was also guided by the published liver microphysiological systems roadmap from the IQ MPS affiliate¹. In the presentation, we will describe the logic of drug choice for our “calibration set,” the experimental and data-analytic steps required to compare the different systems’ predictive validity, DILI classification performance across 25 drugs, and the implications for DILI decision quality. We will also discuss the implications of the results for the future positioning of other Organ-Chip models in safety or efficacy settings.

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S23-04**Human iPSC-derived retinal organoid model for *in vitro* toxicity screening**

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The number of patients with non-treatable visual impairment caused by retinal degeneration is on the rise affecting millions of people worldwide. Application of animal models in the study of disease progression and in drug discovery is limited by the structural and functional differences when compared to human retina. Retinal organoids derived from human induced pluripotent stem cells (hiPSCs) are increasingly used for a range of applications, including disease modeling, safety assessment and in the study of retinogenesis. Using cells obtained from healthy donors we generated methods for large-scale production of retinal organoids and tested their applicability in predicting a physiologically relevant response after treatment with known retinotoxins. To achieve this, we treated the organoids with a range of known toxins, including Thioridazine and Tamoxifen demonstrating a dose-response effect on cell viability and structural and functional changes within the retina. Organoids contain the majority of the cell types organised in laminated structure, however current organoids lack immune cells that may contribute to important functional properties. To complement our current model, we developed an enhanced retinal organoid containing hiPSC-derived microglia-like cells and tested their retinal invasion capacity and function. We incorporated microglia-like cells into retinal organoids at 13 weeks and tested their effect on function and development. Our key findings showed that microglia-like cells were able to respond to endotoxin challenge in monocultures and when co-cultured with the organoids. Single cell RNA-Seq transcriptomic analyses, protein expression, electron microscopy imaging and electrophysiological recordings showed that retinal organoids developed normally, expressed key markers, and retained their ability to generate spiking activity in response to light. Thus, this new co-culture immunocompetent *in vitro* retinal model provides a complementary platform with greater relevance to the human retina.

S24 | Building confidence in the use of New Approach Methodologies for safety decision-making

S24-01

Strategies to evaluate *in vitro in silico* physiologically based kinetic (PBK) models as essential tool in next generation (animal-free) risk evaluations

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Physiologically based kinetic (PBK) modelling plays a crucial role in the transition towards next generation (animal-free) testing strategies for chemical safety evaluations to convert *in vitro* toxicity data into *in vivo* dose–response or potency information. To facilitate the application of PBK models in next generation risk assessment, it is important to gain confidence in the predictive value of these models without the use of *in vivo* data. In this study different *in vitro* and *in silico* input strategies to develop PBK models have been evaluated with respect to their performance to predict the peak plasma concentration (C_{max}). To this end, C_{max} predictions were made for 562 studies (i.e. exposure situations described in the literature with reported C_{max} values) for 43 different compounds. Adequate predictions within 5-fold of the reported *in vivo* C_{max} could be made for 34 out of the 43 compounds when comparing the median predicted C_{max} with the median reported C_{max} values. However, larger deviations predicted and observed C_{max} values also occurred, ranging up to five orders of magnitude for some of the compounds, depending on the input approach that was applied. Differences between calculation methods for partition coefficients contributed most to this wide range in C_{max} outcomes. Chemical characteristics were evaluated of chemicals that could not be adequately predicted with 5-fold. Overall, the results obtained can be used to define strategies to reduce uncertainties in internal dosage predictions based on PBK models and to define the applicability domain of these models.

S24-02

In silico approaches to link adverse outcomes to molecular initiating events through AOPs

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The adverse outcome pathway (AOP) framework has been explored as way to organize and display mechanistic information in the context of toxicity assessment. Although there is a clear benefit of AOPs in giving structure to the knowledge by establishing the relationship between the biological events that lead to a given adverse outcome, the use of AOPs as part of safety assessment programs is still limited. Based on this, we have been studying if the association of (quantitative) structure activity relationships ((Q)SAR) predictions, assay data, and reasoning between the evidence in the context of an AOP database, can enhance the application of AOPs to enable informed decision making on chemical safety. Taking carcinogenicity as a starting point, we have developed 37 AOPs, covering genotoxic and non-genotoxic mechanisms, and created prototype software where we combined *in silico* predictions from an expert rulebased system (Derek Nexus), and *in vitro* and *in vivo* assay data when available (Vitic). A reasoning method was developed to allow a hazard classification,

based on the revisions being considered for the ICH S1 guideline for pharmaceuticals, and indicate whether there is any added value in running a 2-year carcinogenicity assay in rodents. While the reasoning method in this case was based on the ICH S1, the principles may be applicable to other contexts, including non-pharmaceutical compounds. When assessing the performance of this tool against a carcinogenicity database containing 382 chemicals with an IARC carcinogenicity classification of 1, 2a or 2b, a high sensitivity was achieved, predicting 94% of IARC carcinogens in the ICH S1 class 1 category. These results indicate that the coverage of this approach for known carcinogens is good, while the specificity still requires further investigation. This work shows so far that the application of *in silico* approaches to AOPs can support decision-making as part of the safety assessment of chemicals. The prediction of molecular initiating events and key events using machinelearned models is currently being explored and additional test sets developed, to see how the performance of this framework is affected. Similar approaches have been also developed for other endpoints, such as Developmental and Reproductive Toxicity.

S24-03

Strategic Use of High-Throughput Transcriptomics and Phenotypic Profiling Data in Support of Regulatory Decisions

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New Approach Methodologies (NAMs) are any technology, methodology, approach or combination thereof that can be used to provide information on chemical hazard and risk assessment that avoids the use of intact animals [1]. The US EPA has developed a NAMs-based tiered hazard evaluation strategy for which high-throughput profiling (HTP) assays conducted in human whole transcriptome TempO-Seq assay [3,4] and imaging-based high-throughput phenotypic profiling (HTPP) using the Cell Painting assay [5,6]. The data from these assays are appropriate for use in a variety of fit-for-purpose risk assessment approaches. First, *in vitro*-to-*in vivo* extrapolation (IVIVE) can be used to convert *in vitro* potencies from HTTr and HTPP to administered equivalent doses (in units of mg/kg-day) and establish molecular point(s)-of-departure (POD_{NAM}) [7]. In a study of chemicals from the Accelerating the Pace of Chemical Risk Assessment (APCRA) project, we found that POD_{NAM} is equivalent to or more conservative than traditional *in vivo* effect data (POD_{TRAD}) for approximately 83% of the tested chemicals when information from multiple cell types (MCF-7, HepaRG, U-2 OS) is combined. The percentage was less (66–68%) when considering each cell type individually. The POD_{NAM} can then be compared to exposure estimates to identify and prioritize chemicals where bioactivity is observed within the range of potential human exposures. Second, HTP data can be used to inform chemical grouping and read across via comparison of biological response profiles. For example, in a HTPP screen of ~1200 chemicals, we discovered that structurally similar chemicals tend to display a greater degree of similarity in biological phenotypes than what would be

expected by chance. In addition, structurally diverse sets of fungicides (strobilurins and conazoles, respectively) produced hallmark phenotypic profiles that were distinct from one another. Third, HTP data can be used to predict putative mechanisms of toxicity and distinguish biological effects that are due to activity at a discrete molecular target from those that are due to generalized cell stress. Participants in this session will gain a broader understanding of emerging high-throughput technologies and their potential applications for NAMs-based chemical safety assessment. *This abstract does not reflect USEPA policy.*

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S24-04

An industry perspective on strategies for integrating new approach methodologies for next generation risk assessment

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Several theoretical frameworks describing a tiered approach for NGRA have been published over the past few years, but concrete next generation risk assessment (NGRA) examples of how to analyze, integrate and interpret all the data obtained from new approach methodologies (NAMs) in order to inform a safety decision have been lacking. In this presentation, we set out to showcase how currently available NAMs can be integrated to make a safety decision without using any *in vivo* animal data for a hypothetical safety assessment of 0.1% coumarin in in face cream and body lotion. PoDs derived from ToxCast, PubChem, a cell stress panel (CSP) and high throughput transcriptomics (HTTr) were compared to exposure estimates (plasma C_{max}) to calculate a margin of safety (MoS) with associated uncertainty and used in the risk assessment decision. In this case study, we were able to demonstrate that a safety decision could be reached with the use of NAM data only, however a need to evaluate the approaches applied in this example was highlighted, in order to give greater confidence. Recently, we have been evaluating three NAMs (HTTr, *in vitro* cellular stress (CSP) and *in vitro* pharmacology profiling panels) which, together with approaches to estimate the relevant human exposure, could form a core toolbox within an NGRA framework for

systemic toxicity. To build confidence in the use of this toolbox, a pilot study has been conducted generating data for 12 chemicals to assess the reproducibility of these assays and optimize the protocols. Future work will generate data for 40 benchmark compounds with identifiable human use scenarios and human risk classification (compounds will be classified as either low risk or high risk from a consumer goods perspective). For each compound exposure scenario, the risk classification and the obtained MoS (ratio between PoDs and the predicted internal exposure) will be used to develop a statistical model for inferring the level of risk of a new chemical/exposure scenario based on the MoS. The output of this model can then be used as part of a weight of evidence NGRA. In conclusion, in this presentation we aim to present the principles behind our strategy to build confidence in NGRA and the preliminary results of this evaluation with 12 pilot chemicals.

S25 | Safeguarding female reproductive health across disciplines

S25-01

New insights into how early-life exposure to industrial chemicals can disrupt female reproductive development

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In recent times, we have witnessed an increase in female reproductive disorders such as precocious puberty, polycystic ovary syndrome, early menopause, and gynaecological cancers. Because this increase has taken place over a relatively short period of time environmental factors must play a role. Developmental exposure to industrial chemicals, including endocrine disrupting chemicals (EDCs), have been suggested to contribute to disease causation, and forms the basis for the ovarian dysgenesis syndrome (ODS) hypothesis. The ODS hypothesis proposes that early life exposure to industrial chemicals can interfere with programming of the reproductive system and give rise to lifelong reproductive disorders in women.

Female reproductive development depends on a complex series of events during early life, from embryonic stages through to puberty, and involves numerous signaling pathways that direct differentiation and function of both germ and somatic cells of the ovaries. Since early female reproductive development is largely independent of sex hormone signaling, endocrine disruption may not be induced by disrupted steroid hormone synthesis or hormone receptor binding, but rather through as yet unidentified events. In addition, the cause-effect relationships between chemical exposure and female reproductive disorders have proven problematic to determine. This has made it difficult to assess the risks chemical exposures pose to a woman's reproductive development and function. To address this knowledge gap, the EU Horizon 2020 project FREIA investigates two classic EDCs that cause adverse reproductive outcomes in both humans and experimental animals: diethylstilbestrol (DES) and ketoconazole (KTZ). The aim was to identify novel biomarkers for female reproductive toxicity.

In addition, we use the adverse outcome pathway (AOP) concept to summarize current knowledge about how chemical exposure can affect female reproductive health, with a special focus on effects on the 1) ovarian toxicity and 2) disruption of pubertal timing by interfering with the central hypothalamic trigger of puberty, GnRH neurons. This way, we pinpoint modes of action that could be targeted for alternative test method development.

S25-02**Female fish are a critical model for linking molecular mechanisms of action and population-wide impacts of industrial contamination**

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Many urban and coastal waterways have a legacy of contamination with industrial wastes. The impacts of endocrine disrupting and toxic compounds in the environment can be readily studied using fish models in the lab and on-site in the field. We examine the molecular, cellular, organismal, and population-level impacts of several industrial contaminants on fish reproductive health. We find that female fish are uniquely affected by exposure to some chemicals, and that often the underlying changes to adult reproductive damage occur during early life stage exposure to chemicals. Our studies seek to identify mechanisms impacting reproduction in fish as well as to translate these findings into impact assessments for entire populations. We test the hypothesis that the mechanism of impacts to the reproductive system of the females may involve classically known toxicology pathways, as well as gene and development pathways not typically associated with endocrine disruption. We employ next generation sequencing, pathohistology, morphometrics, and endocrinological methods to investigate these questions. Two cases where female fish are specifically most vulnerable to the impacts of industrial contamination will be discussed, as well as the broader implications to the entire population, and how this knowledge could be applied in a management context. Due to a high degree of genetic conservation among animals and the ubiquitousness of many chemicals in the environment, the results discussed offer insight into mechanisms of action and population-wide impacts to all vertebrates, including humans.

S25-03**Influence of endocrine disrupting chemicals on female puberty – evidence from human epidemiology**

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Purpose: Pubertal timing in healthy girls has changed towards lower ages at onset of breast development during the last 30 years. Several individual studies have reported that exposure to endocrine disrupting chemicals (EDCs) may be associated with pubertal timing. However, a systematic joint evaluation of all existing results has been lacking. We therefore conducted a systematic review and meta-analysis to iden-

tify and evaluate current evidence on the timing of pubertal onset in girls following prenatal or postnatal exposures to EDCs.

Methods: We performed a systematic literature search following PRISMA guidelines and identified approx. 700 articles reporting on exposures to EDCs measured in maternal or child samples and pubertal onset.

Results: A total of 41 publications fulfilled our inclusion criteria for the qualitative trend synthesis and 21 publications for the meta-analysis. According to statistically significant associations in the qualitative trend synthesis, we found that postnatal exposure to phthalates may advance onset of thelarche and delay onset of pubarche. But overall, we did not find consistent evidence for associations between either prenatal or postnatal exposures to xenobiotic EDCs and timing of pubertal onset in girls in our metaanalysis. Nor were we able to identify pre- or postnatal windows of exposure as particularly critical and susceptible for effects of EDCs. Current evidence on specific exposure-outcome associations remains too scarce to firmly confirm EDC exposure as a risk factor for global changes in age of pubertal onset.

S25-04**EDCs and female fertility – what can we learn from human clinical samples?**

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Industrialization has brought progress and development in our economy throughout the decades but these advantages come with disadvantages as well. Exposure to industrial chemicals is now ubiquitous in everyday life. Currently, there are over 350,000 chemicals and mixtures registered on the global market. Some of these chemicals, which are called endocrine-disrupting chemicals (EDCs), cause adverse effects in humans through an endocrine mode of action. While there are numerous studies on EDCs and the male reproductive system, investigations on EDCs and the female counterpart are disproportionately less.

Human clinical samples are a great source of information to understand how the body is functioning, ancillary to physical signs and symptoms in determining diagnoses. While they are routinely utilized in medical management, their potential as valuable resources in field of toxicology remains to be fully realized, especially regarding EDCs and female fertility.

We have measured 9 organochlorine pesticides, 10 polychlorinated biphenyls, 3 polybrominated diphenyl ethers, and 6–9 perfluoroalkyl substances in blood and follicular fluid of women living in Sweden. We also measured these chemicals in fetal tissues with their corresponding placenta and maternal serum samples. Our studies show that women living in Sweden are exposed to mixtures of EDCs^[1,2,3,4]. These chemicals pass the ovarian follicular basement membrane and expose maturing oocytes directly. They also pass the placenta, exposing the fetuses to a mixture of chemicals throughout gestation^[1,2]. We have also found associations of these chemicals with lower ovarian reserve^[3], higher ovarian response to stimulation, lower embryo quality, higher odds for infertility^[3,4] and longer time-to-pregnancy^[4]. To assess their effects on appropriate endpoints for female fertility and investigate further their mechanisms of action, there is a need to develop relevant *in vitro* systems, where ovarian tissue cultures and primary cell cultures may play an important role. In this talk, I will discuss the implications of chemical exposures on fertility in women and how we can maximize use of human clinical samples not only for exposure determination but also their potential tool as experimental models to study the effects of these chemicals on fertility.

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S26 | Computational toxicology – new advances and acceptance in academia, industry and regulation

S26-01

Developing and Assessing *In Silico* Profilers for Organ-Level Toxicity Using Non-Standard Data

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In silico approaches to toxicology encompass a number of techniques and approaches, from read-across to machine learning. Predicting the effects following repeated-dose exposure is one of the most challenging for *in silico* toxicology due to the complexity and subtlety of the endpoint. One potentially viable method to determine effects following chronic exposure is to consider organ-level toxicity. This may be captured through the development of structural alerts, i.e. molecular fragments associated with specific adverse outcomes. Compilations of alerts, serving a common purpose such as identifying chemicals with a specific adverse effect or mechanism of action, are often termed “*in silico* profilers”. Although the characteristics of the alerts for specific purposes have been little explored, there are a number of potential uses of such profilers – including the direct prediction of toxicity, creation of molecular fingerprints and the identification of analogues for read-across. In this study, a series of profilers for effects relating to liver toxicity are presented. The knowledge underpinning these alerts was gained from a variety of data sources, including repeated dose *in vivo* studies, human clinical and adverse reaction reports as well as from New Approach Methodologies (NAMs). The anchor for many structural alerts was the molecular initiating event (MIE) from the relevant Adverse Outcome Pathway (AOP). Using MIE as the starting point, profilers have been developed for phospholipidosis, cholestasis, steatosis and general liver toxicity. The development of alerts using the MIE has several advantages, namely the direct relevance to mechanism of action and the capability of using diverse types of data. It is important to note that such *in silico* profilers do not address regulatory endpoints specifically, but can be used to build up a weight of evidence to support a regulatory decision. Criteria to assess the quality of structural alerts have been

applied, notably to assess their coverage and specificity. These two aspects were found to be closely related, i.e. highly specific alerts have limited coverage, and vice versa. In order to further interrogate alerts, a novel approach based around the commonly used medicinal chemistry technique of Matched Molecular Pairs Analysis (MMPA) was applied. Compounds considered as MMP differ by the presence of a single, localised chemical transformation – typically consisting only of a small quantity of atoms. MMPA was performed using a KNIME workflow, to investigate the coverage of an alert within a profiler by its presence across molecules forming a toxicological database. This analysis was able to demonstrate how close the compounds sat in chemical space – establishing clusters of like molecules within which the presence of activity cliffs could be visualised. This analysis gives confidence to alerts, describing their overall specificity and will direct the development of new, more specific alerts.

S26-02

Artificial Intelligence in Drug Discovery and Computational Safety: What is Realistic, What are Illusions?

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Artificial Intelligence (AI) has had a profound impact on areas such as image and speech recognition; however, comparable advances in drug discovery are still rare. This contribution will explore some of the reasons why this is the case, starting firstly by embedding the application of AI in drug discovery into its historical context. Next, we will explore which types of improvements – namely to speed, cost, or quality of decisions – are able to have the most profound impact on the success of drug discovery projects, and we will see that it is *quality of decisions in clinical phases* that matters most for project success. However, while computational algorithms are tremendously powerful today, much of the *data* we have available in drug discovery projects comes from the *preclinical, proxy* domain – which possesses only limited utility to make predictions for clinical stages. Hence, while numerically we are at least to an extent becoming better at modelling *proxy endpoints*, this leads to a ‘models of models’ situation, which only translates to a very limited extent to the safety and efficacy of compounds which will reach patients in a *clinical* setting. Even more fundamentally, there are intrinsic aspects of chemical and biological data which make labelling data much more difficult than in the image- or speech-domain, such as its conditionality (e.g. the dependence of effects on dose), which pose conceptual problems to being able to apply computational algorithms in the drug discovery domain. After presenting some current areas of research using data for anticipating compound safety, by using -omics, waveform, and other data, the contribution will conclude with aspects of model validation and perceptions of AI in society which pose additional problems for their unbiased evaluation and development, and point out developments which are needed for applications of AI in drug discovery to further contribute to the clinical safety and efficacy of future medicines.

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S26-03**Industrial perspectives on *in silico* tools – early screening to regulatory applications**

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Computational methods have gained considerable interest in many industrial chemical-oriented sectors over the past number of years. In the pharmaceutical industry, such methods are widely used in every phase of drug discovery and development and are strategically integrated into the risk assessment process, to complement experimental methods. The applications are heavily tailored to support the decision making process at the various stages and range from supporting traditional chemical design, predicting off-target effects, or more recently, predicting toxicologically driven histopathology findings using deep learning image analysis. Considerable interest in regulatory applications was triggered with the adoption of the ICH M7 guideline that specifies using (Q)SAR methods to predict bacterial mutagenicity of drug impurities, leading the way towards other potential applications. This presentation will provide practical case studies of machine learning applications and how they are integrated into the workflow at various stages. It will also discuss maximizing information gain from limited data and give examples of advances towards novel regulatory applications.

S26-04**Open source computational toxicology tools in food and feed safety: integrating historical data, meta-analysis and species-specific generic models**

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Open source computational toxicology tools are becoming increasingly important in food and feed safety. These tools allow the implementation of New approach methodologies (NAMs) providing a mechanistic basis to predict hazard properties particularly in data poor situations and when data gaps are identified. Furthermore, NAM-based data can be implemented in hazard assessment and risk assessment (RA) through the integration of different lines of evidence in the overall weight of evidence while supporting the reduction of animal testing. Here, an overview of EFSA activities in this area for the last decade is provided to illustrate current and future applications of such open source tools in the food and feed safety area. A brief description of the EFSA weight of evidence guidance document and its application in the chemical risk assessment sets the scene. OpenFoodTox is then described as an open source database providing historical hazard data for over 5000 chemicals, used by EFSA in its risk assessment, together with species-specific QSAR models for the prediction of hazard properties in data poor situations. Integration of human kinetic data in risk assessment is illustrated through a) Bayesian meta-analyses of human variability in kinetics for a range of probe substrates of phase I, phase II enzymes and transporters b) derivation of pathway-related variability and pathway-related uncertainty factors, c) integration of isoform-specific *in vitro* data and pathway-related variability for the refinement of quantitative *in vitro* to *in vivo* extrapolation models. The development of species-specific generic physiologically-based models in humans and farm animals is discussed and illustrated for forward and reverse dosimetry applications through EFSA's TKplate platform. Future perspectives in this field include further development and implementation of species-specific generic PBK models in RA, the development of relevant guidance documents and case studies to illustrate their use in different RA contexts and in training programmes. All contributors to this work and relevant publications are acknowledged.

S27 | Predictive systems to identify etiological factors and pathogenic mechanisms of neurodegeneration**S27-01****Translation of mechanistic data into *in vivo* systems to predict risk for neurodegeneration**

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Environmental exposures are linked to neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis. Difficulty in early-stage diagnosis, increasing incidence, limited efficacy of treatments, and identification and mitigation of risk factors are critical to public health. The adverse outcome pathway (AOP) has been an exciting development that utilizes mechanistic conclusions to inform risk assessment. However, increased translational value of the mechanistic data used to construct AOPs; thereby increasing predictive power is needed. Proposed strategies include: 1) Utilization of a comparative biology approach. Here, similarities and differences in neurotoxic responses across species inform both mechanistic conclusions and putative risk, along with clear integration of the relative advantages and disadvantages of each model system in the formation of mechanistic conclusions; 2) Achievement of relevant internal doses. Measurement of internal toxin/toxicant dose is critical to determine relevance to human exposures; 3) Biochemical, neuropathological, and functional assessments to establish relevance to a single (selectivity) or multiple (overlap) neurodegenerative diseases. In this context, heterocyclic aromatic amines (HAAs), organophosphates (OPs), and per- and poly-fluoroalkyl substances (PFAS) are chemical classes that have been linked to adverse neurological outcomes and possibly major neurodegenerative diseases. The level of scientific support and mechanistic insight is highly variable across chemicals within these classes. To translationally advance mechanistic understanding, we have conducted experiments across cellular, nematode, amphibian, and rodent models; in many cases, overcoming translational deficiencies through the creation of novel models, achieving relevant internal doses, and neurotoxic evaluation across a scale of endpoints. Specifically, for these chemical classes, this approach has identified new mechanisms of neurotoxic action, such as: 1) Interaction with neuromelanin (catecholamine breakdown product) that increases intracellular dose and exacerbates mitochondrial toxicity, targeting both mitochondrial respiration and mitophagy; 2) Identification of subcellular import pathways that mediate mitochondrial toxicity; 3) Overlapping mechanisms across neurodegenerative diseases, such as interactions between Alzheimer's and Parkinson's disease neurotoxicity pathways. These mechanistic inferences have been drawn from external exposure regimens that produce internal doses relevant to human plasma levels. Thus, we postulate that to increase the predictive power of AOPs, increased rigor and translational value of the mechanistic studies are critical.

S27-02**Altered neurotransmitter homeostasis as a mechanistic biomarker of neurotoxicity progressing to neurodegeneration**B.S. Cagle¹, R.A. Crawford¹, N.N. Paranjape², *J.A. Doorn¹¹ University of Iowa, Pharmaceutical Sciences and Experimental Therapeutics, College of Pharmacy, Iowa City, USA;² University of Iowa, Occupational and Environmental Health, College of Public Health, Iowa City, USA

Epidemiological evidence implicates exposure to environmental agents, such as organochlorines (OC) and organophosphates (OP), in the pathogenesis of neurodegenerative disease such as Parkinson's Disease. While the correlation between human interaction with these agents and later neurodegenerative disease appears substantial, the mechanistic link is unknown, and there is a dire need for biomarkers indicating initiation of pathogenesis in the absence of overt, diagnosable symptoms. Increased translational value for the mechanistic findings and identified biomarkers is essential for: 1) constructing adverse outcome pathways to inform risk assessment; 2) determining overlap between mechanisms of toxicity for structurally diverse compounds associated with similar diseases; 3) identification of new therapeutic targets and novel biomarkers for earlier disease diagnosis. Here, we present evidence that multiple environmental contaminants, including OCs and OPs, disrupt neurotransmitter homeostasis, and in particular, the metabolism and trafficking of dopamine yielding elevated levels of harmful species. Our hypothesis is that disruption of dopamine homeostasis by environmental insults is an early, shared, and mechanistic underpinning relevant to the pathogenesis of neurodegenerative conditions such as Parkinson's Disease. Dopamine metabolism is tightly regulated via the neuron-glia axis and involves transporters and specific enzymes responsible for the generation and detoxification of reactive intermediates. Biotransformation of monoamines via monoamine oxidase produces reactive oxygen species plus a biogenic aldehyde, 3,4-dihydroxyphenylacetaldehyde (DOPAL) that can be further oxidized to a quinone. DOPAL is a reactive and toxic intermediate that requires carbonyl metabolism via aldehyde dehydrogenases and/or reductases, both of which are sensitive to lipid peroxidation products and environmental agents. Functional consequences of increased biogenic aldehyde load, such as DOPAL in context of dopamine neurons, includes protein modification and aggregation, enzyme inhibition and oxidative stress. A technical challenge involves measuring the free and protein-bound parent compound (dopamine) and associated metabolites, such as DOPAL. In our search for scavengers of biogenic aldehydes, we have discovered high reactivity of DOPAL to amino acid and peptides, producing stable conjugates that can be readily measured via LC/MS. In particular, the Cys amino acid adduct of DOPAL is generated intracellularly but exported extracellularly. This conjugation reaction may represent a novel mechanism of detoxification but also a stable, measurable biomarker for a transient, reactive species relevant to impaired catecholamine metabolism. In summary, these findings implicate disrupted dopamine homeostasis as a shared target for structurally diverse environmental agents (e.g., OCs, OPs), and indicate measurable biomarkers for assessment of insult.

S27-03

Application of an adverse outcome pathway-based *in vitro* testing battery for neurotoxicity evaluation

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Parkinson's disease (PD) is the second most common neurodegenerative disorder of the elderly, affecting approximately 1% of the population over the age of 60. The prevalence of PD in Egypt is high-

er than the international average, thus representing a major health and economic burden due to its chronic and progressive nature. Only 5–15% of PD cases are attributed to genetic mutations, while the rest are idiopathic. Egypt is an agricultural country which heavily relies on the use of pesticides, resulting in a high incidence rate of pesticide intoxication in Egypt. Numerous epidemiological and animal studies have supported the hypothesis that exposure to pesticides, in particular organophosphate pesticides (OPs), can increase the risk of developing PD, however, the results of these studies are not conclusive. Hence, the European Food Safety Authority (EFSA) recommended the reassessment of the hypothesis that some pesticides might cause Parkinsonian motor symptoms (PMS) using the adverse outcome pathway (AOP) concept.

Based on the recommendations of EFSA, we established an AOP-informed, mode-of-action-based human *in vitro* testing battery for hazard identification and investigation of the neurotoxic potential of the most commonly applied OPs in Egypt; chlorpyrifos, malathion, and parathion as well as their metabolites. The *in vitro* test battery is based on dopaminergic neurons differentiated from Lund human mesencephalic (LUHMES) cells. This human cell model was used to establish assays representing early key events (KEs) of the proposed AOP using the two PMS model compounds rotenone and paraquat. Those KEs are represented in a multiplexed assay assessing cell viability, cytotoxicity, and apoptosis, as well as two additional assays assessing the level of adenosine triphosphate (ATP) and the proteasomal activity, respectively. In order to verify the sensitivity of the test battery, we incorporated saccharin and diethylene glycol as negative compounds. All OPs induced a concentration- and time-dependent reduction of cell viability, ATP levels, and proteasomal activity, as well as an increase in cytotoxicity. Of note, the metabolites of the tested OPs were less toxic than their respective parent compounds. Metabolomic analyses using high-resolution nuclear magnetic resonance spectroscopy (HR ¹H NMR) revealed disturbance of energy metabolism, mainly by interference in the citric acid cycle, both after model compounds and OP exposure. In conclusion, LUHMES-derived dopaminergic neurons are a promising model system for identifying compounds that affect early KEs of PD and consequently disturb the dopaminergic system. In the future, we will complement our testing battery with assays representing later KEs of the proposed AOP, i.e. alpha-synuclein aggregation and specific dopaminergic cell death.

S27-04

In vitro neurotoxicity test methods: from development to degeneration

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Human epidemiological and animal *in vivo* data indicate an association between early exposure to environmental pollutants and impaired neurodevelopment. Such exposures may make the nervous system prone to subsequent neurodegenerative damage. Importantly, neurodegeneration shows a sex-specific prevalence, with males and females being more likely to develop respectively Parkinson's and Alzheimer's Diseases. As only a fraction of the environmental pollutants has been studied for adverse neurodevelopment effects, efficient *in vitro* tools are required to investigate the neurotoxic potential of environmental pollutants, taking into account genetic sex. Our research therefore aimed to assess sex-specific acute and developmental neurotoxicity of environmental pollutants, thereby paving the way for subsequent assessment of neurodegeneration.

To that aim, primary rat cortical cultures, derived from male or female pups, were grown on microelectrode arrays (MEA) as *in vitro* model to investigate effects of acute and developmental exposure. Cortical cultures were acutely exposed for 30 min to bisphenol A (BPA), estradiol (0.001–100 μ M), aldicarb or endosulfan (0.01–100 μ M) on Day *In Vitro* (DIV) 10 to assess effects on network function. Additionally, cultures were exposed from DIV 7–21 to assess developmental effects on network formation and maintenance.

Our data demonstrate that acute exposure of rat cortical cultures to BPA or estradiol induced a concentration-dependent inhibition of neuronal activity at ≥ 1 μ M and 10 μ M, respectively, with complete cessation of activity at 100 μ M. Acute exposure to aldicarb was without effect, whereas endosulfan induced a biphasic effect, with excitation at low concentrations (0.1–10 μ M) and cessation of activity at 100 μ M. Interestingly, endosulfan-induced overexcitation was most pronounced in female cortical cultures.

Developmental exposure to BPA (0.01–10 μ M) had little effect up to DIV17. However, BPA (0.1–10 μ M) induced an apparent hyperexcitation from DIV21–28. Exposure to 100 μ M BPA induced an inhibition of activity during the entire exposure. On the other hand, preliminary data indicate that developmental exposure to aldicarb had little effect. Interestingly, the clear hyper- and hypo-excitation observed following acute endosulfan exposure was strongly attenuated during developmental exposure, although a clear hyper-excitation could still be observed at 1 μ M, especially in female cortical cultures.

These data demonstrate that acute and developmental exposure to environmental pollutants may result in different effects in female and male cortical cultures. The changes in neuronal network properties can be long-lasting changes, which may leave the network more vulnerable to neurodegeneration.

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S28 | Preclinical immune-safety evaluation of immuno-oncology therapies

S28-01

Current nonclinical evaluation of immune-related safety risks for IO biopharmaceuticals

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The explosion of immuno-oncology (IO) targets and modalities in recent years has revolutionized cancer treatment and with it, new challenges in ensuring the conduct of clinical trials that protect patients from unnecessary risk. Nonclinical safety data are intended to characterize exposure-response relationships for adverse events that can inform selection of safe starting doses and translational risk. Yet traditional toxicology studies are often not relevant or predictive for several safety concerns that have emerged with IO agents, such as infusion reactions and mechanism-based immune-related adverse events. This talk will discuss modifications to nonclinical safety testing strategies for IO candidates, with attention to monoclonal antibodies that induce anti-cancer immune responses and the usefulness and limitations of systems currently employed.

S28-02

Current nonclinical evaluation of immune-related safety risks for engineered T cell therapies

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Immunotherapy has established itself as an important approach in the treatment of cancer. Engineered T cell therapies, namely chimeric antigen receptor (CAR) T cells and T cell receptor (TCR) T cell therapies, are therapeutic modalities that have demonstrated remarkable efficacy in several hematological malignancies and are pursued also for the treatment of solid tumors. While engineered T cell therapies offer opportunities among immunotherapies, they also present challenges. This presentation provides an overview of the current important nonclinical safety assessment considerations for the development of these therapeutic modalities including for immune-related safety risks (including cytokine release syndrome, neurotoxicity, on-target off-tumor toxicities, alloreactivity) and discusses approaches used for hazard identification or risk assessment.

S28-03

Clinical toxicology of immune checkpoint blockers

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Immunotherapy with monoclonal antibodies targeting immune checkpoint molecules, including programmed death-1 (PD-1), PD ligand-1 (PD-L1), and cytotoxic T-lymphocyte-associated antigen (CTLA)-4, has become prominent in the treatment of many types of cancer. However, this can also result in the development of treatment-induced immune-related adverse events (irAEs) in multiple tissues. Immune checkpoint blockers (ICB) induce inflammatory and autoimmune events mediated by innate cells but also by antigen-specific adaptive immunity leading to tissue destruction. Most irAEs are clinically manageable; however, some irAEs can result in mortality or long-term morbidity. Basic science and clinical insights on the mechanisms responsible for ICB-induced irAEs will be presented as well as approaches that may uncouple irAEs from ICB clinical efficacy.

S28-04

Regulatory considerations and establishing FIH dose across immunomodulators

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In recent years cancer immunotherapy has revolutionised the field of oncology. In general, immunomodulatory treatment of cancer aims at stimulating the immune system which can be achieved by multiple mechanisms; e.g. direct activation of immune effector cells such as T cells by bispecific T cell engagers or adoptive transfer of tumour-targeting CAR-T cells; immune check-point therapy blocking inhibitory check-point receptors or ligation of co-stimulatory agonists to bolster an endogenous anti-tumour T cell response. These approaches may be further enhanced by the administration of immunomodulatory cytokines such as IL-2 or GM-CSF or adjuvants targeting pathways of the innate immune system.

While having clinical efficacy, immune stimulation has been shown to be associated with immune-related adverse effects in the clinic such as cytokine release or vascular leak syndrome or more delayed reactions such as autoimmune inflammation. A regulatory challenge is the poor predictivity of non-clinical safety data for some of these immune-related adverse effects in humans.

The present talk will present regulatory considerations regarding the non-clinical safety assessment of stimulatory immuno-modulators. In addition, it will discuss study design and how to establish a starting dose for first-in-human clinical trial, considering the need to find a dose that is low enough to be safe but also sufficiently high as to minimize exposure to sub-therapeutic treatment.

S29 | Is there a human risk to PFAS exposure?

S29-01

Investigating developmental effects of PFAS using a 3D human induced pluripotent stem cell differentiation model

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We have developed and applied a novel human toxicity assay 'PluriBeat' based on human induced pluripotent stem cells (hiPSC) that we hypothesized would be valuable for studying developmental toxicity of legacy and new PFAS.

In our 'PluriBeat' assay, hiPSCs go through the early stages of embryonic development in 3D cultures of embryoid bodies that mimic the human blastocyst, until they finally form beating cardiomyocytes [1]. The assay mimics the first weeks of embryonic development in which the heart develops and starts beating and may be used to test for developmental toxicity. We showed the *in vivo* relevance of the differentiation model for developing embryos in a mammalian species that develops very similar to the human, namely the pig [2]. By investigating pig embryos of relevant developmental stages before and after the first heartbeat, we found that a number of marker proteins overlapped in expression with our *in vitro* system. These findings indicate that many developmental stages of the heart are recapitulated in our *in vitro* model.

Polyfluoroalkyl substances (PFASs) including perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) are persistent pollutants routinely found in human blood that cause developmental toxicity in humans such as decreased birth weight of newborns. Substitutes to these PFASs such as ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoate (GenX) have been introduced, although hazard information is limited. We investigated PFOS, PFOA and GenX for effects on early embryonic development *in vitro*. Both PFOS and PFOA showed marked effects on cardiomyocyte differentiation at non-cytotoxic concentrations, with PFOS being more potent than PFOA. Moreover, both compounds decreased the volume of the embryoid bodies at the highest test concentrations. GenX induced a weak concentration-dependent effect on differentiation of one hiPSC line, but not of another, illustrating variable sensitivity between individual hiPSC lines. Transcriptional analysis of mRNA from the cardiomyocytes showed that PFOS increased expression of the early cardiac marker ISL1, whereas PFOA decreased expression of the cardiomyocyte marker MYH7. This suggests that PFOS and PFOA perturb cardiomyocyte differentiation by disrupting molecular pathways similar to those taking place in the developing embryo.

Moreover, we have created a luciferase reporter hiPSC line termed the 'PluriLum' assay with an improved readout and showed that luminescence intensity follows cardiomyocyte differentiation [3]. Overall, our data suggest that the luciferase reporter cell line increases the throughput and ease of handling of the assay. Based on these findings, we conclude that our PluriLum assay has the potential to become a valuable, sensitive model system for elucidating embryotoxic effects of PFASs in future.

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S29-02

Epidemiological approaches to PFAS toxicity

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Epidemiology has been ridiculed as a black-box endeavour that contemplates associations but leaves causality as an elusive mystery. In regard to PFAS toxicity, we have to ask: Do our findings appropriately represent the specific hazard and its plausible effects? Unfortunately, so-called Type 3 errors are common, and we often fail to recognize that imprecision causes bias. As a result, non-informative studies are considered "negative". We need to sharpen our epidemiology designs and the translation. The question that we often forget to ask is: Given our possibilities for epidemiology research, what could we have discovered, and what could the implications of PFAS exposures be in a worst-case scenario? These persistent substances tend to accumulate in the body, and the greatest blood concentrations are achieved in infancy, when some organ functions may be in a sensitive developing phase. The PFASs have therefore emphasized the need for prospective epidemiology research, with an emphasis on early life.

S29-03

Exposure to real-life PFAS mixtures present in several human matrices, assessed with *ex vivo* effect biomarkers

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Perfluorinated alkyl acids (PFAAs) are used in many household products as food contact materials. Hence, humans are continuously and lifelong exposed to those chemical contaminants. PFAAs compounds have half-lives up to 8.8 years, favouring their accumulation in human being, and increasing the risk of adverse health outcomes. PFAAs readily pass through the placenta, being the functional group and carbon chain length important determinants for their transfer efficiency [1]. Prenatal exposure to PFAAs may disrupt several hormonal systems increasing the risk of several developmental and reproductive health outcomes as longer time to pregnancy [2], lower foetal growth [3,4], higher risk of congenital cerebral palsy in boys [5], and

poor semen quality^[6]. High PFAA serum levels have also been associated with an increased risk of breast cancer^[7,8].

In vitro cell studies suggest that some PFAAs can transactivate the estrogen receptor (ER), antagonize the androgen receptor^[9], and interfere with thyroid hormone^[10,11] and aryl-hydrocarbon receptor functions^[12]. PFAAs also show cellular oxidative stress potential^[13]. Humans are exposed to an array of PFAAs, and the quantity and combination of PFAAs in human matrices differs among individuals. Single PFAA compounds have the potential to interact additively^[14]. Hence, the toxicological studies of single PFAAs and simple mixtures might be insufficient to predict how complex mixtures of PFAAs may affect humans. To get a better evaluation of the real-life mixture effects, we developed a solid phase, liquid/liquid extraction, HPLC fractionation and weak anion exchange methodology^[15] to extract the mixture of PFAAs present in human serum samples. This method was used to investigate the association between the combined xenoestrogenic (XER) activity of PFAAs extracted from serum of 702 Danish pregnant women and birth outcomes (weight, length, and head circumference) of their offspring. We found an interquartile range increase of XER associated with 48 (95%CI: 6; 90) g lower birth weight and 0.3 (95%CI: 0.1; 0.5) cm shorter birth length, what suggest that real-life PFAA mixtures can affect fetal growth through disruption of the ER function^[16].

Moreover, upon extraction of PFAA mixtures from 25 Spanish human placenta homogenates we evidenced estrogenic activity of the extracts using two cell-culture bioassays, E-screen and ER-transactivity. The PFAA placenta extract could further enhance the transactivity of 17 β -estradiol^[17], suggesting that PFAAs present in the placenta may contribute to the xenoestrogenic environment of the fetus.

The proposed PFAA extraction/fractionation methodology, could be used to study the effects of the real-life PFAA mixtures on steroid hormone actions, as well as, other hormonal systems e.g. thyroid hormone function. We will discuss how the developed PFAA extraction method might be used in future research to assess the endocrine impact of PFAAs on human health.

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S29-04

Wide-spread PFAS contamination of drinking water in Sweden – exposure and health risk assessment

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In 2011, PFAS contamination of municipal drinking water was discovered in Botkyrka County, Stockholm, caused by leakage of PFAS from a former military installation with extensive use of PFAS-containing fire-fighting foam^[1]. Following this, several similar contamination cases were discovered, most notably in Ronneby where PFAS concentrations >1000 ng/L were detected^[2]. Over 20% of the population in Sweden have municipal drinking water with PFAS concentrations >10 ng/L, in many cases dominated by PFOA, PFNA, PFHxS and PFOS^[3]. The current EFSA tolerable weekly intake of these four PFASs (4.4 ng/kg body weight/week)^[4] is exceeded by young women consuming drinking water with PFAS4 concentrations of about 20 ng/L, without taking into account contribution from other sources of exposure. In Sweden, consumption of locally caught fresh water fish may cause intakes of PFAS4 well above the TWI, without exposure from drinking water^[5]. Biomonitoring of children/adolescents and young women in Sweden show that drinking water exposure to PFAS4 significantly contributes to measured serum concentrations already at drinking water concentrations below 10 ng/L^[6,7]. Studies on populations from Sweden with background PFAS exposure report suspected PFAS effects on birth weight and childhood growth^[8,9], and liver function^[10]. Evidence is limited for diabetes^[11,12], coronary heart disease^[13], cholesterol levels^[14], hypertension^[14], preeclampsia^[15], and overweight in children^[16]. Conflicting associations for miscarriage. The highly exposed population from Ronneby so far shows evidence of effects on cholesterol^[17], but limited evidence for thyroid disease^[18] and inflammatory bowel disease^[19].

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S30 | Revisiting paracetamol-induced multisystem toxicity: novel mechanistic insights

S30-01

Paracetamol hepatotoxicity: Discovering new drugs based on mechanistic insight from animal studies

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Paracetamol (acetaminophen, N-acetyl-p-aminophenol, APAP) is a widely used analgesic and antipyretic drug. Although generally considered safe at therapeutic levels, an overdose can cause liver injury and acute liver failure, which is a serious clinical problem in the western world. Early mouse studies showed formation of a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI) by P450 enzymes, especially Cyp2E1. NAPQI reacts with the sulfhydryl group of GSH and later, after GSH depletion, with cysteine residues on proteins, which correlates with cell death. Based on this mechanistic insight, N-acetylcysteine (NAC) was successfully introduced as the first antidote against APAP toxicity in patients. NAC is highly effective when treatment is initiated within 8 hours after APAP ingestion mainly because the newly synthesized GSH enhances the capacity to detoxify NAPQI but it also scavenges reactive oxygen and peroxynitrite inside mitochondria. However, the efficacy of NAC is limited in patients presenting late and in patients with a very severe overdose. In addition, NAC treatment can have side effects (e.g. anaphylactoid reactions) and in mice NAC can delay regeneration. Despite more than 40 years of research, NAC is still the only clinically approved antidote against APAP hepatotoxicity. However, significant progress has been made in better understanding the mechanisms of APAP-induced liver injury in the mouse model during the last decade.^[1,2] Importantly, studies in primary human hepatocytes and use of mechanistic biomarkers in APAP overdose patients showed that the mechanisms in mice translate to the human pathophysiology. Based on these investigations, several promising therapeutically relevant targets were identified including cytochrome P450, c-jun N-terminal kinase (JNK) and the mitochondrial oxidant stress and peroxynitrite formation.^[1,2] These studies identified 4-methylpyrazole (4MP, Fomepizole), which is a clinically approved antidote against methanol and ethylene glycol poisoning, as potent inhibitor of both Cyp2E1 and of JNK resulting in effective protection against APAP toxicity after early and late treatment in mice and in human hepatocytes.^[3,4] Furthermore, unlike NAC, 4MP did not delay but promoted liver regeneration in

mice. Moreover, 4MP effectively eliminated the formation of oxidative metabolites by Cyp2E1 in healthy volunteers who consumed a moderate overdose of APAP.^[5] In addition, mechanistic studies showing the critical role of mitochondrial peroxynitrite for the pathophysiology by using the mitochondria-targeted SOD mimetic Mito-Tempo^[6] provided the rationale for the development and clinical testing of the SOD mimetic calmagafodipir.^[7] Based on these studies clinical trials are under way and it is expected that these new drugs may complement NAC as antidotes for APAP hepatotoxicity by expanding its therapeutic window or in the future even replace it.

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S30-02

Paracetamol-associated adverse reactions in kidney: Different mechanistic pathways compared to liver

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The most widely used antipyretic and analgesic paracetamol (PAR) is safe when ingested at the therapeutic levels. However, severe and fatal hepatic injury occurs at overdoses. Since it was realized in the early 70's, the mechanism of PAR-induced hepatic injury has been well documented, although few details still remain to be uncovered. Mechanism of hepatotoxicity involves CYP2E1 catalyzed formation of reactive N-acetyl-p-benzoquinonimine (NAPQI) from PAR in hepatocytes, alkylation of mitochondrial proteins by NAPQI and induction of mitochondrial oxidative stress. In turn, cytosolic C-Jun N-terminal kinase (JNK) is phosphorylated by increased hydrogen peroxide and translocated into mitochondria, where it induces release of endonuclease G and apoptosis inducing factor (AIF) to cytosol and translocation of these proteins to nucleus and fragment nuclear DNA. In addition to the liver injury, a significant proportion of PAR overdose patients also develop nephrotoxicity. While mechanisms of PAR-induced liver injury have been well studied, molecular mechanisms involved in PAR-induced kidney injury are not characterized yet. We recently conducted a study on the potential of 4-methylpyrazole (4MP) as an antidote against PAR-induced nephrotoxicity. Data of this study revealed that PAR-induced nephrotoxicity does not fol-

low a similar cascade of molecular events as in PAR-induced hepatotoxicity; JNK is not activated and not translocated to mitochondria in kidney tissue^[1]. One of the alternative possible hypotheses is *in situ* formation of reactive metabolite(s) from PAR-Cys via renal *cysteine conjugate β -lyase*, which is abundant in proximal tubular cells where PAR-induced nephrotoxicity occurs. *Cysteine conjugate β -lyases* also have mitochondrial forms, and possible activation of PAR-Cys may occur in this critical organelle, as many other drugs or chemical^[2]. Although at moderate level, we demonstrated that APAP-Cys serves as a substrate for renal *cysteine conjugate β -lyase* in *in vitro* mouse kidney cytosol, as well as mitochondrial fractions. Its role as a substrate was confirmed in human kidney fractions as well. Further experiments revealed positive results when the same incubations were performed in the presence of 4-(4-nitrobenzyl)pyridine and *o*-phenylenediamine as nucleophilic trapping agents indicative of generation of reactive electrophilic intermediates from APAP-Cys. In conclusion, the present data suggest an alternative mechanism for PAR-induced nephrotoxicity: *cysteine conjugate β -lyase*-catalyzed local bioactivation of PAR-Cys. Further studies on possible contribution of *β -lyase* mediated activation of renal *cysteine conjugate* to APAP-induced kidney injury *in vivo* are underway.

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S30-03

Uncovering the role of platelets in human paracetamol-induced hepatotoxicity

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The human liver has evolved to cope with exposure to a diverse range of metabolic, inflammatory, and toxic compounds that include medicinal agents and alcohol. However, in the context of overdose, the metabolic and regenerative capabilities of liver cells are compromised leading to significant hepatocellular damage. Human acute liver failure, whether from alcoholic hepatitis or injury such as paracetamol overdose has a very high mortality. Global incidence is increasing, and treatment options are limited meaning that in severe cases many patients die or require transplantation. Novel pharmacological therapies are required for patients who present late, or for those for whom transplantation is not an option. We have discovered a new immunomodulatory pathway that is implicated in the development of hepatocellular injury and inflammation in both alcohol and paracetamol-induced liver injuries. This pathway is dependent upon the interaction between two cell surface receptors CLEC-2 and Podoplanin expressed on platelets and non-parenchymal cells respectively. Platelets are increasingly being recognised for their complex and often contradictory roles in liver injury^[1]. In this presentation we provide evidence from murine models and human systems to demonstrate that activation of platelets via their surface receptor CLEC-2 initiates a cascade of signalling events that influences the inflammatory response during acute injury. We discuss the impact of platelet function in the context of liver injury and show evidence that inhibition of the CLEC-2: podoplanin interaction modifies the

outcome of injury and enhances resolution^[2]. Thus, we suggest that this pathway presents a tractable route for the development of new therapies in liver disease.

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S30-04

Hepatotoxicity in NAFLD: acetaminophen and other xenobiotics

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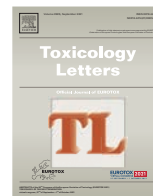
Some xenobiotics including drugs and environmental toxicants can be more toxic in obese patients with nonalcoholic fatty liver disease (NAFLD)^[1,2]. This liver disease refers to a large spectrum of hepatic lesions which include fatty liver (steatosis), nonalcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma. Actually, some xenobiotics may cause more severe and/or more frequent acute liver injury in obese individuals whereas others may trigger the transition of simple fatty liver to nonalcoholic steatohepatitis (NASH), or may worsen pre-existing hepatic lipid accumulation, necroinflammation and fibrosis^[1,2]. Noteworthy, the latter situation requires repeated or prolonged exposure to xenobiotics.

Acetaminophen (APAP) overdose is suspected to cause more frequent acute liver injury in patients with NAFLD^[3,4]. Importantly, different experimental studies in obese rodents confirmed that NAFLD can favor APAP hepatotoxicity^[3,5] and further *in vitro* investigations in lipid-laden HepaRG cells attempted to disclose the involved mechanism(s)^[6]. A particular attention was paid to cytochrome P450 2E1 (CYP2E1), the main enzyme responsible for the generation of N-acetyl-p-benzoquinone imine (NAPQI), the toxic metabolite of APAP. Indeed, hepatic CYP2E1 expression and activity are commonly enhanced in obese patients with NAFLD^[7,8]. In obese rodents, APAP hepatotoxicity was correlated with CYP2E1 induction but not with the severity of steatosis^[5]. In agreement with this finding, investigations in HepaRG cells showed that APAP cytotoxicity was higher in cells treated for one week with stearic acid, which induced steatosis and enhanced CYP2E1 activity, while APAP cytotoxicity was not increased in cells treated with oleic acid, which caused steatosis but not CYP2E1 induction^[6].

Regarding prolonged exposure to xenobiotics, recent investigations in obese zebrafish larvae showed that co-exposure for 7 days to benzo[a]pyrene (B[a]P) and ethanol triggered the transition of fatty liver to NASH, whereas each individual compound was less hepatotoxic^[9]. Notably, B[a]P/ethanol co-exposure also caused the transition of steatosis to a steatohepatitis-like state in HepaRG cells incubated with stearate and oleate for two weeks [8]. Further investigations in this cellular model of NAFLD disclosed that this steatohepatitis-like state was associated with mitochondrial dysfunction and reactive oxygen species (ROS) generation. In addition, B[a]P/ethanol-induced impairment of mitochondrial respiration in HepaRG cells was dependent on the activation of the aryl hydrocarbon receptor (AhR)^[10]. Finally, other investigations suggested that nitric oxide overproduction and plasma membrane remodeling in liver cells could also be involved in B[a]P/ethanol-induced hepatotoxicity in fatty liver^[11,12]. Altogether, these studies on B[a]P/ethanol co-exposure reveal that liver toxicity in NAFLD could involve different mechanisms, which might be intertwined.

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Short Oral Communications

SOC01 | Short Oral Communications I

SOC01-01

Development of a kidney-on-a-chip model that replicates an antisense oligonucleotide-induced kidney injury biomarker response

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The pharmaceutical industry increasingly investigates novel therapeutic modalities, including antisense oligonucleotides (ASOs). There are examples of ASOs that have induced kidney toxicity in preclinical and clinical studies (e.g. SPS5001). In recent years, we and others have validated the potential of *in vitro* kidney models to predict kidney toxicity induced by small molecules, while such validation data for ASOs is sparse. Therefore, we used SPC5001 as a tool to evaluate toxicity in chip-cultured human-derived renal proximal tubule epithelial cells (HRPTEC). Exposing chip-cultured HRPTEC to nephrotoxic SPC5001 ASO and safe 556089 ASO for 48 h downregulated their respective target mRNAs, *PCSK9* and *MALAT1*, referred to as productive uptake. Continuous exposure to SPC5001 ASO for 20 days induced cytotoxicity, measured as lactate dehydrogenase release into the chip lumen. Moreover, kidney injury biomarkers were released into the perfusate, including kidney injury molecule 1, neutrophil gelatinase-associated lipocalin, clusterin, osteopontin and vascular endothelial growth factor, each showing unique time-dependent profiles. In conclusion, the currently developed kidney proximal tubule-on-a-chip replicates the release of injury biomarkers upon exposure to a nephrotoxic ASO. Its full translational value will be evaluated in the future by expanding the validation set of ASOs.

SOC01-02

Pyrrolizidine alkaloids affect miRNA expression in human HepaRG cells

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Pyrrolizidine alkaloids (PAs) are secondary plant metabolites that occur as food and feed contaminants and exert toxic potential upon metabolic activation in the liver. Humans are exposed to PA via honey, herbs and tea. Acute and subacute PA poisoning can lead to severe liver damage in humans and animals, comprising liver pain, hepatomegaly and the development of ascites due to occlusion of the hepatic sinusoids (human sinusoidal obstruction syndrome). Chronic exposure to low levels of PA can induce liver cirrhosis and liver cancer. The molecular modes of action, especially PA-induced changes in gene expression, have been well investigated in the liver. However, other regulatory mechanisms, for example at the post-transcriptional level, have not been elucidated so far. Therefore, in this study, the influence of the PAs lasiocarpine and senecionine on miRNA gene expression was investigated and associated with a PA-specific gene expression data set from primary human hepatocytes. The PAs lasiocarpine and senecionine were chosen as they are known to be very toxic in the human liver cell line HepaRG, which is considered metabolically competent comparable to primary human hepatocytes.

In order to identify the optimal treatment conditions for analyzing differential miRNA expression patterns, HepaRG cells were treated with the most toxic PA lasiocarpine at different concentrations and incubation times. The miRNA was isolated and expression was analyzed using a liver-specific miRNA expression array. Differentially regulated miRNAs (e.g. miR-3653-3p, miR-430, miR-4516) were compared with the gene expression profile of primary human hepatocytes incubated with different PAs as published in Luckert *et al.* [1] using bioinformatic methods. Predicted gene expression targets (e.g. *SLC41A1*, *MMGT1*) were verified in HepaRG cells using additionally the PA senecionine.

In conclusion, 1,2-unsaturated hepatotoxic PAs affected miRNA expression patterns in HepaRG cells. Altered miRNAs possess many target genes involved in several biological cellular functions, like the induction of cholestasis, cancer, cellular growth and the regulation of transport processes. The results add a new level of regulation to the known processes and confirm already known biological functions that are affected by PAs, e.g. the disturbance of bile acid homeostasis, but also provide hints to yet unknown targets that can be investigated in future studies.

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SOC01-03**Novel *in vitro* model captures drug-induced inflammation as a mechanism contributing to hepatotoxicity***F. Tasnim¹, X. Huang^{1,2}, Y. T. Soong¹, H. Yu^{1,2,3}

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In vitro liver models which lack immune components cannot capture inflammation, a key feature of hepatotoxicity which has been shown to alter the activity of enzymes involved in drug metabolism [1,2]. Human liver macrophages – Kupffer cells (hKCs), key liver immune component, play critical roles in the response of the liver to inflammation and injury caused by drugs and other xenobiotics [3,4]. KCs exert such effects by both direct cell-cell contact with hepatocytes as well as release of a variety of inflammatory cytokines, growth factors and reactive oxygen species upon activation [5]. Incorporation of hKCs into *in vitro* liver models has been restricted by the cell source: donor availability and variability, cost, low yield and/or purity, tedious isolation procedures and inability to expand the cells in culture. To address this, we recapitulated *in vivo* ontogeny of KCs to generate human iPSC – derived KCs (hiKCs) and co-cultured them with human iPSC-derived hepatocytes (hiHeps) to develop a human inflammatory liver co-culture model. Importantly, hiKCs and hiHeps in the co-culture model showed mature cell functions which is a major challenge in state-of-the-art stem cell-based models [6].

The co-culture model was exposed to increasing concentrations of several inflammation-associated hepatotoxic drugs including Acetaminophen (APAP) and Trovafloxacin (TVX) in the presence and absence of endotoxin stimulation. Changes in cell viability, cytokine production by hiKCs and their consequent effects on cytochrome P450 (CYP) drug-metabolizing metabolic enzymes in hiHeps were assessed. Treatment with TVX and APAP resulted in a concentration-dependent toxicity which was exacerbated by addition of endotoxin to the culture. Endotoxin treatment caused >5-fold upregulation of cytokine production by hiKCs including Interleukin-6 (IL6) which correlated with the downregulation of cytochrome P450 CYP activity in hiHeps (≥2-fold). Interestingly, co-administration of endotoxin and drugs resulted in a decline in IL6 by >4-fold which reversed the downregulation of CYPs. This phenomenon was not observed with Levofloxacin, the non-toxic analog of TVX or in mono-culture of hiHeps without the presence of hiKCs. The shift in the cytokine profile in co-cultures, combined with the corresponding changes in CYP expression suggests that our model can capture immune-mediated chemical-induced hepatotoxicity. The model was further validated with 19 compounds and showed changes in cytokine production which was highly correlated to their respective clinical/*in vivo* responses. The predictivity of the model was 89.5%. This is the first *in vitro* liver model comprising of renewable, mature and functional human cells for detecting immune-mediated hepatotoxicity upon treatment with a wide range of drugs and hence can be very useful in warranting further investigation of new drugs that demonstrate drug-induced cytokine imbalance.

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SOC01-04**Scientific Validity of Non-Animal-Derived Antibodies**

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Affinity reagents are binding molecules that have a high specificity for their unique target (antigen). They are crucial tools for research, diagnostics, therapeutic and regulatory applications. Based on their recognition properties and binding specificity, protein-based antibodies are currently still the most important tools for the specific detection of proteins or other molecules. Animals are still widely used for the development and production of monoclonal and polyclonal antibodies as well as other affinity reagents despite the availability of alternative non-animal technologies for more than 20 years. In line with the legal requirements of EU Directive 2010/63/EU on the protection of animals used for scientific purposes, animals should not be used in procedures, where a non-animal alternative exists, which provide the same or higher level of information as obtained from animal procedures. For this reason, the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) mandated its Scientific Advisory Committee (ESAC) to review the available evidence and deliver an opinion on the scientific validity of antibodies and non-antibody affinity reagents produced using animal-free technologies. The review focused on non-animal-derived antibodies generated by phage-display technology since this is the most mature technology and already widely used. Taking into consideration the available evidence, the ESAC concluded that non-animal-derived antibodies are mature reagents generated by a proven technology, being able to replace animal-derived antibodies in the vast majority of applications. They have no general or systematic disadvantages with respect to affinity, stability, shelf life and specificity, and offer significant scientific and economic benefits as compared to most animal-derived antibodies. Based on the ESAC Opinion, EURL ECVAM issued its own recommendation that the provisions of Directive 2010/63/EU should be respected and the use of animals for the development and production of antibodies should no longer be authorised in the absence of robust and legitimate scientific justification [1]. An overview of the ESAC findings and of the EURL ECVAM Recommendation on non-animal-derived antibodies will be presented.

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SOC01-05***In Vitro* Toxicity Screening of an Inclusive Panel of Engineered Nanomaterials using an Advanced 3D Liver Model.**

*S.V. Llewellyn, G.E. Conway, U.-K. Shah, G.J. Jenkins, M.J. Clift, S.H. Doak, Research funded by EU Horizon 2020 project PATROLS (EU Grant Agreement #: 760813).

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Hepatic toxicology is key when considering engineered nanomaterial (ENM) exposure, as the liver is vital in metabolic homeostasis and detoxification as well as being a major site of ENM secondary deposition and accumulation post exposure. The PATROLS (Physiologically Anchored Tools for hazard assessment of nanomaterials; EU Grant Agreement #:760813) project is partly aimed at advancing existing 3D *in vitro* liver models to enhance their physiological relevance, whilst using known adverse outcome pathways to better understand hazards associated with both acute and long-term ENM exposure. Furthermore, it is important to consider the realism of ENM exposures, as the manner in which ENMs are dosed onto cells within *in vitro* cultures may influence their biological interaction. To achieve this, cell-line (HepG2) based 3D liver spheroid models have been developed which are viable for long-term culture (≥ 14 days) and able to support both long-term and repeated ENM exposures. Several toxicological endpoints (e.g. liver function, (pro-)inflammatory response, cytotoxicity and genotoxicity) have been evaluated using ENMs (TiO₂, ZnO, Ag, BaSO₄ and CeO₂). Both short- (24hr) and long-term (120hr) single-bolus and fractionated-repeated daily ENM exposure regimes were explored. Neither acute nor long-term exposure to either ENMs significantly ($p \geq 0.05$) reduced liver functionality nor induced a dose dependent IL-8 (pro-)inflammatory response in HepG2 spheroids. Whilst no cytotoxicity was observed across either exposure regimes, acute exposure to all ENMs induced a significant increase ($p \leq 0.05$) in genotoxicity, albeit not dose-dependent. ZnO was the only material to exhibit genotoxicity at both an acute and long-term exposure in 3D HepG2 liver spheroids. For the materials selected in this study, there was no significant difference between long-term bolus or repeated exposure regimes, indicating that the added complexity of fractionated dosing was not necessary for this experimental set up. In conclusion, 3D *in vitro* hepatic spheroid models have the capacity to be utilised for evaluating more realistic ENM exposures, thereby providing a future *in vitro* approach to better support ENM hazard assessment in a routine and easily accessible manner.

SOC01-06**Development of a Liver Carcinoma Biomarker Panel in 3D HepG2 Liver Spheroids Following Exposure to Ag and TiO₂ Nanomaterials**

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Due to the rapid development of engineered nanomaterials (ENM), exposure is inevitable, and the development of robust, predictive *in vitro* hazard testing systems is essential. Adverse Outcome Pathways (AOP) describe the sequence of key events (KEs) that are required to result in a pathological event, or Adverse Outcome (AO) and are there-

fore considered to be useful mechanistic tools for the development of novel endpoint targets for human and environmental risk assessment. This study aimed to develop a novel panel of biomarkers to detect KEs that indicate liver carcinogenesis to better support predictive toxicology using an advanced *in vitro* 3D liver model.

3D HepG2 liver spheroids were exposed to either TiO₂ or Ag for up to 120hrs before RNA extraction. 88 genes associated with liver carcinogenesis were screened using RT-qPCR arrays. Following 120hr exposure to TiO₂, IGFBP3, TGFB1 and WNT5A genes were upregulated. In contrast, at 120hrs, TGFB2, MMP3, IL10 were downregulated. Of the 88 genes screened, 11 genes were selected for further investigation by RT-qPCR across ENM dose-ranges.

Our data demonstrates that longer-term exposure to Ag and TiO₂ results in transcriptional alterations that are important in driving hepatocellular carcinoma. Changes that occur after 24hr Ag exposure appear to be reversed by 120hrs suggesting that long-term effects are not imposed by ENMs that readily dissolve. For ENM with slower dissolution profiles such as TiO₂, transcriptional changes are more prominent over longer exposure times and could be associated with driving AO. We identified that short-term changes were mostly related to inflammation and fibrosis, whereas, after longer exposures, transcriptional changes appeared to be more relevant to carcinogenesis. This study enhances our understanding of biological alterations induced by ENM exposures while also demonstrating the potential for the identification of new biomarkers that could predict clinical outcomes.

SOC01-07**Combining single cell gene expression analysis and a 3D *in vitro* liver model to investigate cell type-specific responses to pro-fibrotic TGF- β 1.**

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3D cell culture systems are widely used to study disease mechanisms and therapeutic interventions. Multicellular liver microtissues (MTs) comprising three human cell lines (HepaRG, hTERT-HSC and THP-1) maintain multicellular interactions and physiological properties required to mimic the key events described in the liver fibrosis adverse outcome pathway (AOP). However, the discrimination of cell type specific responses is often hindered by the inherent complexity of multicellular 3D-systems. Here, we aimed at investigating the individual responses of cells involved in the development of fibrosis elicited by TGF- β 1 using state-of-the-art single cell sequencing (scRNA-seq) technology.

As a first step, isolation of viable cells from multicellular, self-assembled MTs was established. Optimised enzymatic dissociation of liver MTs allowed us to retrieve cell suspensions containing all three cell types showing good viability. Cells could be re-plated and cultured in 2D, and expressed cell-type specific markers determined by scRNA-seq, qRT-PCR, ELISA and immunostaining. For in depth analysis of the cell-specific responses the scRNA-seq data was used. Analysis of untreated liver MT showed that the three cell populations could be successfully clustered using supervised and unsupervised methods. The data also indicate that the HepaRGs could be further subdivided into sub-groups, which show gene expression patterns indicative of functional differences. As expected, treatment of liver MT with TGF- β 1 led to hepatocellular damage and activation of stellate cells in the MTs, detected as decreased albumin and increased α SMA immunostaining, respectively. Single cell analysis uncovered

cell-type specific responses to the treatment including: i) HepaRG damage characterized by a decrease in cellular metabolism, ii) prototypical inflammatory responses in THP-1s and iii) extracellular matrix remodelling in HSCs. Finally, we identified novel cell-specific markers in hTERT-HSC (COL15A1), and THP-1 (ALOX5AP and LAPTM5) that could further improve our understanding of liver fibrosis.

In conclusion, scRNA-seq allowed us to characterize multicellular liver MTs, to assess the individual cell responses to TGF- β 1, and to identify potential novel cell-type specific markers of fibrosis. In addition, the observed differences in HepaRG subpopulations may reflect functional diversity of hepatocytes, potentially associated with physiological liver zones.

SOC02 | Short Oral Communications II

SOC02-01

Towards a personalized drug development using autologous iPSC-derived Multi-Organ-Chips

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Until now, efficacy and toxicity of newly developed drugs are analyzed in preclinical studies with the help of animal testing and static cell culture studies. However, animal models are known not to adequately represent the human organism. Moreover, static cell culture experiments are not complex enough to represent important physiological interactions between the human organs. Due to these limitations along with the high failure rates and development costs, the drug development pipeline needs to adapt to a more efficient, cost-effective, animal-free process that applies human cells under physiological conditions.

An alternative to the traditional models are microphysiological systems. These enable the combination of human-derived miniaturized tissues cultivated in physiological communication with each other and allow for complex tissue-tissue communication as present in the human body. The Multi-Organ-Chip is such a microphysiological system which currently combines the culture of up to four human organ models. A cell culture medium is circulated within the channel system which supplies the organ models with nutrients and oxygen and assures their communication via messenger metabolites.

So far, predominantly cell lines or primary tissues from different donors have been used for the co-cultivation in microphysiological systems. However, the different genetic background of the tissues during the co-cultivation is a drawback. This problem was solved by generating several organ models with the same genetic background using induced pluripotent stem cells from a single donor. Additionally, we introduce a newly designed proprietary PBPK-compliant HUMIMIC Chip4 to generate human-relevant ADME profiles.

A successful co-cultivation combining a 3D small intestine, liver, kidney and brain model was achieved in the PBPK-compliant HUMIMIC Chip4. All four tissue models were pre-differentiated from induced pluripotent stem cells from a single healthy donor. A further differentiation into the organ models was achieved by utilizing the technological advantages of the HUMIMIC Chip4. The medium circulation through the microfluidic channel system enabled a cross talk between the tissues.

The autologous HUMIMIC Chip4 is the first step towards the development of a Patient-on-a-Chip. Genome editing or the usage of blood donations from patients can help to generate disease models on the HUMIMIC Chip4 in the future. The Patient-on-a-Chip provides

the unique opportunity to accelerate the drug development and to intensify the research of individualized therapies. As a result, most laboratory animals, as well as healthy volunteers in Phase I clinical trials, could be replaced by the Patient-on-a-Chip.

SOC02-02

Prediction of inotropic effect based on calcium transients in human iPSC-derived cardiomyocytes using novel waveform parameters and a modified random forest algorithm

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Functional changes to cardiomyocytes have been recognised as a common cause of failure in drug discovery. Understanding and anticipating the inotropic effects of compounds is necessary in such cells and hence it is advantageous in the early stages of drug discovery to decrease the potential risk of cardiovascular adverse effects. Established approaches to detect inotropic effects via *in vitro* or *in vivo* assays are often time consuming and low throughput. Recently, approaches leveraging calcium transients in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) have been developed to predict cardiac activity, such as contractility changes, induced by a variety of mechanisms. Most of the current methods analysing calcium transients are based on common waveform parameters such as peak frequency and peak amplitude. Although these approaches have been able to provide some predictive performance, the hypothesis behind the current work was that by using additional waveform parameters both better insights, as well as predictivity can be achieved. In this study, we derived 26 parameters from each calcium transient waveform and developed a modified random forest method to predict the inotropic effects of compounds. In total data for 48 compounds was available (out of which 26 were annotated as inotropes), and which were tested at 8 different concentrations. The results show that the random forest model with a modified purity criterion performed better than an unmodified algorithm in terms of the area under the curve (0.84 vs 0.81 using selected parameters and 0.81 vs 0.76 using all parameters in cross validation). The final model provides an accuracy of 0.84 during cross-validation. The feature importance of the models shows that PW10 (peak width at 10% from peak maximum) and average amplitude at highest concentration play key roles in the prediction. Our study hence provides evidence that more advanced parameters derived from waveforms, in combination with additional machine learning methods can be better in predicting cardiovascular risk.

SOC02-03

High-throughput phenotypic profiling within the NAMs-based, tiered hazard evaluation strategy at the United States Environmental Protection Agency

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The U.S. Environmental Protection Agency (USEPA) has outlined a tiered testing strategy for human hazard evaluation based on new approach methodologies (NAMs)^[1]. The first tier consists of two high-throughput profiling assays: high-throughput transcriptomics (HTTr) and image-based high-throughput phenotypic profiling (HTPP). In HTPP, cultured cells are exposed to chemicals, followed by fluorescent labeling of cell organelles and high-content imaging^[2]. Using automated image analysis, the distribution and intensity of the different labels are quantified, currently yielding 1300 features per cell^[3]. The collected data can then be used for two applications: first, to derive for each chemical an *in vitro* potency estimate as a lower bound for bioactivity^[4]. This value can be compared to legacy *in vivo* data or to human exposure estimates^[5] in order to prioritize chemicals for higher-tier testing. Second, to predict mechanisms of toxicity. For this purpose, comparing the phenotypic profiles of chemicals to profiles of chemicals with annotated mechanisms can elucidate putative mechanisms for data-poor chemicals. To date we have screened >1200 chemicals from the ToxCast library at multiple concentrations in U-2 OS osteosarcoma cells, with approximately 40% of chemicals eliciting bioactivity below 100 μ M in HTPP. For ~300 chemicals, reverse dosimetry was used to compare *in vitro* potencies to *in vivo* effect values. While HTPP estimates were higher than other NAMs (ToxCast assays, threshold of toxicological concern), the values were closer to the *in vivo* point-of-departure (POD) (RMSE=1.75 log₁₀-mg/kg/day), and for 78% of chemicals within two orders of magnitude of the *in vivo* POD. When compared with estimated general population median exposures^[6], for 49% of chemicals the estimated dose necessary to produce bioactive concentrations was 1000x higher than the estimated upper limit of predicted exposure. Profile comparison of all active chemicals showed that structurally related chemicals tend to result in similar phenotypic profiles. However, phenotypic similarity was also observed for structurally unrelated chemicals in some cases. A subset of chemicals induced highly specific phenotypes, characteristic for glucocorticoids and retinoids, respectively. However, approximately half of the active chemicals resulted in a non-specific phenotype, perhaps indicative of general cell stress. Thus, this shows that HTPP can be used both to identify specific modes-of-action as well as general cell stress. Overall, this work shows how HTPP could be used as an integral part of USEPA's tiered hazard evaluation strategy and help in prioritization of chemicals. In future efforts, this HTPP assay will be applied to diverse cell types^[7] to increase biological coverage.

This abstract does not necessarily reflect USEPA policy.

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SOC02-04

Mutations in the filaggrin gene determine immune response after dermal chemical exposure

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Background: Filaggrin (*FLG*) loss-of-function (null) mutations impair skin barrier function. *FLG* null carriers have, compared to wildtype (wt) carriers, a higher dermal uptake of chemicals that may lead to systemic effects, such as inflammation.

Aim: To investigate inflammatory protein changes, including Vitamin D, between *FLG* null and wt carriers after dermal exposure to chemicals common in the environment.

Methods: We performed a dermal exposure experiment where 23 *FLG* null and 31 wt carriers were exposed during 4 h to pyrimethanil (a fungicide), pyrene (a polycyclic aromatic hydrocarbon), oxybenzone (a UV-filter/absorber) and nickel (a metal used for alloying). We measured metabolite or parent organic compounds in 48 h urine using liquid chromatography-mass spectrometry (LC-MS/MS) and calculated the area under the curve (AUC_(0-40h)). Nickel in tape strips (corresponding to the outer stratum corneum) of exposed skin was analysed using inductively coupled plasma (ICP) MS. We measured in pre- and post-exposure serum samples 25-hydroxyvitamin D3 (25(OH)D; biomarker for vitamin D) using LC-MS/MS and 92 inflammatory proteins (*n*=22 null and *n*=22 wt) using proximity extension assay (PEA) technology.

Results: *FLG* null carriers had both pre (19%) and post (26%) exposure significantly higher 25(OH)D concentrations than wt carriers. Post exposure, 30 inflammatory proteins were up- or down-regulated; for *FLG* null 7/11 (64%) were up-regulated; and for *FLG* wt 22/22 (100%) were down-regulated. Twelve proteins showed significantly different protein expression between *FLG* null and wt, of which 4/4 were significantly upregulated among *FLG* null and 8/8 were significantly downregulated among wt. Among *FLG* null carriers, protein changes were associated with activation and among wt carriers with inhibition of immune functions. The vitamin D receptor (VDR) was identified as an upstream regulator for 2 proteins among *FLG* null, and 6 proteins among *FLG* wt. **Conclusions:** *FLG* genotype influenced the immune response after dermal exposure to three organic chemicals and a metal. This gene-environment interaction may lead to differences in susceptibility to systemic effects of dermal exposure to chemicals.

SOC02-05**The impact of pooling animal histopathology control data on the statistical detection of treatment-related findings**

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The use of virtual control groups (VCGs) has the potential to reduce animal usage by up to 25%^[1], however, the application of VCGs to preclinical regulatory toxicity testing has not occurred primarily due to the hitherto unquantified variability of control animal data in relation to experimental conditions (sex, strain, administration route, etc.). The main barrier to such analysis has been the lack of access to sufficiently large datasets. Here we aim to address this through a retrospective analysis of preclinical histopathology data in the eTOX database^[2]. Firstly, we curated the original 6,111 studies which, owing to their origination within 13 distinct eTOX partner organisations, consisted of a heterogeneous range of experimental conditions and histopathological findings. The curated dataset contained studies conducted across 8 species, comprising between one and eight different strains each, making up 800,000 individual data points of observations across 28 selected histopathological findings. We then quantified the variability in histopathological finding prevalence with respect to experimental conditions. Changes in experimental conditions were related to significant differences (chi-squared test FDR p -value ≤ 0.05) in the prevalence of a subset of control animal histopathological findings which varied according to species, organ class, and finding type. For example, Sprague-Dawley rats were observed to have significantly higher prevalence rates across a range of liver histopathologies compared to Wistar Han rats in short-term studies (time point of observation ≤ 28 days). Finally, we analysed the impact of pooling control animal data investigated under different experimental conditions (pseudo-VCGs) on a data-driven treatment-relatedness relabeling of histopathological findings and compared these to the labels assigned by the original study pathologist. We observed an increased propensity to assign treatment-related labels (leading to a concurrent increase in recall, but a decrease in specificity compared to the study pathologist labels) as the VCG became larger and less similar in terms of experimental conditions to the dose group animals, with the derived label also becoming increasingly dissimilar to the label assigned when only the concurrent control group was used. The results highlight the effect experimental conditions can have on control animal histopathology prevalence rates and how pooling control data across such conditions can lead to a divergence in preclinical finding labeling compared to when only the concurrent control group is used.

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SOC02-06**Novel biomimetic membranes for nanoparticle transport studies at biological barriers**

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Several nanoparticles (NPs) can penetrate protective tissue barriers and elicit adverse effects in various organs. Therefore, the design of safe and effective NPs for commercial and medical applications requires a profound understanding of NP translocation and biological effects at biological barriers. To achieve predictive mechanistic insights, physiologically relevant and reliable human *in vitro* biobarrier models are indispensable. However, most current model systems utilize polymeric microporous membrane inserts to cultivate cells, which are not suited for NP transfer studies because they do not appropriately mimic the natural extracellular matrix, constitute a major barrier to the free transfer of NPs, and do not deliver predictive results.

Here, we aim to develop new membranes for predictive NP interaction and transfer studies in advanced biological tissue barrier models *in vitro*, with a focus on the placental barrier. We exploit different electrospun nanofibrous membranes, capitalizing on their high porosity and structural similarity to the extracellular matrix.

We successfully developed the methodology to achieve free-standing chitosan membranes on cell culture inserts and identified optimal electrospinning parameters to obtain reproducible, thin and nanofibrous membranes as evidenced by scanning electron microscopy analysis. Viability assays (MTS and LDH) confirmed the absence of cytotoxicity to BeWo trophoblast cells and successful formation of a tight barrier was verified by TEER measurements, ZO-1 tight junction stainings and Na-fluorescein exclusion. Translocation studies with differently sized molecules and NPs (0.3 kDa Na-fluorescein, 40kDa Dextran, 30 nm PMMA NPs and 70, 180 and 500 nm polystyrene NPs) across empty and cell containing membranes revealed a considerably enhanced permeability compared to commercial microporous membranes. Importantly, the transfer data of NPs were highly similar to data from perfusion studies of intact human placental tissue, highlighting the improved predictive value of the newly developed model system.

In future studies, we will exploit the newly developed chitosan membranes for integration in microphysiological systems as well as their potential to improve translocation studies at additional key biological barriers relevant to NP exposure (e.g. lung, intestine).

SOC02-07**Validation of the ToxProfiler reporter assay for toxicological profiling and determination of the underlying mode of action**

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Classical *in vitro* chemical safety assessment often relies on simple cytotoxicity endpoint measurements. However, the underlying mechanisms are usually poorly understood from these general assays. In order to gain further understanding of cell stress mechanisms

we have developed the ToxProfiler assay, a unique new approach method (NAM). ToxProfiler can be applied to accurately quantify chemical-induced stress response pathways at a single cell level to reveal the toxicological Mode of Action (MOA) of novel medicines, (agro)chemicals, cosmetics and food ingredients.

ToxProfiler consists of a collection of 7 unique genetically engineered human liver HepG2 cell lines. Each cell line contains a green fluorescent protein reporter that reflects a specific cellular stress response pathway; oxidative stress, genetic stress, endoplasmic reticulum stress, ion stress, protein stress, autophagy and inflammation. To visualize activation of the different cellular stress response reporters, we developed an automated live cell confocal imaging and data analysis pipeline to derive a unique quantitative toxicity fingerprint of chemicals. A point of departure (POD; lowest concentration at which a significant response was observed) is determined for each endpoint in order to directly compare the potency from one chemical to another. Here we present the validation of the ToxProfiler technology including assessment of specificity. We also provide two examples on the use of the ToxProfiler in chemical read-across and Drug Induced Liver Injury (DILI) prediction.

To summarise, we developed and validated a new mechanism-based *in vitro* reporter assay, ToxProfiler, that can provide mechanistic toxicity information for early chemical safety testing, read-across, adverse outcome pathways (AOP) and weight-of-evidence (WoE) approaches.

SOC03 | Short Oral Communications III

SOC03-01

Rat biodistribution of cerium dioxide and titanium dioxide nanomaterials after single and repeated inhalation exposure

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Introduction: Poorly soluble Engineered Nanomaterials (ENMs), after inhalation, may translocate to secondary organs due to their small size and may accumulate there. To accommodate the need for safety testing many different types of materials, alternative models are being developed. In the EU funded project PATROLS (Physiologically anchored tools for nanomaterials risk assessment), we work on developing a Physiologically based kinetic (mathematical) model. To improve this model, biodistribution *vivo* data is needed.

Methods: Rats were exposed nose-only to these ENMs or clean air for 1 day (30 minutes, 2 and 6 hours per day) or 2 weeks (similar exposure times per day, 5 days per week). Organ burdens in lung, liver, spleen, kidney as well as cerium and titanium levels in blood, urine and feces were determined by inductively coupled plasma-mass spectrometry at different time points. ENMs metal content was determined in the bronchoalveolar lavage fluid (BALF), BAL cells and in the lavaged lung.

Results: A dose dependent increase in lung burdens for both ENMs was found with faster clearance for titanium compared to cerium

after 60 days. Over 90–99% of total lung cerium was detected after lavage. Around 1–9% is taken up in phagocytic cells such as macrophages and less than 1% freely available on the alveolar surface. It takes several days to detect an increase in cerium content in the liver and several weeks before it levels off. A dose dependent increase in urine cerium content 18 hours after exposure was found that returned to background level after 1 week. Titanium was detected in liver and spleen tissue above background levels, but not in urine.

Conclusion: Two datasets on poorly soluble nanomaterials have been generated following a similar study design. This data provides a basis to parametrize a physiologically based kinetic model that is under development elsewhere in the project. This work contributes to reducing the need to test every nanomaterial *in vivo* and predict their behavior in the body using mathematical models. It will advance our understanding of the cause of adverse effects by ENMs at the site of deposition as well as in secondary organs.

SOC03-02

Estimation of uncertainty in multi-level *in silico* models predicting biomarkers of drug-induced proarrhythmic risk

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In silico methods are a valuable complement to animal testing that can contribute to the 3R's principle, speed-up risk assessment, and reduce development costs. Modern approaches have demonstrated an excellent predictive accuracy for many endpoints. However, before *in silico* predictions can be used for regulatory decision-making, the uncertainty associated with each individual prediction must be estimated and provided together with the result.

In previous works^[1,2] we described a multi-level *in silico* framework for the prediction of drug-induced QT-interval prolongation, a well-known cardiotoxicity biomarker. The QT-interval of the electrocardiogram, representing the duration of the ventricular depolarization-repolarization phase, is mainly regulated by an interplay between sodium, calcium, potassium channels. Drug-induced blockade of one or more of these ion channels can induce ventricular arrhythmias which can further progress to the lethal Torsade de Points (TdP). Relying on classic biomarkers, such as hERG IC50, may be insufficient to correctly classify high- and low-risk TdP substances and therefore, the use of the arrhythmogenic index (Tx) has been proposed to enhance the current cardiotoxicity risk assessment.^[3] The Tx represents the ratio between the drug concentration that triggers 10% prolongation of the ventricular action potential at 90% repolarization in control conditions and the effective free therapeutic plasma concentration of the drug. A Tx of 8 is considered the threshold for separating torsadogenic from non-torsadogenic compounds. Values lower than this threshold predict an increased proarrhythmic risk.

Our predictive methods are based on electrophysiological simulations describing how the blockade of these ion channels translates into arrhythmogenic risk. Ion channel inhibition can either be measured experimentally or predicted using *in silico* Quantitative Structure-Activity Relationships QSAR methods. In either case, the uncertainty levels of the model inputs should be quantified and considered in the simulations to provide reasonable estimates of the prediction uncertainty.

Here, we propose an approach that integrates conformal regression^[4,5] and probabilistic methods to estimate how the uncertainty

associated with model inputs is propagated onto the model outputs and what impact this has on the final risk assessment. This approach was implemented in the open-source modelling framework Flame (<https://github.com/phi-grib/flame>). The method was validated by comparing the predicted and observed arrhythmogenic and torsadogenic properties of a panel of drugs with well-known clinical effects.

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SOC03-03

Next generation risk assessment for skin sensitization combining non-animal data and read-across: A case study with resorcinol

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Since animal testing has been prohibited in the EU, new approach methodologies enabling a Next Generation Risk Assessment (NGRA) have to be used for the risk assessment of cosmetic ingredients. A NGRA framework for evaluation of skin sensitization has been proposed by Gilmour *et al.* [1]. It relies on data integration from NAMs covering various key events of the skin sensitisation AOP in defined approaches (DA) and read-across.

This study illustrates the evaluation of the resorcinol skin sensitisation potential according to the NGRA framework when used at 0.2% in a face cream as well as defining the maximum acceptable concentration to avoid the sensitisation of consumers.

The DA applied in this case study is a stacking meta-model integrating several *in silico*, *in chemico* and *in vitro* methods with a combination of 5 statistical models [2]. This DA is a two-tier strategy with sequential prediction of skin sensitisation hazard and potency.

Resorcinol, predicted as sensitizer in the first tier hazard prediction (Cat.1) with a high probability of 92% was classified as moderate/weak (Cat.1B) in the second tier. To increase the confidence in the potency categorization, read-across was performed in addition to the DA to set the point of departure for the risk assessment. Search for analogues based on structural features followed by selection on the basis of protein reactivity profiling and skin sensitisation data availability identified 39 suitable analogues. Three approaches were then applied to select the final set of suitable analogues and the outcome was compared to gain insight into potential differences in analogue selection and POD determination. All three approaches for analogue selection resulted in the conclusion of a moderate potency for skin sensitisation and determined nearly identical POD values (3.8% in approach 1 and 2, 3.6% in approach 3). The POD of 3.6% was used for the final risk assessment as it was derived from the EC3 values of 3 analogues and was therefore considered to be more robust than the EC3 value from a single analogue determined in approach 1 and 2.

Consequently, a higher maximum safe concentration of 0.36% in face cream was identified for resorcinol. The case study confirms that the applied NGRA is sufficiently protective for the consumer and allows an exposure led skin sensitisation risk assessment of cosmetic ingredients without generating new *in vivo* data.

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SOC03-04

Expanding the H295R steroidogenic assay using LC-MS/MS and an ER-alpha reporter gene assay as read-outs using azole fungicides as test compounds

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There is great concern on gynaecologic and female reproductive disorders related to exposure to endocrine disrupting chemicals (EDCs) that interact with steroid hormone synthesis, e.g. via food or personal care products. Our EU-funded FREIA project intends to improve testing strategies to assess female reproductive disorders caused by EDCs for implementation in regulatory frameworks.

We have developed a platform that combines both a reporter gene bioassay of estrogen receptor (ER) activation and quantification of steroid levels using LC-MS/MS. H295R cells were pre-incubated with forskolin (10 µM, steroidogenic inducer) for 24 hours. Then, cells were exposed to various concentrations of selected azole fungicides. Subsequently, exposure media were transferred to an ER-reporter gene assay (VM7Luc4E2) to determine ER transactivation and steroid profiles were assessed using LC-MS/MS. For the LC-MS/MS analysis, medium samples (100 µl) were extracted with solid phase extraction with Agilent Bond Elut Plexa 30 mg in a 96 well format and analysed with a SCIEX 6500+ triple-quadrupole mass spectrometer with electrospray ionization. Lastly, the samples are derivatized with dansylation and re-analyzed to improve the sensitivity for estrogens.

The azoles alone displayed no ER agonism and moderate ER-antagonistic activity in the ER reporter gene assay with IC50 values >10 µM. All azoles caused a concentration-dependent inhibition of estrogen production in forskolin pre-induced H295R cells, as shown by decreased ER activation in the reporter gene assay. The order of potency for the fungicides to inhibit estrogen formation (IC50) was letrozole (0.1 µM) > triflumizole (0.2 µM) > clotrimazole (0.4 µM) > flusilazole (0.6 µM) > ketoconazole (0.8 µM) > tebuconazole (5.3 µM) > propiconazole (25 µM). LC-MS/MS analysis showed that exposure to ketoconazole (1 µM) compared to vehicle control decreased both dehydroepiandrosterone and androstenedione levels by 42%, 17-hydroxyprogesterone by 30%, 17-hydroxypregnenolone by 15%, testosterone by 67%, 17β-estradiol by 24% while 17α-estradiol, estrone, estril levels were unchanged. Levels of 5α-androsterone and 5α-androstanedione, associated with the backdoor steroidogenic pathway, were reduced more than 50% upon ketoconazole exposure. Moreover, we found that deconjugation with β-glucuronidase and arylsulfatase increased the levels of progesterone by 60-fold, pregnenolone by 25-fold, dehydroepiandrosterone by 6-fold, 11-deoxycorticosterone by 2-fold, indicating phase II metabolism in H295R cells.

Together, these data show that a quantitative analysis of steroid hormones using LC-MS/MS provides a more in-depth information on interaction of chemicals with steroidogenesis. The H295R assay followed by a reporter gene assay seems a straightforward approach to explore interaction with steroidogenesis, provided that direct interaction of the test compounds with the nuclear receptor is accounted for.

SOC03-05

A novel prediction model to evaluate genotoxicity based on a gene signature in metabolically competent human HepaRG™ cells

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Genotoxicity testing is essential to ensure the safety of newly developed substances for human health. Traditionally, a stepwise standardized approach is applied, starting with a battery of *in vitro* tests. Despite its wide applicability and high sensitivity, the current *in vitro* genotoxicity battery is facing several limitations, among which the high number of misleading positive results which provoke unnecessary and costly follow-up *in vivo* animal studies^[1–3]. To improve the predictive capacity of genotoxicity testing, mechanistic information at the molecular level is needed which can be obtained via new approach methodologies including gene expression biomarkers^[4–7]. In this context, we previously developed GENOMARK to identify genotoxic substances in metabolically competent human HepaRG™ cells. GENOMARK consists of 84 biomarker genes, derived from whole genome transcriptomics data and selected to cover diverse modes of action, including bulky adduct formation, DNA alkylation, cross-linking, radical generation causing DNA strand breaks, inhibition of tubulin polymerization and base analogues^[8,9]. Briefly, cells are exposed for 72 hours to a concentration of the test chemical inducing a low level of cytotoxicity. Afterwards, gene expression levels are evaluated with RT-qPCR and results are automatically analyzed with a support vector machine (SVM)-based model to classify chemicals as genotoxic or non-genotoxic. In the present study, an improved prediction model was developed based on a different algorithm called random forest (RF). To this extent, the existing reference dataset of 24 chemicals was enlarged to 38 by selecting additional *in vivo* genotoxic and non-genotoxic chemicals for which new test data were generated. Next, two supervised machine learning algorithms in R software, i.e. SVM and random RF were applied on the extended dataset and their predictive capacity was compared. Both prediction models showed the same predictive accuracy (i.e. 92.3%), although the RF model displayed a higher sensitivity and a lower specificity compared to the SVM model. As the RF model is also less sensitive to outliers, this model was selected and further applied to predict the genotoxicity of 6 misleading positive chemicals. Four of these (2-Methyl-4-isothiazolin-3-one, 4-Amino-3-nitrophenol, Sodium benzoate and Dihydroxyacetone) were correctly classified as non-genotoxic by the GENOMARK biomarker. One chemical was classified as equivocal (hydroxybenzomorpholine) and one as genotoxic (1-naphthol). These results demonstrate that GENOMARK could be useful as a follow-up

of the *in vitro* genotoxicity test battery to de-risk misleading positives in a Weight of Evidence approach and may contribute to the paradigm shift in genetic toxicology to move towards a more human-relevant genotoxicity testing.

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SOC03-06

Emerging role of epigenetic alterations in the lung inflammation and tissue remodeling induced by air pollution-derived PM_{2.5} in mice

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Purpose: Even though clinical, epidemiological and toxicological studies have progressively provided a better knowledge of the underlying mechanisms by which air pollution-derived particulate matter (PM) exerts its adverse health effects, further studies are still needed. Emerging data suggested the possible alterations of some critical epigenetic marks by air pollution in general, and, in particular, its fine particle fraction (i.e., aerodynamic diameter ≤ 2.5 μm , PM_{2.5}). Hence, in this work, we tried to better determine whether epigenetic marks (i.e., DNA methylation, histone acetylation/deacetylation, miRNA) occurred and persisted in the lungs of mice exposed to urban PM_{2.5} (Lille, France), 24 h after their acute exposure, and 24 h and 2 months after their subchronic exposure.

Methods: A/J mice (male, specific and opportunistic pathogen free, 10 weeks, n=6/group) were exposed either for 24 h by one intranasal instillation, or for 28 days by 3 intranasal instillations/week, to 0, 10, 50 or 100 μg of urban PM_{2.5} suspended in 30 μl of sterile saline. Mice were sacrificed either 24 h after their acute exposure, or 24 h or 2 months after their subchronic exposure. Global DNA methylation, gene promoter methylation of some critical cell cycle regulators, DNA methyltransferase (DNMT) activity, histone H3 post-translational modifications (i.e., H3K9ac, H3K14ac, and H3K27ac), histone acetyltransferase (HAT) and deacetylase (HDAC) activities, and miRNA profiles were studied in lungs by ELISA, EpiTectMethyl II PCR mouse

cell cycle, and TaqMan® OpenArray® Rodent MicroRNA Panel. Non-parametric Mann-Whitney U-test was used to look at statistical differences versus controls ($p < 0.05$).

Results: Dose-dependent global DNA hypomethylation and DNMT activity decreases, gene promoter hypermethylation of some cell cycle regulators (e.g., *cdk2*, *cdk4*, *cdkn1a*, *cdkn1b*, *gadd45a*), histone H3 acetylation and HDAC/HAT decreases were reported in mice acutely exposed ($p < 0.05$). All these relevant epigenetic marks were highly deregulated 24 h after the subchronic exposure ($p < 0.05$), and most of them persisted, at a lesser extent, 2 months after exposure cessation ($p < 0.05$). Differentially regulated miRNA were found not only in the lungs of mice acutely exposed (i.e., 2 up- and 5 down-regulated), but mostly 24 h after the subchronic exposure (i.e., 26 up- and 1 down-regulated); the deregulation of some of them persisted 2 months after exposure cessation (i.e., 3 up- and 4 down-regulated) ($FC < 0.5$ or > 2 , $p < 0.05$). Accordingly, the alterations of all these epigenetic marks triggered in the lungs by ambient $PM_{2.5}$ and also persisting after exposure cessation, could dramatically contribute to the development and/or exacerbation of an inflammatory response (i.e., deacetylation of histone H3, miRNA) and to a deregulation of cell cycle/induction of tissue remodeling (i.e., gene promoter hypermethylation of cell cycle regulators, miRNA)

SOC04 | Short Oral Communications IV

SOC04-01

Estimating the Kinetics of Titanium Dioxide Nanoparticles in Rats after inhalation using Physiologically Based Kinetic Modelling

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Introduction: *In vivo* to *in vitro* extrapolation (IVIVE) of engineered nanomaterials (ENMs) dose-response data is becoming increasingly important to accurately predict *in vivo* effects, and to comprehend the key mechanisms that dictate the kinetics of ENMs. The development of accurate IVIVE methods can drastically reduce the number of animal studies required for risk assessment and toxicology, thus complying with the three Rs principles. To achieve realistic IVIVE, accurate prediction of delivered *in vivo* doses is necessary, which in turn requires developing, implementing and parametrizing physiologically-based kinetic (PBK) models. Therefore, this study aimed to adapt an existing PBK model to describe the kinetics of titanium dioxide in rats after inhalation exposure, as part of the EU funded project PATROLS (Physiologically anchored tools for nanomaterials risk assessment).

Methods: The distribution of titanium dioxide ENMs (NM-105) in rats was simulated using a PBK model. Initial values for the PBK model parameters were obtained from existing literature on titanium dioxide inhalation studies. These initial values were subsequently optimized using a Bayesian parameter estimation approach in combination with Markov Chain Monte Carlo sampling. The empirical measurement data necessary for this optimization were acquired from an inhalation-distribution study that was specifically designed to obtain kinetic information. Once parametrized, the PBK model was

validated on an external dataset obtained from previously published titanium dioxide inhalation studies.

Results: The parametrized PBK model was able to adequately describe the kinetics of the titanium dioxide ENMs in rats after inhalation exposure. The Bayesian parameter estimation method demonstrated to be a robust method to optimize PBK parameters and to reliably incorporate parameter information from various sources. In addition, the Bayesian approach is able to take the parameter value uncertainties into account, which is particularly useful in order to assess its applicability for IVIVE.

Conclusion: The PBK model implemented and parametrized in this study accurately describes the kinetics of titanium dioxide ENMs in rats after inhalation exposure. This will facilitate future IVIVE approaches to predict *in vivo* effects after the inhalation of titanium dioxide ENMs.

SOC04-02

Glyphosate and T cells: an immunotoxicity *in vitro* evaluation

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Glyphosate is one of the most used herbicides worldwide. The use of glyphosate is currently a debated topic, as several studies indicating its toxicity are emerging. Among them, there is evidence of adverse effects on the immune system. We previously examined the *in vivo* effect of glyphosate on farmers, evaluating several immune parameters. Based on these data, the aim of this work was to investigate if glyphosate could directly affect immune cells, using peripheral blood mononuclear cells obtained from healthy donors. The phenotyping of T helper cells and the production of cytokines, following glyphosate treatment (0.01, 0.1, 1 and 10 $\mu\text{g/mL}$) and stimulation with T cell mitogens (PMA and ionomycin), were assessed, using flow cytometry and enzyme-linked immunosorbent assay. Results shown an enhancement of IL-4 as well as IL-17A production, and a reduction of IFN- γ . Regarding Th subpopulations, the major effect of glyphosate was on the ratio between Th1 and Th2 cells, which resulted diminished, mainly due to the reduction of Th1 cells. Based on literature evidence, we hypothesized an estrogenic effect behind the observed unbalance between Th1/Th2 cells, and the inhibition of estrogen receptor, through ICI 182,780, reflected in the restore of this condition. To further characterize the mechanism of action of glyphosate, the analysis of several miRNAs contained in microvesicles was performed. Results shown an up-regulation of miR-500a following glyphosate treatment, which we demonstrated to be correlated with the reduced production of IFN- γ , by using a selective antagomir. Overall, these results suggest a direct action of the herbicide on lymphocytes.

SOC04-03

Analysis of murine liver mRNA expression, DNA methylation, and histone after repeated exposure to chemicals

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The Perclome Project aims at reinforcing and replacing the safety factor in toxicology by comprehensively identifying the transcriptional networks induced by xenobiotics in murine organs. "Perclome" method was developed (BMC Genomics 7:64, 2006) 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.

In addition, newly designed repeated dose study which is inspired 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 described above and the liver was sampled at 2, 4, 8 and 24 hours thereafter. Now we obtained data sets on CCl₄, clofibrate, valproic acid, and corn oil in terms of Affymetrix GeneChips for the 48 mice and whole genome bisulfite analysis (WGBA) data and ChIP-seq data against H3K4me₃, H3K27me₃, H3K27Ac, and H3K9me₃ for control and 14 day exposed mice.

Here, mRNA, DNA methylation and histone data for CCl₄, clofibrate, and valproic acid were analyzed. Firstly, 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 CCl₄, i.e. lower BR linked to suppressed TR and vice versa. The link were in some part chemical dependent. However, 14-day CCl₄ pre-treatment did not alter DNA methylation, whereas ChIP-seq revealed that BR and TR of mRNA of some characteristic genes was in good correlation with histone modification. Enhanced mRNA response by 14 day repeated dosing was often explained by alteration in histone status, i.e. increase in H3K27Ac and/or H3K4me₃ (activation marks), whereas suppressed mRNA response by 14 day repeated dosing was often explained by alteration in histone status, i.e. increase in H3K9me₃ and/or H3K27me₃ (repression marks). Analysis on other chemicals will be presented. (Supported by Health and Labor Sciences Research Grant of MHLW, Japan)

SOC04-04

Development of an *in silico* platform to assess developmental and reproductive toxicity (DART)

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Assessment of developmental and reproductive toxicity (DART) uses a large number of mammals. It is particularly in this field of toxicology that alternative test methods that follow the principles of the 3Rs (Replacement, Reduction, and Refinement) are highly warranted, to reduce the amount of animal testing. There are many efforts to design alternate assays, whose results are very encouraging. However, a challenge remains in the interpretation of the data. DART is very complex, involving many diverse biological processes and interconnected adverse outcome networks. It is now possible to address this challenge with the recent developments in molecular biology, omics datasets, database interconnectivity, and data science.

We have designed a user-friendly web-based platform that enables complex data integration. For example, the interface can be used

starting from chemical(s), via (predicted) DART phenotypes, to adverse outcome pathway, and reversely: from an adverse outcome pathway towards DART phenotypes. Molecular fingerprints are used to compute compound similarity, which allows the comparison of toxicity profiles between similar compounds. Additionally, the interface contains DART phenotypic endpoint data for a wide variety of test systems, to compare OECD DART test data with 3R test methodologies (zebrafish, *C. elegans*, slime mold, and cell-based assays). Cross-species phenotype-to-pathway analysis of toxicity endpoints allows the prediction of evolutionary-conserved pathways affected by compounds.

The use of the interface enables the selection of relevant biological tests for DART assessment and supports the prioritization of chemicals and adverse outcome effects for further investigation. By integrating years of scientific research and knowledge that is available in public resources, we aim to boost the acceptance of alternatives to mammalian DART testing. We thank the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) CRACK IT Challenges programme for funding this work.

SOC04-05

An *in vitro* harmonized strategy to assess the toxicity of chemicals using multiple human induced pluripotent stem cell (hiPSC)-derived models

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Most OECD guidelines for chemical risk assessment include tests performed on animals, raising not only financial and ethical concerns but also scientific ones. Thus, the development and use of human-based models for toxicity testing is highly encouraged.

The Marie Curie training network project *in3* proposed an *in vitro* multi-organ platform to assess the toxicity of chemicals. Human induced pluripotent stem cells (hiPSCs)-derived models of the brain (2D and 3D mixed-cell cultures), blood-brain barrier (endothelial cells), kidney (podocytes and proximal tubular cells), liver (hepatocyte-like cell) and vasculature (endothelial cells) were successfully generated from the same two donors, using published or newly developed protocols. Paraquat (PQ), a widely employed herbicide with toxic effects

in lungs, kidneys and brain, was used as a reference compound to test this approach.

Cytotoxicity after PQ exposure (24/48h) was determined by MTT or ATP assay for each system. Then the models were exposed to 2–4 concentrations (at least one below IC10 value for cytotoxicity) for 24 or 48h. Samples were collected for TempoSeq™ analysis with a set of 3565 probes. Raw data were subjected to probe and sample filtering and were carefully quality controlled. Differentially expressed genes (DEG) were detected using DESeq2 R package. Pathway analysis using the full list of DEG per model and the database CONSENSUSPathDB® showed the disruption of “Oxidative stress induced gene expression via nrf2 markers”, as expected since it is the well-known PQ mechanism of action. Furthermore, two other pathways previously involved in PQ toxicity, “ESR-mediated signaling” and “Photodynamic therapy-induced unfolded protein response”, were also deregulated. Genes belonging to these pathways, such as MAFF (MAF BZIP Transcription Factor F), PPP1R15 (Protein Phosphatase 1 Regulatory Subunit 15A), ATF4 (Activating Transcription Factor 4) and GDF15 (Growth Differentiation Factor 15), showed various levels of expression in the distinct models, suggesting cell type- or organ-specific ability to respond to PQ exposure.

This strategy allowed to determine known mechanisms of PQ toxicity, although we used a restricted cost-effective, number of probes in TempoSeq analysis. The main advantages of this strategy are to assess chemical toxicity on multiple organs in parallel, exclusively in human cells, and on cell-type- or organ-specific models derived from the same donors, eliminating the interspecies and genetic background biases, and allowing a better evaluation of the differential sensibility of the diverse organs. Furthermore, although we focused on the common mechanisms of action of PQ, this strategy would allow at the same time for organ-specific toxicity testing, by using an increased number of probes for TempoSeq analyses. In conclusion, we believe this strategy will participate in the further improvement of chemical risk assessment for human health.

SOC04-06

Brain Organoids to Study SARS-Cov-2 Infection of Developing CNS

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Early reports from Wuhan suggested that 36% of COVID-19 patients show neurological symptoms, later European studies showed as much as 60%; cases of viral encephalitis have been reported. This suggests that the virus might be neurotropic under unknown circumstances. This is well established for other coronaviruses. Many questions remain with regard to the current pandemic, including the influence of SARS-CoV-2 on the developing brain. In order to understand why some patients develop such symptoms and others do not and whether developing brain might be more susceptible than adult counterpart, we addressed the infectability of the central nervous system (CNS). Reports that the ACE2 receptor – critical for virus entry into lung cells – is found in different neurons support this expectation. We employed a human induced pluripotent stem cell (iPSC)-derived BrainSphere model. A short-term infection of the BrainSpheres with SARS-CoV-2 led to infection of a fraction of neural cells with replication of the virus evident at 72 hpi. Virus particles were found in the neuronal cell bodies extending into apparent neurite structures. PCR measurements corroborated the replication of the

virus, suggesting at least a tenfold increase in virus copies per total RNA. Immature and more mature cultures have been compared. 12-week BrainSpheres were more sensitive to infection than 5-week ones, suggesting that maturation processes (such as synaptogenesis and network formation) might render more sensitive to the infection. These findings were supported by others in similar brain organoid models. These recent findings will be summarized to understand the advantages and limitations of brain organoids in infectious diseases in particular for the developing nervous system, as brain organoids mimic embryonic stages of development.

SOC04-07

Evaluating new approach methodologies for consumer-based risk assessments: challenges and future perspectives

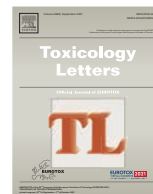
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Using the risk assessment of 0.1% coumarin in a face cream and body lotion as an exemplar case study, we recently demonstrated how new approach methodologies (NAMs) can be applied in Next Generation Risk Assessment (NGRA) to assess the safety of consumer product ingredients [Baltazar et al, 2020]. While this study helps build confidence in the use of NAMs for consumer-based risk assessments, there is an on-going need to demonstrate that these approaches can be used to define low-risk consumer exposures for a wider range of chemicals and scenarios. To that end, we are evaluating a potential toolbox for systemic toxicity, which comprises several NAMs for characterising bioactivity (high throughput transcriptomics, *in vitro* cellular stress [Hatherell et al., 2020] and the Eurofins Safety44® Screen), together with and computational models for estimating relevant human exposures (physiologically-based kinetics (PBK) modelling [Moxon et al, 2020], skin penetration and free concentration models). These tools can be combined to estimate a margin of safety (MoS) for a given chemical exposure. In this presentation we will discuss the overall strategy for evaluating the toolbox, namely to generate data for at least forty chemical-exposure scenarios that are either known to be either associated with adverse systemic toxicity effects, or are known to present a low risk to humans. These data will be used to develop a Bayesian statistical model for characterising uncertainties in the MoS distribution, which in turn could be used to identify appropriate low risk exposures as part of an overall safety assessment for novel consumer ingredients. Preliminary toolbox results for twelve chemical-exposure scenarios (generated as part of a pilot study) will be used to illustrate the overall concepts and future perspectives of the work.

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Poster Presentations

P01 – Adverse outcome pathways

P01-01

5 α -reductase inhibition results in decreases of DHT, E2 and VTG in Zebrafish larvae

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Introduction: Environmental stressors such as changes in steroid hormones are profound regulators of female reproduction. Steroid 5- α reductase (5AR) is responsible for the reduction of steroids to 5- α reduced metabolites including testosterone to active androgen, 5- α dihydrotestosterone (DHT). Adverse outcome pathway (AOP) as the 5AR inhibition to reduce female reproduction in fish (AOP 289) is under development to clarify the anti-estrogenic effects of 5AR inhibitors in female fish.

Methods: To probe the key event relationship (KE) from molecular initiating event to consequence key event (KE) of AOP289, we investigate between DHT and estrogen hormone in zebrafish (*Danio rerio*; ZF) larvae. 5 AR inhibitor, dutasteride was treated in zebrafish larvae. To address these data gaps in the mechanisms in key events and links, we analyzed estrogen receptor (ER) and aromatase gene expressions and 17 β -estradiol (E2) hormones, and a precursor of the egg yolk proteins, vitellogenin (Vtg) in different concentrations of dutasteride treatment. For the sex hormone measurement, the 10-fold diluted samples were used for E2 and DHT quantification using specific ELISA kits according to the manufacturer's instructions. Each sample was normalized to total protein content, quantified using the BCA protein assay.

Conclusions: In conclusion, the results presented in this paper demonstrated that 5 α Reductase inhibition could affect reproductive functions in Zebrafish embryo. By comparing our results for the level of DHT and E2 and VTG in the presence of Dutasteride, it resulted in decreases level compared to the untreated control group. These results suggest that Zebrafish embryo would be of great utility for the evaluation of potential adverse effects caused by anti-androgenic chemicals and warranted to investigate the reproductive adverse outcome in fish.

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P01-02

This abstract has been withdrawn.

P01-03

Adverse outcome pathways for uranium induced kidney toxicity

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The development of adverse outcome pathways in the field of exposure to ionizing radiation is a means of identifying knowledge gaps to assess the health risks of exposure as successfully done for chemicals. Here we propose a review of knowledges, from experimental and epidemiological datas, to build the AOP for the renal impairment pathways identified for a reference nephrotoxicant, uranium (U).

U metal-ions are filtered through the glomerular membrane of the kidneys, then concentrates in cortical and juxtaglomerular areas and binds to the brush border membrane of the proximal convoluted tubules. U uptake by epithelial cells occurs through endocytosis and the sodium-dependent phosphate co-transporter. Molecular initiating events start with U binding to cell proteins lipids or nucleic acid. The formation of reactive oxygen species cause the collapse of mitochondrial membrane potential due to cyt b5/cyt c disruption. In the nucleus, U directly interact with negatively charged DNA phosphate, thereby inducing adduct formation and possibly DNA strand breaks or cross-links. U also compromise DNA repair by inhibiting zing finger proteins. Thereafter U triggers the Nrf2-, NF- κ B or ER-stress pathways. The resulting cellular key events include oxidative stress, DNA strand breaks and chromosomal aberrations, apoptosis, and pro-inflammatory effects. Finally, the main adverse outcome is tubular damage of the kidney's S2 and S3 segments, leading to renal filtration dysfunction and then chronic kidney disease. Attribution of renal carcinogenesis due to U is controversial and need specific experimental or epidemiological studies. A tentative construction of AOP on uranium induced kidney diseases is then proposed.

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P01-04

Mathematical modeling approaches for quantitative adverse outcome pathway: proof of concept for the integration of key events and key event relationships.

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Adverse outcome pathway (AOP) is a sequence of causal biological events that qualitatively explain how the adverse outcome proceeds. AOP framework enables us to perform pathway-based toxicity assessment, thus it is expected to be an effective approach for risk prediction as well as regulatory decision making. However, it is also important to examine the quantitative relationships between each biological event in an AOP, and to assess the extents or probabilities of the adverse outcome incidence. Mathematical modeling is proposed to advance AOP from qualitative to a quantitative tool. In this study, we applied several mathematical modeling approaches to make predictions on the outcome based on predictor variables; linear regression, mixed model, Random Forest, Multivariate adaptive regression splines (MARS), and Gaussian Bayesian network (BN) modeling. The dataset used were obtained from 5 different donors of primary human bronchial epithelial cells (HBEC) exposed to cigarette smoke extract, to include the donor-to-donor variability. Dose-response and the response-response relationship between two molecular initiating events (MIE) and a key event 1 (KE1) with 4 key event relationships (KERs) were then modeled, where KE1 was used as an objective variable for the first instance of the modeling approach. Among the 4 models other than the BN model, the mixed model with donor as the random effect showed the highest performance where the data showed high linearity in the dose-response and response-response relationships, while machine learning models showed lower performance due to relatively small sample size. Alternatively, the BN model is the probabilistic model that provides the prediction of the distribution of KEs. BN model showed a dose-related effect in the probability of KE1 activation when KE1 activation is defined using

a specific cut-off value. We also generated the probability matrix and its visualization to calculate volume under the surface which enables comparative risk assessment among different test substances when there is no evident cutoff or threshold.

In summary, a regression model could be one modeling alternative if the response-response relationship shows a certain linearity, while if the relationship shows non-linear behavior, Random Forest and MARS may be better alternatives. Considering that machine learning models generally require relatively large sample size and complete dataset (i.e., missing values are not allowed), BN model is more flexible because it could make inference on a specific node given evidence on some other nodes, and if the given network structure is simple enough, the sample size does not need to be large. Such specification of models and characteristics of dataset should be considered when building quantitative models for AOP. Extension of the modeling approaches to predict chronic phase toxicity/effects is recommended for future work.

P01-05

Comparing the impact of multi-walled carbon nanotubes between murine and human lung epithelial cell models *in vitro*

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Most literature associated with nanotoxicology focuses on short-term, high-dose exposures of engineered nanomaterials (ENM) using 2D-monocultures or *in vivo* studies. The EU Horizon 2020 project PATROLS (Grant No. 760813) aims to create and characterise *in vitro* model systems for ENM hazard assessment using previously established *in vivo* data on specific ENM. This study aimed to complete an inter-species comparison between a murine and human lung epithelial cell model in order to establish if *in vivo* (murine cell line) exposures can be used to determine potential human effects (human cell line).

A previous study that exposed a murine epithelial cell line (FE1) to MWCNTs (Mitsui-7) (3.9 µg/cm², 7.8 µg/cm² and 31.19 µg/cm²) over 24hrs was used as the basis for comparison against the herein experimental approach^[1]. Using an identical submerged *in vitro* exposure, a human epithelial cell line (A549) was exposed to the same MWCNT for the same duration and concentrations in order to compare across species. Using a real-time Polymerase Chain Reaction (Bio-Rad) array, established Key Events from published Adverse Outcome Pathways (AOP); specifically AOP 302 and AOP 173; were focussed upon. The gene list included those pertinent to fibrosis (CEBPB, and COL1A1), inflammation (CCL2, CCL5, IL-15, IL-33 and IL-6) and oxidative stress (SOD2).

The human epithelial cell (A549) system showed a significant concentration-dependent increase in the expression of BCL2 (apoptosis), and COL1A1 (type II collagen), as well as a decrease in CCL2 (chemotactic for monocytes and basophils) and SOD2 (anti-oxidant) after 24hrs exposure. Compared to the negative control, a significant down-regulation of CASP3 (apoptosis) at all concentrations, and IL-10 (anti-inflammatory) at the lower MWCNT concentrations was also observed.

In comparison to the mouse epithelial cell (FE1) signature previously published, it was observed that Cebpb expression was increased in both models; however, the human model was increased at all concentrations tested, whilst the murine model was only increased at the highest concentration. IL-33 expression was decreased in the

murine model at all concentrations, while in the human model it was only decreased after the higher concentrations. *CCL5* expression was decreased at all concentrations in the human model, while the murine model saw increases at all concentrations. *Sod2* expression was unchanged in the murine model, while in the human model the expression was decreased at all concentrations.

From our results, we identified that the same MWCNT exposure elicits different responses depending on the model species (mouse vs. human) at a gene level. Assessment of these differences at a pathway level is important before determining the relevance and usefulness of these murine models in terms of them being the best tool to predict human health hazards following ENM exposure.

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P01-06

Evaluation of the Skin Sensitization Potential of Silica nanoparticles using *in vitro* and *in vivo* assay

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As nanotechnology has expanded its application to technologies such as electronics, materials, medicine and energy, it is drawing much attention as a major core field of technology development in the 21st century. Silicon dioxide (SiO₂) NPs are widely used in industrial products, including cosmetics, drugs and biomedicine. Even if increasing interest in dermal toxicity of silicon dioxide (SiO₂) NPs, little information has evaluated the potential of their skin sensitization. Aiming to fill this lacuna, we evaluated the skin sensitization of silicon dioxide (SiO₂) using the ARE-Nrf2 Luciferase KeratinoSens™ *in vitro* assay method and the LLNA: BrdU-FCM *in vivo* assay method. The SiO₂ were measured physicochemical properties such as the hydrodynamic size, polydispersity and zeta potential under stock and working solution conditions. After that, the skin sensitization potential of the SiO₂ were assessed using the ARE-Nrf2 Luciferase KeratinoSens™ *in vitro* assay and the LLNA: BrdU-FCM *in vivo* assay. This study demonstrate SiO₂ showed negative responses for skin sensitization potential. These results suggest that the KeratinoSens™ and LLNA: BrdU-FCM assay can be applied to evaluate the potential of nanomaterials for skin sensitization.

P02 – Biomarkers of effect/exposure

P02-01

Quantification of thyroid hormones via online-SPE-LC-MS/MS from different biological matrices

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Thyroid hormones (TH) such as T3 and T4 play a critical role in organ function and biological development across different taxonomic groups. TH deficiency during development can lead to severe disorders

including cretinism and also adults could be affected by TH disorders. TH homeostasis can be disturbed by xenobiotics, which are known as endocrine disruptors (ED). Toxicological assessment of chemicals with potential endocrine disrupting properties requires robust and reliable measurement of TH in different matrices.

We have developed and validated an online-SPE-LC-MS/MS method combined with a simple sample preparation to measure total 3,3'-T2, T3, rT3 and T4 in rat plasma. In a 50 µL plasma sample, the LLOQ was 0.028 nM for T2, 0.28 nM for T3 and rT3 and 7 nM for T4. This method was linear in the concentration range 0.02–2.8 nM for T2, 0.14–21 nM for T3 and rT3 and 0.28–280 nM for T4. By slightly modifying the sample preparation, we could also quantify 3,3'-T2, T3, rT3 and T4 in rat brain. In 15 mg rat brain, the following concentrations were determined: T2 (0.87 ± 0.09 pmol/g), T3 (6.07 ± 0.09 pmol/g), rT3 (0.24 ± 0.01 pmol/g) and T4 (6.65 ± 0.85 pmol/g).

Combined with a suitable sample preparation, our robust online-SPE-LC-MS/MS method has the potential to be broadly applicable for TH analysis in different biological matrices. This could also facilitate the toxicological assessment of potential endocrine disrupting chemicals and help to determine their mode of action.

We will present the analytical methods and their details comprising the SPE, LC and MS parameters. Additionally, the results from the analytical method validation in rat plasma will be shown. Furthermore, the results from the detailed analysis of the potential influence of plastic vials and filters on quantitative results will be shown and solution approaches to avoid them.

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P02-02

This abstract has been withdrawn.

P02-03

CD36 and adiponectin, adequate markers for dyslipidemia during gestation in a prediabetic mouse model

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Gestational diabetes (GDM) is the most common disorder in pregnancy and both, hyperglycemia and dyslipidemia have severe consequences for mother and child. New Zealand obese (NZO) mice exhibit impaired glucose tolerance before and during pregnancy. The adipokine adiponectin (AdipoQ) and the fatty acid translocase (FAT/CD36) are discussed as potential markers for the underlying pathomechanisms of GDM. However, available data are limited and appropriate models are needed for better prediction of disease progression. The aim of this project was to investigate whether alterations in AdipoQ levels and CD36 expression pattern during pregnancy correlate with metabolic changes in this mouse model.

Female NZO mice and healthy NMRI controls were examined pre-conceptional (pc.) and at day 14.5 of gestation (d14.5). Animals were sacrificed at 9–10 weeks of age and plasma, liver and gonadal white adipose tissue samples were collected. Liver weight and fat mass

were measured. Plasma lipid profiles were determined with fluorometric assay kits. Plasma AdipoQ was measured by ELISA. CD36 gene expression was detected by microarray analysis and further assessed and quantified by hepatic immunofluorescence (IF) staining.

NZO mice revealed pc. higher FFA plasma levels relative to NMRI controls. Compared to NMRI controls, NZO mice exhibited hyperlipidemia at d14.5, with significantly elevated TG and FFA profiles. In parallel, AdipoQ was significantly decreased in NZO mice compared to NMRI controls both at pc. and at d14.5. A significantly higher fat pad mass was observed in NZO mice compared to NMRI controls, consistent with the lower AdipoQ levels. However, in contrast to NMRI, AdipoQ levels in NZO mice decreased significantly at d14.5 compared to pc. Both strains showed a significant increase in fat mass and liver weight with a corresponding increase in hepatic CD36 gene expression at d14.5. CD36 was significantly elevated, both pc. and at d14.5 in NZO mice compared to NMRI controls. This is in line with an increased integrated IF-density in NZO mice at d14.5, while NMRI controls showed a moderate decrease. Interestingly, the CD36 IF signal in NMRI hepatocytes was localised to the cell membrane at both time points, whereas no translocation to the membrane, but cytosolic IF was observed in NZO mice at d14.5.

With elevated lipid profiles, NZO mice revealed important characteristics of dyslipidemia during pregnancy. AdipoQ values correlated inversely with increasing fat mass, TG and FFA. In general, AdipoQ was decreased and CD36 was increased at glucose-intolerant states, indicating a marker function. Thus, the determination of AdipoQ and CD36 is suitable for monitoring dyslipidemia, which often precedes the pathophysiology of GDM. Since CD36 translocation is induced by insulin, failure to do so could further reflect hepatic insulin resistance in NZO mice and is the basis for further investigation.

P02-04

Sulfur Mustard exposure: skin and eye animal models for countermeasure and biomarker researches

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Sulfur Mustard (SM), also called yperite or mustard gas, was used for the first time in 1917 during World War I and more recently in Syria, although the use of chemical weapons was forbidden since 1995. SM still represents a chemical threat, particularly in terrorism context. In addition each year, farmers or weapon collectors are exposed by digging up World War I buried shells. SM is insidious since its penetration is painless, the first effects can appear several hours after exposure, thus delaying the decontamination and medical management. Once SM penetrates in organism, it affects almost all organs (lung, eye, skin, brain...). Its mechanism of action is partially known and there is still no antidote and treatment is only symptomatic (supportive care, pain treatment and antibiotic therapy). In order to highlight new biomarkers, and to investigate new skin and eye therapeutics and decontamination lotions, relevant models of SM burns are necessary.

Thus, we developed a model of skin burns by saturated SM vapors. The chosen animal model is the hairless SKH-1 mouse, currently used in dermatology studies. Caps containing SM are fixed on mice back but only vapors are in contact with the skin. This model offers possibility to modulate parameters (exposure or decontamination delays) and to have treated and control areas on the same mice.

The second model developed to evaluated SM toxicity eye is the New Zealand rabbit. Rabbits are exposed by vapors of SM on eyes via caps as the skin model. One eye is exposed and the second one serves as control. Several measurements are performed to assess the corneal thickness, intraocular pressure. Biological fluids are sampled

(urine, blood and tears) to analyze their composition and to detect biomarkers. The decontamination is crucial to prevent a spread of contamination and to limit symptoms since there is no observed effects in the first minutes after contamination. Thus, it is capital to evidence intoxication by SM in order to limit casualties. For this purpose, rapid and non-invasive biomarkers are needed to ensure early decontamination and medical management. Our eye and skin SM-burn models are reproducible, reliable and adaptable according to the aim of the study as regards time of contact, dose of exposure, delay before decontamination. They are relevant to test new therapeutics, decontamination lotions or for biomarker research.

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P02-05

Albuminuria and GM2AP as predictors of patients at risk of developing contrast-induced nephropathy

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The administration of contrast media (CM) is associated with a pathology called contrast-induced nephropathy (CIN), with a high incidence in patients at risk. Scores based on risk factors have been established to predict whether patients will suffer from this pathology before CM administration, but their predictive capacity has many limitations and must be improved.

New markers have recently emerged for the diagnosis of kidney damage, such as neutrophil gelatinase-associated lipocalin (NGAL), kidney damage molecule 1 (KIM-1) or albuminuria. Preclinical studies carried out in our group have identified the M2 ganglioside activating protein (GM2AP) and transferrin as markers of the risk of developing kidney damage after the administration of different potentially nephrotoxic compounds.

The objective of this work was to evaluate the usefulness of these markers to predict CIN in cardiac patients.

A retrospective clinical study was carried out with 154 patients from the Cardiology Service of the University Hospital of Salamanca receiving CM. Prior to the administration of the CM, urine samples, anthropometric data and renal risk factors were collected. In addition, plasma creatinine data were obtained daily up to 5 days after CM. The patients were divided into: (i) Control: they did not develop CIN and (ii) Cases: they did develop CIN.

NGAL, KIM-1, albuminuria and transferrin were determined in the urine sample by ELISA and GM2AP by Western Blot.

Of the total number of patients, 123 were assigned to the Control group and 31 to the Cases group. In both groups, the distribution of sex, age and risk factors was similar. Of the biomarkers measured, albuminuria, transferrin and GM2AP were higher in patients who developed CIN (Cases) while NGAL and KIM-1 did not show differences between both groups.

This study shows that urinary albumin, transferrin and GM2AP are potential biomarkers to predict the appearance of CIN, which could be useful in the preventive management of patients at risk.

P02-06**Usage patterns of facial cosmetic products in Asia**

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Layering practices are widespread in Asia, however limited data are available in the literature on cosmetic products consumption by Asian populations. Moreover, Asia is composed of several populations that may have different usage patterns of cosmetics. These usage patterns information and frequency of use are essential to perform more realistic safety assessments for specific populations. The aim of this study was to characterize skincare habits in 3 Asian countries through a web-questionnaire survey. The types and galenics of facial cosmetic products used, their order of application and the frequency of use were investigated.

The web-questionnaire survey on facial skincare consumption was performed in China, Japan and Taiwan. 11 product categories were investigated including cleanser, tonifier, moisturizer, sunscreen... Women between 16 and 55 years old were recruited as they are supposed to be the main skincare products consumers. They also had to be users of at least one of the 11 product categories. The subjects were selected according to criteria of representativeness: age and region of residence. A total sample size of 1,801 women were recruited: 601 in China, 600 in Japan and 600 in Taiwan.

Data on frequency of use and order of application of skincare products were collected, as well as information related to the impact of COVID on cosmetic consumption habits.

Among the 11 cosmetic product categories investigated, 9 are used by more than 57% of the respondents but disparities between countries are observed. In China, 43% of the participants use all the 11 product categories, compared to 23% in Taiwan and none in Japan. Chinese use an average of 8.7 categories out of the 11, Taiwanese 7.7 and Japanese 6.9.

Percentages of users are similar between the investigated countries for cleanser, tonifier and sunscreen, whereas for the other categories, differences are observed (difference <10%). For example, the facial mask is used by 86% of Chinese, 79% of Taiwanese and 40% of Japanese. The eye contour is used by 72% of Chinese, 46% of Taiwanese and 25% of Japanese.

Furthermore, the number of product categories used at least once a day also varies from one country to another: Chinese use an average of 6.6 categories daily, Taiwanese 5.4 and Japanese 5.

Regarding the impact of COVID, 48% of the subjects in Japan report a change in cosmetic consumption habits, 40% in China and 18% in Taiwan.

This study provides new cosmetic consumption data for 3 Asian populations by highlighting disparities between China, Japan and Taiwan particularly regarding the number of product categories used daily. Therefore, this suggests that layering could be different between the investigated countries. Specific rituals might be identified by analyzing frequencies of use and order of application. All information collected in this questionnaire will help creating a more suitable database for these populations regarding facial skincare habits.

P02-07**Detection of dexamethasone administration in veal calves: transcriptional biomarkers validation in FFPE muscle samples**

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Abuse of growth promoters in meat-producing animals is strictly regulated within the European Community to preserve consumers' safety. Nevertheless, illicit misuse of steroids and other veterinary drugs to increase animal production has still been found by National Residues Control Plans (NRCPs). The setup of complementary diagnostic methods, based on indirect biomarkers of exposure to anabolic substances could be useful to update current tests available in NRCPs^[1].

The aim of the work was to develop a Real Time PCR array analysis for profiling multiple muscular biomarkers applied to FFPE samples, able to identify animals suspected of illicit treatment with dexamethasone (DEX), a molecule belonging to corticosteroids group, one of the most recurrent classes of growth promoters reported by EFSA^[2].

The selection of differentially expressed genes (DEG) suitable as biomarkers of DEX exposure in cattle was defined by analyzing the available datasets from previous transcriptomics studies^[3-5]. A final selection of 28 genetic targets plus 4 reference genes was made to design a qPCR Taqman array suitable for analysis of FFPE muscle samples collected from veal calves. FFPE muscle samples (n=53) were selected from previous animal trials performed to study the biological effects of DEX^[6-7]. Total RNA were isolated from each FFPE sample with miRNeasy FFPE kit (Qiagen), quantified with Qubit BR-RNA kit (Thermo) and then 1.5 µg of total RNA was reverse transcribed with an SSIV VILO Master Mix kit (Thermo). Selective preamplification of targeted cDNA was made by Taqman Preamp Master Mix (Thermo). The qPCR runs were carried out on a StepOne Plus Real-Time PCR System (Thermo). Cq values from all qPCR runs were imported into GeneEx 6.1 software (MultiID) and merged to allow data analysis, filtering and relative quantification (RQ) calculations by the $\Delta\Delta Cq$ method^[8] after reference gene selection by Normfinder and Genorm analysis^[9]. Variance analysis was performed by one-way ANOVA with Dunnet *post hoc* test.

All RQ data collected were then analyzed by Partial Least Squares – Discriminant Analysis (PLS-DA) to identify best candidate biomarkers of the applied treatment^[10]. Multivariate model was built coupling PLS-DA to a variable selection algorithm in backward elimination (BE): at each iteration the variable that least correlates with class belonging is eliminated (smallest VIP score), allowing to achieve a model with the smallest error in cross-validation. Of the 28 biomarkers selected for array design, only 15 transcripts in analyzed FFPE muscle resulted still differentially expressed ($p < 0.05$). However, developed PLS-DA showed the possibility to setup a reliable model able to detect DEX misuse. These preliminary result need to be confirmed by further evaluations on larger sample sets, but they showed that FFPE sampling could ensure potential applications in the frame of current NRCPs, as already reported in recent studies^[11-12].

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P02-08

Occupational welding exposure and lung cancer-related epigenetic modifications

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Welders are occupationally exposed to particles rich in metals such as iron, manganese, chromium, and nickel, which are generated during the welding process. Welding fumes are classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC) and particle exposure is considered to play a role in its carcinogenicity.

The aim of this study was to evaluate changes in (epi)genetic markers in mild-steel welders which had been associated with lung cancer – specifically the methylation status of selected CpG sites in the genes *AHRR*, *F2RL3*, and *B3GNTL1* but also telomere length (TL) and mitochondrial DNA copy number (mtDNAcn).

Data from 338 male non-smokers was evaluated, of whom 171 were welders and 167 controls. Two evaluations spread 6 years apart were available for 174 individuals (78 welders and 96 controls). Respirable dust exposure (adjusted for respiratory protection), cumulative exposure to welding fumes and welding years were chosen as measures of exposure to welding. DNA was extracted from whole blood samples to evaluate the methylation of lung cancer-related genes *AHRR*, *F2RL3*, and *B3GNTL1* using pyrosequencing. qPCR was used to measure the cancer-related biomarkers TL and mtDNAcn in whole blood. For the longitudinal analysis linear mixed models were used.

Adjusted respirable dust median exposure was 0.7 mg/m³ at both timepoints for the welders. A significant decrease in methylation of *B3GNTL1* CpG1 and CpG4 (adjusted for age, BMI, ever smoking: CpG1

$\beta = -0.66$, SE = 0.28, $p = 0.016$; CpG4 $\beta = -0.48$, SE = 0.24, $p = 0.046$) was observed in welders when compared to controls. Sensitivity analysis of never-smokers confirmed the main analysis outcome (adjusted for age, BMI: CpG1 $\beta = -1$, SE = 0.41, $p = 0.015$ and CpG4 $\beta = -0.69$, SE = 0.33, $p = 0.035$). A dose-response relationship, regarding the decrease of methylation in *F2RL3* CpG2, in association with respirable dust and cumulative exposure was observed in welders, the methylation decreased between timepoint 1 and timepoint 2 (adjusted: age, BMI, ever smoking: respirable dust $\beta = -0.67$, SE = 0.23, $p = 0.004$, cumulative exposure $\beta = -0.03$, SE = 0.02, $p = 0.48$). The effect estimates remained negative in association with respirable dust exposure when only including never-smokers, however no longer significant (adjusted: age, BMI: respirable dust $\beta = -0.59$, SE = 0.35). No significant associations with the exposure variables could be observed for *AHRR*, TL and mtDNAcn.

In conclusion, exposure to mild steel welding fumes was associated with a decrease in methylation of some selected CpG sites. However, the effect on the chosen (epi)genetic biomarkers of (lung)cancer was small. We observed significant changes at exposure levels more than three times lower than the Swedish occupational exposure limit (2.5 mg/m³). These findings emphasize the need for better understanding the health effects of occupational welding exposure and finding solutions on how to decrease the health risks among welders.

P02-09

Aminonaphthylcysteine Adducts in Globin and Aminonaphthylmercapturic acids in the Urine of Rats Dosed with Naphthylamine and Nitronaphthalene Isomers

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New aminonaphthylcysteine adducts were identified and quantified in globin of rats dosed with single *i.p.* doses (0.162 mmol/kg b.w.) of 1- and 2-naphthylamine (1-NA and 2-NA) as well as 1- and 2-nitronaphthalene (1-NN and 2-NN) by HPLC-ESI-MS² analyses of globin hydrolysates using authentic standards. Both 1-NA and 1-NN gave S-(1-amino-2-naphthyl)cysteine together with S-(4-amino-1-naphthyl)cysteine whereas 2-NA and 2-NN gave S-(2-amino-1-naphthyl)cysteine (2A1NC). Moreover, in the rat urine corresponding aminonaphthylmercapturic acids were found. Thus, the rats dosed with 1-NA or 1-NN excreted 1-amino-2-naphthylmercapturic acid and 4-amino-1-naphthylmercapturic acid, those dosed with 2-NA or 2-NN excreted 2-amino-1-naphthylmercapturic acid (2A1NMA).

Formation of all the above species included metabolic activation of parent arylamines or nitroarenes to the corresponding arylhydroxylamines and further to reactive arylnitrenium ions and/or the arylhydroxylamine conjugates. These are generally known to bind to nucleophilic sites in DNA leading to mutagenesis and carcinogenesis but an evidence of binding as aminoaryl cysteine adducts in proteins have not been presented until recently. Herein, the extent of globin adduct formation as described by haemoglobin binding index (HBI) [(mmol adduct/mol Hb)/(dose in mmol/kg b.w.)] for 2-NA, 2-NN, 1-NN and 1-NA was 14.0, 0.54, 0.41 and 0.21, respectively. For comparison, HBI values of known sulphinamide adducts of the same parent compounds, which were also determined in this study, were about one order of magnitude lower.

In the subsequent experiment, toxicokinetics of 2-NA was investigated. Level of the globin adduct 2A1NC reached its maximum 7 days post-exposure and then depleted continuously in a sublinear course for ca. 60 days. Urinary 2A1NMA was excreted massively on day 1 as an ultimate product of direct reaction of the reactive

intermediate(s) with glutathione but then it followed a slow depletion reflecting time course of the release of 2A1NC from globin and other proteins and its conversion to 2A1NMA.

Our results indicate that aminoacylcysteine adducts can be formed from both carcinogenic and non-carcinogenic arylamines and nitroarenes but carcinogenic 2-NA afforded notably higher adduct levels. These new globin adducts formed via toxicologically relevant precursors are more abundant than the sulphinamide adducts hitherto used as biomarkers of exposure, and appear to be applicable as alternative biomarkers of cumulative exposure to arylamines, nitroarenes and possibly also to heteroaromatic amines.

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P02-10

***In vitro* and *in vivo* redox-related effects of nanomaterials: A comprehensive approach**

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The emerging field of nanotechnology has attracted widespread interest within the last few years. The technical advantages of nanomaterials, that stem from their unique physicochemical characteristics, have given them prominence over their bulk counterparts. In this regard, a plethora of heterogeneous nanomaterials have been manufactured for the purpose of being incorporated into diverse fields, both for research and industrial use. At the same time, the anticipated intensification of exposure rates provokes safety concerns with respect to human health. On that note, oxidative stress, induced by the excessive generation of reactive oxygen species, might be the principal mechanism of nanotoxicity. Nevertheless, several nanomaterials exert antioxidant activities, either inherent or derived from their functionalization with conventional antioxidants. Considering that the biological effects of nanomaterials are interrelated with their potent redox activities, we propose an integrated approach for the assessment of their redox-related properties at three biological levels (*in vitro*-cell free systems, cell cultures, *in vivo*). To this end, we recommend a battery of translational redox biomarkers, on the one hand to determine the antioxidant activities of nanomaterials *in vitro*, and on the other hand to assess the nanomaterials-dependent alterations in redox biomarker levels both *in vitro* and *in vivo*. In the first step, *in vitro*-cell free systems are utilized to evaluate the anti-radical and reducing capacity of nanomaterials, as well as the ability to prevent from free radical-induced DNA damage. Whereupon, cell lines, that are representative of the potential route of exposure, are treated with real life exposure doses of the tested nanomaterials in order to investigate their impact on cell viability and intracellular redox state. Finally, the effects of nanomaterials on the redox state of blood and the respective tissues of experimental animals are evaluated using biomarkers of antioxidant capacity and oxidative damage. The purpose of the present approach is to unveil the complex biological actions of nanomaterials in the interrelated fields of Redox Biology and Toxicology.

P02-11

Analgesic effect of supraphysiological doses of melatonin in mice is not relayed by aryl hydrocarbon receptors (AhR)

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Aim/objective: When applied in pharmacological doses, the indole derivative melatonin is neuroactive and neuroprotective. Indoles and their metabolites, such as kynurenine, are ligands of aryl hydrocarbon receptors (AhR). This study aimed to evaluate the analgesic activity of melatonin. The possible involvement of AhR and kynurenine in their neuropharmacological effects were also tested.

Methods: After intraperitoneal injection of melatonin (30, 100, or 300 mg/kg) for seven days with or without alpha naphthoflavone (ANF) pretreatment, male mice ICR (25–30 g) were evaluated. Two tests for analgesia, i.e., the hot plate test and the formalin test, were applied. AhR and kynurenine concentrations were measured in brain homogenates.

Results: Dose-dependent analgesic activity of the melatonin in parallel with the increase of AhR and kynurenine levels was observed. However, the antagonist ANF neither possesses analgesic activity nor blocks the melatonin's analgesic effect, despite increasing both AhR and kynurenine dose-dependently.

Conclusion: Our results do not support the involvement of AhR in the demonstrated neurobiological activity. Further studies are needed to elucidate their exact molecular mechanisms.

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P02-12

Human biomonitoring to estimate exposure to deoxynivalenol and zearalenone: two human studies to support the establishment of urinary biomonitoring equivalents

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Human exposure to mycotoxins is generally estimated based on food analysis data combined with food consumption data. Human biomonitoring (HBM) is an alternative way to assess exposure, in which biomarkers of exposure are quantified in biological materials, such as urine or blood, and used to infer exposure. For the exposure assessment of mycotoxins this can be advantageous, as mycotoxins can exist in several modified forms which are often not covered in food monitoring, and as also unknown or unexpected exposure from food, but also other potential routes of exposure are covered. However, it is necessary to link exposure inferred from HBM with external exposure through food. To gain insight in such relationships, two studies with human subjects* were conducted.

24-hour duplicate diet samples and matching 24-hour urine samples were collected from 35 subjects. Duplicate diets were analysed for deoxynivalenol (DON) and related compounds (3- and 15-acetyl-DON, DON-3-glucoside), and for zearalenone (ZEN) and related compounds (α/β -zearalenol(ZEL), α/β -zearalanol, zearalanone), using LC-MS/MS based methods. Urine was analysed using dedicated methods involving enzymatic deconjugation and immunoaffinity cleanup to reach appropriate LOQs (< 0.25 ng/ml for DON biomarkers, <0.02 ng/ml for ZEN biomarkers).

DONs and biomarkers were found in all duplicate diet samples (2–16 ng/g) and urine samples (3–32 ng total-DON/ml). ZENs could be detected in most of the samples, but at lower concentrations (0.2–10 ng/g, and 0.02–0.5 ng/ml, respectively). The median ratio urinary excretion of the biomarker vs. dietary intake was 84% for DON. For ZEN, the median was 67%. Especially for ZEN much higher variance was observed at individual level, which supports the hypothesis that excretion may take longer than 24 hours.

To further study the excretion kinetics of urinary biomarkers of ZEN, 19 subjects ingested ZEN at 0.19 µg/kg b.w. (i.e. 75% of the current TDI), and urine was collected as individual voids for the following 72 hours. Subjects refrained from consuming food items with a high likelihood of being contaminated with ZEN two days before dosing and until the end of the study period.

Urinary excretion of ZEN had a very rapid onset after dosing, and the potent estrogenic metabolite α -ZEL was formed very efficiently, albeit underlying interindividual differences. The largest fraction of biomarkers were excreted in the first 24h, but excretion continued until 48–72h. Interindividual differences in the ratios of ZEN biomarker excretion vs. ZEN intake were observed, possibly implying lower internal exposure in some subjects.

The presented data highlight the value HBM can have as alternative to food monitoring, and provide highly relevant information to support the establishment of urinary biomonitoring equivalents for DON and ZEN.

* Ethical approval was obtained through the METC of Wageningen University, the Netherlands.

P02-13

HPLC-ESI-HRMS² determination of *N*-(2-hydroxyethyl)-L-valyl-L-leucine (HEVL), a new biomarker of ethylene oxide exposure in human urine

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Ethylene oxide (EO), a carcinogenic chemical used as an industrial intermediate and sterilant, forms covalent adducts with DNA and proteins. Of them, the adduct with *N*-terminal valine in blood protein globin [*N*-(2-hydroxyethyl)-L-valine, HEV] has been employed as a principal biomarker of cumulative exposures to EO. However, as sampling of blood is inconvenient in routine occupational health practice, a search was undertaken to find surrogate markers enabling non-invasive sampling. As a result, *N*-(2-hydroxyethyl)-L-valyl-L-leucine (HEVL) was recently identified as an ultimate cleavage product of EO-adducted α -chain of globin in the rat and human urine, with typical values in the unexposed subjects and EO-exposed workers being 1.0 ± 0.4 and 5.0 ± 3.1 µg/g creatinine, respectively (Mráz *et al.*, Toxicol. Lett. 326, 18–22, 2020). HEVL is currently being investigated as a promising human biomarker, therefore, the analytical method for its determination has to be appropriately validated. The procedure consists of spiking urine samples (2 ml) with deuterium-labelled internal standard HEVL-*d*₄ (IS) followed by clean-up on solid-phase extraction columns Strata X-C. These are washed stepwise with water, 80% methanol, and finally with 5% NH₄OH in methanol. After replacement of the solvent by a mobile phase, the samples are analysed by HPLC-ESI-high resolution MS².

In this study, the method was validated according to approved bioanalytical method validation guidelines, utilizing human urine samples with HEVL concentration range covering both the unexposed and occupationally exposed subjects. Method sensitivity

(LOQ < 0.025 ng/mL) and selectivity were found to be excellent, allowing reliable determination of even the background levels of HEVL in unexposed subjects. Accuracy determined as the analyte recovery using a spiking procedure was within 98–100%. Within-day and between-day precision of the method was 1.8–3.0% and 2.2–9.7%, respectively. The stability study on urine samples (–20 °C for up to 3 years, freeze-and-thaw up to 10 cycles) and aqueous solutions (5 °C for up to 4 months) indicated no relevant changes in HEVL concentration ($\leq 4\%$) over the time tested. Analytical responses of both HEVL and IS exhibited a matrix effect correlating with urinary creatinine used as an index of matrix composition but this was mostly eliminated using the HEVL/IS peak area ratio, attaining the IS-normalized relative matrix effect < 3%.

In conclusion, the method met successfully all requested criteria and can be recommended for reliable determination of HEVL in human urine. It is especially proposed for screening purposes to identify subjects with relevant exposures to EO approaching to or exceeding the occupational exposure limit for EO (1.8 mg/m³).

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P02-14

Key elements of neurotransmitter pathways as biomarkers of effect of neuroactive compounds with different primary mode of action

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Neuroactive compounds (NCs), including pharmaceuticals, stimulants, illicit drugs and neuroactive pesticides are emerging contaminants in the aquatic environment posing a great risk for wildlife and human. The primary mode of action (MoA) of NCs in target species is well-known, but the mechanism of toxic action which results in adverse outcomes in non-target organisms is poorly understood. Hence, limited progress has been made in establishment of specific biomarker response patterns for NCs that could be used for development of bioanalytical tools in environmental monitoring and detection of early responses in exposed organisms.

In this study, we investigated the responsiveness of some of the key elements of neurotransmitter (acetylcholine, serotonin, dopamine and GABA) pathways to four NCs with different primary MoA: pesticide diazinon, pharmaceuticals clozapine and sertraline, and stimulant caffeine. Gene expression-based biomarker analyses was conducted on human neuroblastoma SH-SY5Y cells treated by test compounds in concentrations 10 ng/L, 10 µg/L, and 1 mg/L or 10 mg/L, using quantitative real-time PCR analysis (RQ-PCR).

Gene expression of the responsive biomarkers was stimulated by diazinon, and mostly inhibited by tested pharmaceuticals and caffeine. The expression of synaptotagmin 10 (*SYT10*), gene encoding protein involved in exocytosis of neurotransmitters, was affected by all tested compounds, and the inhibiting effect was especially pronounced in sertraline treatment. The expression of serotonin receptor 3A (*HTR3A*) was downregulated by both tested pharmaceuticals and caffeine, while the expression of GABA A receptor subunit beta 3 (*GABRB3*) was stimulated by diazinon. Enzymes responsible for the removal of neurotransmitters from the synaptic cleft also distinguished as potential biomarkers, since changes in gene expression were recorded for acetylcholinesterase (*ACHE*, by sertraline), monoamine oxidase A and/or B (*MAOA/MAOB*, by diazinon and sertraline) and GABA transaminase (*ABAT*, by sertraline and caffeine). Most of these biomarkers were sensitive to environmentally relevant concentrations (10 ng/L and 10 µg/L) of tested compounds.

The results showed that *SYT10*, elements of serotonin and dopamine pathway *HTR3A*, *MAOA*, *MAOB*, and *ABAT* from the GABA pathway are the most sensitive biomarkers of neurotoxic effects regardless of the primary MoA of the tested compound, and also promising candidates for development of bioanalytical tools related to estimation of neurotoxicity. Integration of detected responses into adverse outcome pathways (AOPs) available at the AOP-Wiki can imply to possible adverse outcomes of exposure to tested substances.

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P02-15

Long-term respiratory impact of electronic cigarette

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Context/objectives: Tobacco smoking is responsible for 8 million deaths per year worldwide. Currently, smoking cessation is the only solution to curb this mortality but is made difficult by nicotine addiction. In recent years, electronic nicotine delivery devices have come onto the market, namely, electronic cigarette (e-cig), and could be a new alternative to help nicotine withdrawal. Although e-cig are generally perceived as less harmful than cigarettes, their precise impact on lung remains to be determined. Therefore, the aim of this work was to evaluate the long-term respiratory effects of e-cig emissions on a mouse model and to compare them to those of tobacco smoke.

Methods: Tobacco smoke was produced from a standard cigarette (3R4F) and e-cig aerosols were generated by a third-generation model of e-cig (Modbox) with a resistance of 0.5 Ohm and set at low (Mb18W) or high power (Mb30W). BALB/c mice were directly exposed to either cigarette smoke or e-cig aerosol with a nose-only inhalation exposure system using a smoking machine and following a standardized puffing regimen (Health Canada Intense: puff volume of 55 mL, duration of 2s, two puffs per minute). After 4 days, 3 months or 6 months, lung tissues were harvested to perform genotoxic, epigenetic and transcriptomic analyses.

Results/Discussion: Results pointed out that both 3R4F and Mb30W emissions were able to cause oxidative DNA lesions to lung tissues without leading to chromosomal aberrations or gene mutations, regardless of the time of exposure. Epigenetics analyses showed that Mb18W and Mb30W aerosols were responsible for an hypermethylation of the pulmonary DNA, in the same of way as tobacco smoke, and could also modify the expression of several miRNAs at all exposure time. Furthermore, transcriptomic data obtained for 6-months exposures showed that e-cig aerosols could induce the deregulation of signaling pathways involved in oxidative stress, inflammation and metabolism of propylene glycol. However, the low number of genes involved in each signaling pathway calls into question their biological relevance. For comparison, under the same exposure conditions, cigarette smoke had an impact on additional signaling pathways, related in particular to PAH metabolism or immune cell infiltration, and involving a large number of deregulated genes.

Conclusion: Overall, these preliminary results do not reveal a proven long-term lung toxicity of e-cig emissions on exposed mice. However, a more in-depth study of our transcriptomic data is ongoing and complementary results will be necessary to further assess the potential health effects of e-cig emissions in comparison to tobacco smoke.

P02-16

Health effects of red meat products containing phytochemicals and reduced levels of nitrite: the PHYTOME project

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Purpose of the study: It has been proposed that endogenously formed N-nitroso compounds (NOCs) are partly responsible for the link between red meat consumption and colorectal cancer (CRC) risk. As nitrite has been indicated as one of the critical factors in the formation of endogenous NOCs, it is of high importance to reduce the nitrite levels in meat. Therefore, the PHYTOME project was initiated (Phytochemicals to reduce nitrite in meat products; www.phytome.eu (EU-FP7 grant agreement no. 315683), a major EU funded research project aiming to develop innovative meat products in which the food additive nitrite has been replaced by natural compounds originating from fruits and vegetables.

Methods: A human dietary intervention study was conducted in which healthy subjects consumed 300 grams of meat for two weeks, in subsequent order: normal processed red meat, white meat, and red processed meat with normal or reduced levels of nitrite and added phytochemicals (standard-nitrite or reduced-nitrite PHYTOME meat, respectively). Before and after each intervention period, colorectal biopsies, urine and faeces were collected for analyses of different markers of effect.

Results and conclusions: Consumption of standard-nitrite PHYTOME meat products leads to a significant reduction in Apparent Total N-nitroso Compounds (ATNC) levels in faecal water, a surrogate marker of endogenously formed NOCs, as compared to the consumption of conventional processed red meat products. A reduction of nitrite in the PHYTOME meat lowered these levels even further. In addition, DNA strand breaks induced in *ex-vivo* faecal water exposed Caco-2 cells and O⁶-methyl-guanine adducts levels in colonic DNA were significantly higher after consumption of normal processed red meat as compared to white meat intake. PHYTOME meat intake resulted in reduced levels of these genotoxic markers; however, these were not statistically significant. Whole genome gene expression analyses identified differentially expressed genes and genes associated with ATNC, which are related to molecular pathways which may explain cancer risk initiation after intake of processed red meat and cancer risk prevention after intake of the PHYTOME meat. Together these results indicate that addition of natural extracts to conventional processed red meat products results in reduced endogenous formation of NOCs, and may therefore contribute to a reduced risk of CRC, which is mechanistically supported by gene expression analyses.

P02-17

Varenicline increases histone H3 acetylation, while affecting global DNA methylation on differentiated SH-SY5Y neuroblastoma cells

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Several pharmaceuticals originate mood and cognitive adverse events (MCAEs) that are only detected at later stages of clinical trials (e.g. Phase III) or during post-marketing surveillance, posing major safety concerns. Varenicline (an agonist of $\alpha 2\beta 4$ nicotinic acetylcholine receptor), stands among the pharmaceuticals for which MCAEs have been frequently reported^[1]. However, the molecular mechanisms triggering such MCAEs remain unclear, hindering their early detection. Here, we aimed at assessing the ability of this pharmaceutical to promote epigenetic changes, as a potential trigger of MCAEs, in a neuronal cell model, while evaluating its neurotoxicity.

SH-SY5Y neuroblastoma cells, differentiated into a dopaminergic phenotype with 10nM retinoic acid, for 72h, and 10nM retinoic acid and 80nM 12-O-tetradecanoylphorbol-13-acetate for another 72h, were exposed to varenicline at 0.1, 1, and 10 μ M for 24, 48, and 72h. Metabolic activity was assessed by the MTT reduction assay. Fluorescent staining with Hoechst 33342/propidium iodide was used for apoptotic/necrotic cell death quantification. Neurite outgrowth was assessed by measuring differentiation ratios (number of neurites per total cell number) by phase contrast imaging. Intracellular calcium levels were quantified using Fluo-4 AM fluorescent labelling. Global DNA methylation (% 5-methylcytosine) and histone H3 acetylation were determined using commercially available kits (Abcam) based on colorimetry and fluorometry, respectively.

Varenicline did not significantly alter the metabolic activity, differentiation ratios, intracellular calcium levels or apoptotic/necrotic cell death up to 10 μ M and 72h exposure, suggesting no apparent neurotoxicity at the tested concentrations and timepoints. However, 1 μ M varenicline decreased global DNA methylation levels at 24h, and increased them at 48h, compared to the vehicle (saline solution). Exposure to varenicline at 1 μ M for 24h also resulted in higher histone H3 acetylation levels, compared to the respective vehicle control.

Altogether, our data suggest that varenicline has the potential to induce epigenetic modifications, namely DNA methylation and histone acetylation, at non-cytotoxic, clinically relevant concentrations. Still, further research is required to understand how these epigenetic changes may trigger MCAEs.

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P02-18

Lanthanum, cerium, praseodymium and neodymium in hair of a healthy group of Spanish adolescents (13–16 years-old)

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Rare earth elements (REEs) have arisen as a public health concern due to their numerous applications in the modern technological industry. Human biomonitoring is a robust tool for assessing and managing chemical threats, which can provide invaluable information on current exposure of a specific population to chemical contaminants to support policy making and environmental intervention. We studied

the exposure to lanthanum (La), cerium (Ce), praseodymium (Pr) and neodymium (Nd) in a teenage population group living in Alcalá de Henares, one of the biggest populated cities in the Community of Madrid (Spain). Scalp hair samples were appropriately collected from 96 adolescents (13–16 years-old; 28 boys and 68 girls), who were recruited following strict selection criteria. These four REEs were analysed by ICP-MS after appropriate removal of exogenous contamination using Triton X-100 with bath sonication. The limits of detection were (in ng/g): La (0.011), Ce (0.020), Pr (0.003) and Nd (0.010 ng/g). Presence of these elements were detected only in a few samples monitored, specifically: La (8.2%), Ce (6.2%), Pr (5.2%) and Nd (3.1%). Owing to the high levels of censored data obtained (non-detects), sample statistics were determined by means of the methods available in the statistical package 'NADA' using the free software R-project. 95th percentiles and ranges found in scalp hair for these four REEs are provided in ng/g, as follows: La 0.012 (<0.011–0.048); Ce 0.021 (<0.020–0.098); Pr 0.003 (<0.003–0.011); except for Nd, for which the high sample percentile was not possible to be determined because of the very low number of samples in which Nd was detected (3.1%; concentration range was 0.015–0.030). None of these elements showed influence due to sex, except for Ce, which showed a slight but significant higher presence in male participants (0.020–0.098 vs. 0.026–0.080; $p < 0.05$). Although unclear, the higher presence of Ce in male teenagers living in Alcalá de Henares might be attributed to behavioural differences between sexes of this age group. Thus, young male individuals could be significantly exposed to Ce through inhalation from intense traffic, as they generally spend more time outdoors than young females in general, e.g. practising sports. Moreover, although data is scarce, different studies have suggested that men may be more sensitive to REE than women. Thus, higher levels of these emerging contaminants have been found in plasma in a recent study carried out in a general Spanish population living in the region of Andalusia. To our knowledge, this is the first study reporting levels of La, Ce, Pr and Nd in hair of a teenage population group living in the Community of Madrid. In general, our results may indicate that the studied group would have had a low exposure to these four REEs, as their presence in the hair monitored were lower than those reported in environmentally exposed groups.

P02-19

Dietary exposure assessment of sweeteners within their re-evaluation by the European Food Safety Authority (EFSA)

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Purpose: To present an overview on EFSA's work on the exposure assessment of sweeteners within the process of their re-evaluation.

To re-evaluate the food safety of sweeteners, two protocols have been developed: (i) on the assessment of the hazard identification

and characterisation of sweeteners, and (ii) on the exposure assessment.

According to Regulation (EC) No 1333/2008, all food additives, including sweeteners, permitted before 20 January 2009 should be subject to a new risk assessment by EFSA and a programme for the re-evaluation of approved food additives has been set up by Commission Regulation (EU) No 257/2010. The sweeteners to be re-evaluated include 15 substances: sorbitols (E 420); mannitols (E 421); acesulfame K (E 950); cyclamates (E 952); isomalt (E 953); saccharins (E 954); sucralose (E 955); thaumatin (E 957); neohesperidine DC (E 959); neotame (E 961); salt of aspartame-acesulfame (E 962); maltitols (E 965); lactitol (E 966); xylitol (E 967) and erythritol (E 968).

Methods: As for the other food additives under re-evaluation, sweeteners have authorised uses and maximum permitted levels in food defined in the European legislation (Regulation N°1333/2008). To estimate the most realistic exposure to these food additives, data on current use levels and/or analytical results are needed. Therefore, EFSA launched a call for data to collect concentration data for the 15 sweeteners in subject, i.e. use and use levels from the interested business operators (i.e. food industry) as well as analytical data mainly provided by EU national authorities.

The EFSA Comprehensive European Food Consumption Database is used as providing the essential consumption data for estimating dietary exposure. This database covers 22 countries out of the 27 members of the EU. Thanks to the FoodEx2 nomenclature defining the foods recorded in the database, it was possible to select foods for which a facet indicates the presence of a sweetener.

These two sources of data are combined at the individual level to estimate dietary exposure. This was performed according to different scenarios using either maximum levels as defined in the European legislation or levels submitted to EFSA. These are calculated on consumers only of at least on food category containing the sweetener; an additional scenario will consider consumers only for each food category separately.

Results: This protocol defines a new exposure assessment methodology as targeting the foods and the population of interest. Outcomes of the 1st dietary exposures estimated for some sweeteners will be presented.

Conclusions: The methodology has been developed for the sweeteners, as being specific additives. This would allow more realistic exposure assessment to sweeteners.

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P02-20

Biomarkers of effect of three pesticides on a human breast cell line

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Agrochemicals, including acaricides, insecticides, herbicides and fungicides, are used on plant production against insects, weeds, fungi and rodents; however, the pesticide exposure can represent a risk for human health, mainly linked to neurological, respiratory and repro-

ductive diseases. In children, the pesticides exposure can be associated with premature sexual maturation, including the premature thelarche, defined as breast development in girls below the age of eight years without other signs of sexual development. Indeed, several pesticides are considered as endocrine disrupting chemicals since they can interfere with the dysregulation of sexual, thyroid and neuro-endocrine hormones, acting via nuclear receptors. The present study aims to evaluate a panel of nuclear receptors as biomarkers of effect of three widely used pesticides such as glyphosate, imidacloprid and chlorpyrifos on a human breast cell line (MCF-7), representing the target organ of the idiopathic premature thelarche. Cytotoxic effects of the three pesticides were evaluated by the MTS and CyQuant assays at 48 and 72 hours(h), using five 10-fold dilutions spanning real exposure concentrations (100 pM–1µM). Proliferation was not affected whereas a dose-dependent decrease of metabolic activity was observed at 72h in all three pesticides, being statistically significant in imidacloprid treated cells. Three concentrations, including the real exposure level occurring in children, were selected for each pesticide to evaluate the gene expression of the estrogen receptor α (ER α), the estrogen receptor β (ER β), the androgen receptor (AR), the aryl hydrocarbon receptor (AhR) and the progesterone receptor (Pgr), by Real Time PCR (qPCR). Preliminary results will be shown.

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P02-21

This abstract has been withdrawn.

P03 – Computational toxicology

P03-01

Can we de-risk sub-classes of nitrosamines as pharmaceutical impurities?

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In light of the recent discovery of nitrosamine impurities in several marketed pharmaceuticals, there has been increased interest in their safety assessment. Nitrosamines as a class are considered highly potent, whereby even an intake below the conservative threshold of toxicological concern (TTC) has the potential to pose a significant genotoxic and carcinogenic risk. As such, they are included within the 'cohort of concern' within ICH M7.^[1]

Whilst there are some exceptionally potent (Ames positive) carcinogens within this class, one example being nitrosodiethylamine (NDEA), this does not appear to be a universal property of all nitrosamines.^[2] Of an Ames data set of 282 nitrosamines, 22% were judged to be non-mutagenic, which may suggest that the cohort of concern is overly sensitive.

In vitro mutagenicity and carcinogenicity data were analysed to identify trends within the structure-activity relationship, and potential mechanistic rational investigated. It was found that sub-classes of nitrosamines may be de-risked, due to a lack of activity or significantly reduced potency compared to NDEA. Initial proposed restrictions to extremely conservative structural alerts within Derek Nexus allowing the exclusion of known experimentally negative sub-classes improved the positive predictive value (from 78% to 82%) and negative predictive value (from 0% to 92%) against the aforementioned Ames data set. For the carcinogenicity endpoint against a data set of 186 compounds, the negative predictive value was improved

(from 0% to 100%) with a minor concomitant decrease in the positive predictive value (from 75% to 73%). In both cases 100% sensitivity is maintained, which is particularly important for the identification of such cohort of concern compounds.

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P03-02

Intravenous lipid emulsion as a treatment in buprenorphine overdose – pharmacokinetic and pharmacodynamic evaluation in the rabbit model

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In several countries, the opioid overdose is among the most common causes of drug-related deaths (Rudd *et al.*, 2016; Helmerhorst *et al.*, 2017). Apart from analgesics and neurosuppressive effects, these drugs may also lead to life-threatening respiratory depression that requires the use of specific antidotes (Wanger *et al.*, 1998). Therefore, alternative and easily accessible rescue protocols may be of great value in live threatening conditions. One of such protocols proposes the use of intravenous lipid emulsions (ILE). Although these agents have been confirmed to be effective in poisonings with some lipophilic substances, their efficacy in opioid overdose is speculated based on a few case reports (Orr & Bailie, 2010; Jamaty *et al.*, 2010).

This study aimed to evaluate the effects of ILE (Intralipid 20%) in the acute buprenorphine overdose in a rabbit model. To describe possible effects and mechanisms of action, both pharmacokinetic (PK) and pharmacodynamic (PD) data were collected.

In a cross-over experiment 10 New Zealand rabbits were anaesthetised with isoflurane. Five min after intravenous buprenorphine bolus (0.3 mg/kg), normal saline or ILE was administered according to resuscitation protocol (initially 1.5 ml/kg bolus, afterwards 0.25 ml/kg/min infusion for 29 min). Blood samples and cardiorespiratory parameters were collected up to 240 min after the drug administration.

Noncompartmental analysis revealed that ILE had a profound effect on the PK profile of buprenorphine. The calculated median (range) area under curve from time 0 to 240 min were 4399.5 (3804.4–6553) and 7529.5 (5834.6–9277.8) min×ng/ml in saline and ILE group, respectively ($p = .01$), indicating higher drug retention in blood when ILE was co-administered. Steady-state volume of distribution was much higher [23047 (15025–51424) ml/kg] in non-ILE group, comparing to ILE group [7475 (6456–12226) ml/kg] ($p = .003$), and clearance was 183.4 (149–252.3) ml/min/kg and 141 (105.8–188.9) ml/min/kg in control and ILE group, respectively ($p = .01$). The elimination phase looked very different in the two groups: elimination half-life was 194.7 (130.7–283.7) min in control group and 58 (46.6–100.2) min in ILE group ($p = .0003$).

Among the PD parameters, the end tidal CO₂ was significantly lower in the group receiving ILE (the average value during the last

136 min interval was 33.76 mmHg vs. 47.64 mmHg, $p = .03$). This difference was significant starting from 102 min until the end of the experiment. Other parameters (heart rate, blood pressure, tidal volumes) seemed not to be affected.

The collected data suggest that ILE limits tissue distribution of buprenorphine by retaining the drug in the central compartment. Dynamic redistribution between plasma and tissues was indicated by the presence of “shoulders” on individual PK profiles. The observed differences may suggest a beneficial effect of ILE infusion in acute buprenorphine overdose. To further confirm that hypothesis the PK/PD model will be developed.

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P03-03

Cell morphology descriptors and gene ontology profiles improve prediction for mitochondrial toxicity

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The mitochondrial toxicity of drugs is a major cause of late-stage drug withdrawals and can be linked to cardiotoxicity, hepatotoxicity and toxicity of the central nervous system. On the other hand, the vital role of mitochondria in cancer progression also renders them a novel target for enhancing chemotherapy. Previously, mitochondrial toxicity has been predicted using compound structural features. Recently, high-dimensional cell morphology data such as from the Cell Painting (CP) assay has been shown to contain information about cell health phenotypes. We hypothesized that such descriptors may contain signals for mitochondrial toxicity. We analyzed 382 distinct compounds from the Tox21 assay (AID 720637) for small molecule disruptors of the mitochondrial membrane potential and annotated them with cell morphology annotations from the Cell Painting assay and Gene Ontology profiles. We found toxic compounds are more similar intra-class and different from other non-toxic compounds in morphology space (median Pearson correlation 0.37 vs 0.06) while being indistinguishable in chemical space (median Tanimoto Similarity of Morgan fingerprints 0.11 vs 0.10). Further, when considering toxic compounds, CP descriptors corresponding to granularity and correlation between DNA and mitochondria object correlated to Gene Ontology (GO) profile: side of the membrane; hence could contain information about DNA fragmentation and heterogeneity in mitochondrial content. In terms of predictive modelling, we trained Random Forest models on CP descriptors, GO profiles, Morgan Fingerprints, and combinations thereof using 50 repeated 4-fold nested

cross-validations. We performed feature selection separately on CP descriptors and GO profiles using a combination of statistical tests. Models were evaluated on an external test set of 236 compounds. When classifying imbalanced binary datasets for toxicity, it is well recognized that sensitivity and AUC-PR (which combines both precision and recall) are more insightful and less likely to exaggerate model performance, unlike balanced accuracy and AUC-ROC. We found that late-stage fusion (by averaging prediction probabilities) of CP descriptors, GO profiles and Morgan Fingerprints relatively improved sensitivity, AUC-PR and balanced accuracy compared to models using Morgan fingerprints. Further, our models could extrapolate to new chemical space while Morgan fingerprints failed when test compounds were structurally dissimilar to training datasets. Overall, this suggests that combining CP and GO with structural fingerprints leads to improved sensitivity to mitochondrial toxic compounds, while also making mechanistic analysis easier by investigating the respective GO profiles and CP descriptors.

P03-04

How the EMMC MODA can be used for physic-based and data-based models for risk assessment?

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Computational modelling methods provide useful tools that support the development of novel materials (including nanomaterials) as well as assist in their risk assessment. Wide opportunities offered by these techniques attracted the interest of scientific communities and industry. Benefits from the application of modelling techniques in the risk assessment of nanomaterials were recently highlighted in the discussions at the level of the EU NanoSafety Cluster and in “EU-US Nanoinformatics 2030 Roadmap”. In parallel, the European Materials Modelling Council (EMMC) declared that, due to the significant reducing R&D time and costs, the modelling would have great impact on innovation and improvement of competitiveness and sustainability of industry.

To make the modelling methods more useful for industry, in 2017 the EMMC proposed, the standard scheme for collecting information across the computational models named the MODelling Data fiche (MODA). The general objective is to introduce a uniform and informative description of the models. In principle, the provided information should be as instructional as possible to make it usable for the industry. It should also allow for linking particular models together and, consequently, using outputs from one model as input data for another one.

Proposed PATROLS MODAs were developed separately for (i) physics-based models (PBM), and (ii) data-based models (DBM). The PBM refers to models and simulations based on physics/chemistry equations at different levels of materials composition. The “Review of Materials Modelling (RoMM)” published by EMMC provides a description of PBM. However, the taxonomy proposed for this PBM (i.e.: electronic, atomic) may be inadequate. For example, it is not clear what should be described as a ‘geometry’, where models are focused on the proper-

ties of materials. The ontology developed by EMMC refers mainly to predicting properties of the materials based on the materials models at various scales (e.g. electronic, atomistic), whereas models could also predict properties of the materials based on models of various phenomena (e.g. metabolism, physiological transport). These are not only physical phenomena but also biological and physiological ones. The DBM refers to the approaches that describe relationships between data from the experiment/databases and descriptors/predictor variables. Surprisingly, DBM and the related ontology are discussed in RoMM at a general level with providing only few examples of such models. In our opinion, the work done in PATROLS would open up the discussion on possible revisions of PBM and DBM definition and on the extension of the templates applicability and further discussion on models’ ontology and classification of models for new types (e.g. “biology-based models” or “physiology-based models”).

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P03-05

How to use AOP to prioritize NanoQSAR model development?

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There is an urgent need for developing faster, cheaper, and feasible animal alternatives to address the safety assessment of nanomaterials that are produced and placed on the market.^[1] Among methods that follow this need are machine learning (ML) based predictive models that by defining the relationships between the structural properties and toxicological responses allow predicting the biological effects in a timely manner.^[2] However, the majority of ML-based models developed so far focus on predicting phenotypic changes in cells/organisms observed following nanomaterial exposure.^[3] They are insufficient owing to the nanomaterials’ sophisticated properties and the property-specific toxicity mechanisms at play. The relevance of predicting an apical or a phenotypic change to an eventual adverse effect manifested at the organism level is not clear and as a result, the mechanistic basis for the selection of specific features for inclusion in the models as predictors is also not justified. Thus, instead of targeting the apical changes, the ideal path forward for the new generation of predictive models need to be to target the early or upstream biological changes of the toxicity mechanism as endpoints that are biologically plausible and essential for the occurrence of an eventual adverse outcome (AO) at the organism level. Combining the Adverse Outcome Pathway (AOP) with the predictive models may support a better understanding of the adverse effect of nanomaterials on human health and form the basis for comprehensive and realistic risk assessment.^[3,4] The goal of this study was to provide the ML-based models for predicting toxicity nanomaterials towards effects anchored in AOP.

The AOP173 was applied and a model allowing to predict of pulmonary pathology induced by carbon nanotubes was delivered. Here, both supervised as well as unsupervised modeling techniques were used to link the characteristics of nanotubes with the 162 transcriptional pathways associated with lung pathologies. The pathway associated with early upstream key events (KEs) crucial for the occurrence of lung fibrosis (AO in AOP173) was selected. The ML-based model provides new insights into predicting the AO by quantitatively expressing the changes in structural properties of nanotubes that

are detrimental to inducing perturbation in transcriptomic pathways essential for lung fibrosis was developed.

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P03-06

This abstract has been withdrawn.

P03-07

Using multiple *in silico* models to improve robustness of predictions for bacterial mutagenicity

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Purpose: The approval process for pesticides is one of the most complex in the world and many toxicological properties of the active substances (a.s), metabolites or impurities have to be investigated. Among those properties, mutagenicity is of particular importance. While legal data requirements for experimental studies with the a.s. are comprehensive, those for metabolites or impurities are limited. For this reason, and also to reduce animal experiments, *in silico* studies are becoming more popular. Relevant guidance documents recommend using a combination of at least two models, ideally an expert and a statistical tool.

The aim of the present study is to investigate whether robustness of *in silico* predictions for bacterial mutagenicity and thus their regulatory acceptability in pesticide regulation can be improved by using multiple models.

Methods: In a pilot study, 15 mutagenic and 15 non-mutagenic compounds were selected from pesticide regulation. All experimental data was obtained in studies performed in compliance with OECD TG 471. *In silico* predictions for bacterial mutagenicity were conducted with the freely available expert system Toxtree (v. 3.0.1) as well as two commercial tools implementing expert and statistical models within the Leadscape Model Applier (v. 3.0.2-4) and Lhasa Nexus platform (Derek Nexus, v. 6.1.0 and Sarah Nexus v. 3.1.0). For pragmatic reasons, expert review was omitted but specific acceptance criteria for judging predictions *mutagenic* or *non-mutagenic* were defined.

Results: A number of 8/15 positive compounds were correctly predicted by Toxtree, leaving 7 false-negative. With the Leadscape Model Applier or Lhasa software, the number of correctly predicted compounds was to 9/15 in both cases. Using our acceptance criteria, the number of false-negatives was 0 and 4 for Lhasa Nexus and Leadscape.

For the non-mutagenic compounds, 12/15 were correctly predicted by Toxtree and 3 were false-positives. Leadscape Model Applier correctly predicted 11 compounds also with 3 false-positives. Using Lhasa software, 13/15 non-mutagenic compounds were correctly identified while 1 false-positive prediction was generated.

For the remaining compounds, the prediction was either equivocal or not acceptable.

Prediction outcome depends on the choice of the model. As robustness and sensitivity are of great concern from the regulatory

perspective, we explored the model combination approach. When applying the strict criterion that at least one positive prediction by either Toxtree, Leadscape or Lhasa is sufficient to consider a compound mutagenic, 11/15 compounds are correctly predicted to be mutagenic. Further model combination approaches are explored.

The results demonstrate that the use of multiple *in silico* tools for pesticide assessment improves robustness and thus regulatory acceptance.

P03-08

Web application to predict the 'Agranulocyte adhesion pathway and diapedesis' in AOP 173 induced by MWCNTs

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Nano-QSAR models are useful tools for predicting biological activity of new, untested nanomaterials. In addition, they promote alternative, time and cost efficient methods for assessing the toxicity of nanomaterials without the use of animals. Unfortunately, their use is usually associated with the requirement of expert knowledge in the field of cheminformatics. Due to this, most of the developed models are never put to use in practice by scientists who do not specialize in this field. In order to popularize QSAR tools that are accessible for everyone, we have developed an application that uses the recently published AOP-anchored nano-QSAR model by Jagiello *et al.* [1]. The application requires only basic information on two properties of MWCNTs: their length and diameter. Based on those, one can carry out the prediction of responses at the 'Agranulocyte adhesion pathway and diapedesis' level (BMDL_{aa} values), which are essential for the early key event caused by multiwalled carbon nanotubes (MWCNTs) in the Adverse Outcome Pathway for lung fibrosis (AOP173). Additionally, the user will receive specific information on which pathway associated genes will be down or up-regulated. The application provides a set of interactive charts, tables and menus that support users predictions with additional detailed information on how to interpret and use their results. With further development of models allowing for prediction of specific transcriptomic pathways and early key event identification, the application will be continuously developed to provide more functionalities.

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P03-09

Using mechanisms of toxic action (MechoA) to strengthen skin sensitisation predictions for chemicals and their metabolites

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iSafeRat[®] MechoA Scheme model [1] (MechoA) is an *in silico* model based on structural alerts, which can detect 26 classes of toxic mechanisms of action. Among these, MechoA classe 3 and sub-classe 4.3 detect electrophilic parent substance and metabolites, respectively. Sub-class MechoA 4.4 detects chemicals which can undergo cyclic oxido-reduction reactions, generating reactive oxygen species (ROS).

These reactive substances are responsible for hapten-protein complex formation as the molecular initiating event (MIE) of the adverse outcome pathway (AOP) for skin sensitisation. In this study, MechoA, which is equivalent to MIE, is tested for its performance to identify SS for the substances listed in Cosmetics Europe skin sensitisation database (CESSD)^[2] and the ring trial for GARD[®]skin^[3].

In order to compare the models, two hypotheses were used:

1. When the experimental assay was not capable of metabolism (i.e., DPRA), only MechoA 3 was considered as positive output (SS).
2. When metabolism may occur in the experimental assay, chemicals triggering MechoA 3 and/or 4.3 and/or 4.4 were considered positive as SS.

Positive predictions, i.e., protein binders:

A correlation was observed between chemicals triggering MechoA 3 and/or 4.3 and/or 4.4 and positive results observed for SS in the experimental studies. The probability of this positive prediction being correct was 62% relative to human clinical data^[2] (51 positives out of 82 total) and of 57% relative to LLNA data^[2] (51 positives out of 90 total). Positive predictivity relative to *in vitro* methods was between 49–78% of which, only MechoA 3 correlated with 49% DPRA positives.

Negative predictions, i.e., non-protein binders:

A strong correlation was observed between negative results identified in SS studies and chemicals triggering other MechoA classes than 3, 4.3 and 4.4. The probability of these negative predictions being correct was 83% relative to human clinical data^[2] (33 negatives out of 40 total) and 78% relative to LLNA data^[2] (25 negatives out of 32 total). Negative predictivity relative to *in vitro* methods was between 57–95%. Chemicals triggering MechoA classes other than MechoA 3, correlated with 74% of negative results in DPRA.

Therefore, MechoA can be used to reliably predict non skin sensitizer (78–83%), and to predict with moderate reliability (57–62%) potential skin sensitizers based on the protein-binding mechanism.

While MIE/MechoA is a necessary first step to evaluate the potential for skin sensitisation, it is not sufficient in itself. The dermal absorption of a substance should be estimated upstream of the AOP as a limiting factor for SS whatever the reactive potential of a substance.

Use of MechoA can help in the decision making of the *in vitro* testing strategy. We are currently working on combining MechoA together with our dermal absorption QSAR model to further improve performance for skin sensitisation prediction.

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P03-10

A Tool for Tanimoto Similarity Clustering of Chemical Compounds

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Grouping is an important step in the read-across evaluation of metabolites and impurities. We developed a simple computational grouping tool based on Tanimoto similarity scores. A binary substructure fingerprint is calculated for each fragment of a metabolite from the structure as defined by the SMILES code. The fingerprint integrates a number of physicochemical properties, for example, atom neighbourhood pattern, atom pairing, lipo/hydrophilicity, acidity,

molecular weight, size, etc. Different fingerprints can be used for the comparison and the exact information integrated depends on the type of fingerprint used, e.g. Pubchem, MACCS, etc. Metabolites are then compared pairwise to give a Tanimoto score. The scores are compared in turn in a Tanimoto distance matrix. A low Tanimoto score, e.g. 0.01 indicates low similarity of 1% compared to a high score, e.g. 0.9, which is indicative of 90% similarity. A dendrogram is generated to visualise the relative relatedness of each of the metabolites to each other. A threshold of similarity can also be selected for grouping. The software is written in R using the Shiny Framework, which is open source software and is freely available upon request (also at <http://vm-zooglow/apps/ShinyGrouping/>). It must be noted that this software is in pre-alpha status and the result does not replace expert review and is only meant as a quantitative basis for grouping.

P03-11

Characterising potential gene-environment interactions in nervous system diseases for pesticides with different modes of action

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Interactions between environmental factors and genetics underlie the majority of chronic human diseases. However, connecting chemicals, single nucleotide polymorphisms (SNPs), and diseases to assess how chemicals exert effects through gene-environment (GxE) interactions is a challenge. New approach methodologies (NAMs) are necessary to incorporate genetic variability into chemical risk assessment to protect sensitive populations. We integrated diverse datasets, including chemical activity data from ToxCast high-throughput screening, and human SNP-disease data from DisGeNET to form new chemical-disease associations through gene enrichment, and identified SNPs that may be involved in GxE interactions. This established a unique dataset of possible chemical-SNP-disease relationships. We describe the risk of these associations through a novel value, the disease quotient (DisQ). Here, we use this dataset to characterise potential GxE interactions for 3 illustrative pesticide groups with distinct insecticidal modes of action (MoA): acetylcholinesterase inhibitors (AChE group, 19 chemicals in dataset), GABA-gated chloride channel blockers (GABA group, 12 chemicals), and sodium channel modulators (Na group, 22 chemicals). Through this, we assess the likelihood for GxE interactions in nervous system disease for different pesticide groups, and characterise SNPs implicated in differential population susceptibility. In total, these 53 chemicals formed 804 unique chemical-disease associations with 1124 total SNPs implicated (median 2 SNPs per chemical-disease association). Despite little difference in diseases enriched by these groups (e.g., all had high risk values for Alzheimer's disease), only 168 of the SNPs implicated in chemical-disease intersections overlapped across all groups, with 728 SNPs unique to one group. This suggests that pesticides may interact with different genetic regions depending on their MoA. For example, despite having the fewest chemicals, the GABA group had the most SNPs (925) implicated, and many SNPs in high-risk chemical-disease intersections were exclusive to this group (e.g., rs4680 in Alzheimer's disease). This suggests that GABA group chemicals may contribute more to differential disease progression in a population than the AChE group, which had fewer SNPs (214) implicated. The Na group (532 SNPs) also showed high-risk for GxE interactions (e.g., cypermethrin is high-risk for Alzheimer's disease with 63 SNPs implicated, including the well-studied rs7412), which has high relevance as pyrethroids are applied in large quantities in agriculture across Europe.

This analysis demonstrates how pesticides may have different risk for GxE interactions based on MoA, and identifies specific genetic regions implicated in differential population susceptibility. This represents a NAM to characterise potential GxE interactions for chemical exposures and shows promise for incorporating interindividual variability into chemical risk assessment.

P03-12

Cardiotoxicity mechanism of *Veratrum lobelianum* alkaloids: *in silico* study

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Veratrum lobelianum is a plant widely distributed throughout the territory of the Russian Federation. It is authorized for medical use as an over-the-counter anti-lice drug, Veratrum Aqua. All parts of the plant contain toxic alkaloids, jervine, protoveratrine A (proA), and protoveratrine B (proB) being the predominant ones. *Veratrum lobelianum* alkaloid intoxications occur as a result of the erroneous ingestion of Veratrum Aqua or the plant itself, as well as its use in traditional medicine as an anti-alcohol treatment. Oral intake produces pronounced cardiotoxic effects, i.e. hypotension, arrhythmia, and conduction disorder, and in severe cases results in a fatal outcome. These effects are caused by the overall toxic action of *Veratrum* alkaloid sum. The mechanism of biological action might be different for various alkaloids, however, some of the clinical effects are mediated by the interaction of *Veratrum* alkaloids with the voltage-gated sodium channels. The major isoform of these channels in the heart, Na_v1.5, is of special interest due to *Veratrum* alkaloid cardiotoxicity.

The purpose of the study was to investigate the affinity of major *Veratrum lobelianum* alkaloids to Na_v1.5 sodium channel *via* molecular docking. Virtual structures of jervine, proA, and proB were obtained from the PubChem database. Geometries of ligands were optimized by the quantum mechanics method (RHF/HF-3c). Rat cardiac sodium channel structure (PDB ID: 6UZ0) was chosen as a target. Homology between a rat and a human protein is 94%. Molecular docking and native ligand re-docking (flecainide) was realized in AutoDock Vina 1.1.2. We used affinity energy of AutoDock Vina for ligands rating. Flecainide score was -7.7 kcal/mol, proB -8.0 kcal/mol, proA -8.2 kcal/mol, jervine -10.8 kcal/mol. *Veratrum* alkaloids interact with Na_v1.5 mainly *via* Van der Waals forces due to their steroidal structure.

Additionally, pK_a value for each compound was calculated in MarvinSketch 20.21. pK_a values were 9.25, 9.64, 7.28, and 7.28 for flecainide, jervine, proA, and proB, respectively. Considering this, we assume that *Veratrum* alkaloids, being weak bases just like local anesthetics (lidocaine, etc.), could similarly cross the cell membrane. Since their pK_a values are higher than extracellular pH values, non-ionized forms of alkaloids would readily penetrate the cell membrane and become ionized inside the cell. To support this, *Veratrum* alkaloids and local anesthetics also share similarities in causing cardiotoxic and neurotoxic effects.

In conclusion, we have found that out of the three studied *Veratrum* alkaloids jervine was the most active one that could account for cardiotoxicity symptoms.

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P03-13

The differences in the 2D and 3D cytotoxicity testing results – an *in silico* approach

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Nano-QSAR (Quantitative Structure-Activity Relationships) models are a great alternative to experimental methods. They are cheaper, faster and additionally they allow the prediction of the biological activity of nanomaterials that have not been experimentally tested before without the use of animals and chemicals. However, in order for the model to be characterized by goodness-of-fit, stability, robustness as well as predictive ability, the descriptors that are used to predict the activity must be appropriately selected. Additionally, it happens that developed models are characterized by a lack of interpretability.

For these reasons data from high-throughput screening bioassays of 2D and 3D cell cultures measured at two distinct time points for five endpoints was utilized in statistical analysis and QSAR modeling. The data contained a broad set of nanomaterials for which we collected descriptors from existing sources. Additionally, to expand the area of nanomaterials characteristics, we have employed computational methods to generate a set of new descriptors. Statistical analysis consisting of several statistical tests (Levene's, Shapiro-Wilk, Kruskal-Wallis) was performed to examine the effect of time and type of cell culture on the response for individual endpoints. Next, to investigate how individual descriptors may affect examined endpoints two-way hierarchical cluster analysis (2D-HCA) was utilized. Based on the results of this analysis we have selected the pair of descriptor-activity most suitable for building well-rounded predictive models (ROS production – Cytotoxicity). Finally, we have employed ordinary least squares Linear Regression to build nano-QSAR models for nanomaterials cytotoxicity. It is simple, yet with the right set of descriptors, a powerful method, whose biggest advantages are ease of interpretability and applicability. We have built four distinct models one for each combination of time and type of cell culture. Models developed for 2D endpoints showed not only a good fit to training data but also good predictive properties for data they had not seen before. As only one descriptor was ultimately used to build the models, this further facilitates the interpretation of the link between the descriptor and the cellular response. In the case of 3D models, as they were not characterized by goodness-of-fit and the predictive ability at the same time, we plan to expand the models with additional descriptors in the future.

P03-14

Predicting nuclear receptor activity of chemicals using a suite of complementary *in silico* methods for endocrine disruption hazard identification

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Endocrine-disrupting chemicals are suspected to cause adverse effects in the endocrine system by interfering with the synthesis, transport, degradation, or action of endogenous ligands. In 2018 a guidance describing how to perform hazard identification for endocrine-disrupting properties by following the scientific criteria which are outlined in EU 2017/2100 and EU 2018/605 for biocidal products and plant protection products, respectively, was published by ECHA and EFSA^[1]. In this guidance computational approaches are proposed as line of evidence for endocrine activity assessment. An *in silico* screen-

ing protocol for endocrine disruption which follows this guidance is described in this study. It employs 108 freely available and commercial models covering 27 receptors: 62 (Q)SARs, 19 rule-based profilers, 18 models of receptor interactions and 6 ToxCast pathway models. All the models are applied and the results are combined with an algorithm that considers the reliability of the model itself and the uncertainty of each prediction. If the substance is included in the training set of a model, both the predictions and the experimental data are used. The results of the QSAR are rated according to the sensitivity or the specificity of the model and the reliability of the prediction in terms of applicability domain. The results of the profilers are primarily used to confirm the results of the QSAR as these approaches are known to give high rate of false positives. The molecular modelling and ToxCast results are rated according only to the reliability of the models as the applicability domain is not an issue with these approaches. The predictions are then combined by receptor and an overall conclusion for each receptor and for each receptor sub-type is given. When models with the same level of uncertainty disagree an expert assessment of the nearest neighbours is carried out to get to a final conclusion. If the final result is still uncertain, a read-across approach is applied. Positive predictions can be used to give indication on the mechanism of action for the endocrine disruption. The workflow was implemented in KNIME to avoid possible transcription errors. Two examples are demonstrated: butylparaben, correctly predicted as active towards the estrogen receptor, and triclosan, correctly predicted to be active towards the androgen receptor and towards the constitutive androstane receptor.

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P03-15

Development of a predictive model for individual susceptibility to hearing loss using human genetic, exposure and epidemiological data

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The US Air Force Total Exposure Health (TEH) initiative is aimed at developing a comprehensive understanding of an individual's exposures over a lifetime, and how those exposures interact with genetic factors to affect health risks. To apply TEH information toward clinically actionable recommendations for disease prevention and human performance improvement, we are developing a mathematical framework – the Individualized Exposure Health Risk Profile (IEHRP) model – incorporating genetic, demographic and exposure data into a prediction of relative risk for individuals in a population. The pilot IEHRP model focuses on sensorineural hearing loss (SNHL) and uses public data for key risk factors. The global shift to nonanimal data collection methods, and the need to incorporate genetic susceptibility and long-term, aggregate exposures underpin our computational approach. The initial structure is based on a published cumulative genetic risk score algorithm^[1] and used data from the UK Biobank (n=250,389) and National Health and Nutrition Examination Survey (NHANES; n~3,500) for 41 single nucleotide polymorphisms (SNPs) (p<0.04), gender, age, race/ethnicity, highest level of education, and self-reported exposure to occupational and firearm noise. The mod-

el estimates approximately 10.5% of the US population with elevated (8.1%) or high (2.4%) risk (measured prevalence: 20–30%). Age is the primary determinant of risk (OR for 60–69 yr old: 39.5), followed by education (OR: 2.4–4.2), self-reported noise exposure OR (1.0–2.16) and gender (OR:1.8). Genetic predisposition had negligible effect on overall risk. Because the framework is limited in scalability, we are expanding the infrastructure and adding exposure to chemicals, additional noise sources, and more quantitative noise dosimetry (e.g., noise monitor data). To facilitate model access, we developed a user-friendly application for identification of at-risk individuals and prioritization of risk factors for accelerated implementation of a reasonable hazard protection plans as needed.

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P03-16

Application of conformal prediction for *in silico* definition of molecular initiating events linked to endocrine disruption

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The adverse outcome pathway (AOP) paradigm has brought mechanism of action in the spotlight of regulatory toxicology, linking biochemical interactions on cellular level (i.e. molecular initiating event, MIE) via key events to adverse outcomes (AOs) on population level. Developments on mechanistic understanding of endocrine disruption (ED) has brought forward MIEs associated with early neurodevelopmental interference^[1] and metabolic disruption^[2], describing agonistic and antagonistic interactions with receptors such as constitutive androstane receptor (CAR), estrogen receptor alpha (ER α), farnesoid X receptor (FXR), and glucocorticoid receptor (GR). High confidence on *in silico* predictions is dictated by high quality training data on mechanistically relevant endpoints, where well-defined chemistry is covered. Based on Tox21 *in vitro* assays describing events of agonism and antagonism for 13 receptors linked to ED, 23 *in silico* models were developed using Random Forest Classification. To quantify measures of uncertainty per prediction a Conformal Prediction framework was employed. In order to assess whether currently available models can confidently predict endocrine disrupting chemicals (EDCs), screening of EURION reference chemicals was conducted. The EURION cluster is a constellation of 8 research consortia aiming to improve endocrine disruption identification. Preliminary results revealed strengths in the use of *in silico* models for screening of current ED chemical landscape, and data gaps that need to be considered for next steps.

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P03-17**Development and evaluation of QSAR models to screen endocrine disrupting chemicals***A. Roncaglioni¹, M. Marzo¹, E. Mombelli², E. Benfenati¹¹ Istituto di Ricerche Farmacologiche Mario Negri, Department of Environmental Health Sciences, Milano, Italy;² Institut National de l'Environnement Industriel et des Risques (INERIS), METO, Verneuil en Halatte, France

The ability to identify the role of chemicals in endocrine perturbation is an important step in hazard and risk assessment of chemicals. In this regard, *in silico* predictive models can play a decisive role as a first-tier screening tool to cover data gaps and prioritizing chemicals for higher tier investigations.

Software tools codifying predictive models addressing endocrine activity were identified in the public domain. Most of them (e.g. VEGA and the Danish QSAR database) employ (Quantitative) Structure-Activity Relationship method while docking approaches are less commonly used in these tools.

These software tools were used to investigate a group of industrial chemicals (e.g. bisphenols, perfluorinated compounds) for their potential endocrine effects.

This assessment allowed to:

1. Compare estimations provided by different tools: results were highly influenced by the underlying experimental data at the basis of the models and by the modelling method (QSAR vs. docking);
2. Identify those effects widely addressed (such as estrogen and androgen mediated effects) in comparison to those where fewer models are currently available, including potential targets relevant for their influence on metabolic disorders;
3. Develop new QSAR models for those targets characterized by a paucity of models (such as thyroperoxidase inhibition for thyroid pathway and steroidogenesis) to increase the availability of *in silico* tools for screening endocrine disrupting chemicals.

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P03-18**Application of QSAR modeling to predict the toxicity of nanomaterials – principles of obtaining reliable and valuable results***E. Wyrzykowska¹, A. Mikolajczyk^{1,2}, M. Gromelski¹, T. Puzyn^{1,2}¹ QSAR Lab Ltd., Gdańsk, Poland;² University of Gdańsk, 2Group of Environmental Chemometrics, Faculty of Chemistry, Gdańsk, Poland

The identification of relationships between the chemical structure of the substances with their exhibited toxicological effects is a basis for the safer-by-design of new chemicals with the expected properties and safety. This idea is covered by the Quantitative Structure – Activity Relationships (QSAR) modeling approach, which enables to link the structural features of chemicals expressed in a numerical way (called descriptors), with the observed toxicity responses. Nevertheless, in the case of nanomaterials, identifying these kinds of relationships is rather challenging due to several reasons – mainly related to the appropriate reflection of nanostructure characteristics, as well as, addressing the OECD rules. To ensure reliable QSAR modeling, the OECD principles require i) a defined endpoint, ii) an unambiguous algorithm, iii) a defined applicability domain, iv) appropriate measures of goodness-of-fit, robustness and predictivity, and v) a mechanistic interpretation. However, in the context of nanomaterials specificity, additional extensions are recommended to provide reliable modeling and valuable results for nanotoxicity predictions.

The goal of the presentation is to present relevant aspects that should be taken into account when using QSARs to predict nanotoxicity. The presentation will focus on directions when addressing the OECD principles for nanomaterials modeling, and present new perspectives for developing valuable QSARs models which meet the safer-by-design principles.

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P03-19**Multi-modal approach for prediction of skin toxicity in small molecules**

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Purpose: Every year, the testing of chemicals for drug toxicity results in numerous animal sacrifices, wastage of chemicals, and billions of dollars in costs^[1]. The ability to predict the toxicity of molecules *in silico* can significantly help in this regard by increasing the speed and reducing the number of iterations in preclinical chemical testing^[2]. In recent times, deep learning^[3,4] has enabled the shift in predictive toxicology studies from *in vivo* to *in silico*. Toxicity prediction can benefit from the application of deep learning^[5] and the excellent results being obtained in natural language processing, image recognition, computational chemistry, and bioinformatics. This work presents a multi-modality approach, which integrates three learning algorithms to predict skin toxicity in small molecules.

Methods: Our goal is to build a multi-modality classification model, which leverages data from *numbers* (molecular descriptors & fingerprints), *images* (image features), and *text* (SMILES) for skin-sensitization prediction in small molecules studies. We train three learning models using different algorithms: XGBoost^[6] (*numbers*), convolution neural network (CNN)^[7] (*images*), and graph neural networks (GNN)^[8] (*text*). We adopt an ensemble strategy, where we train a fully connected neural network using the concatenation of features from individual learning models. We use the SMARTS pattern matching technique to highlight reactive sub-structure. This further helps in identifying the mechanism behind sensitization.

Results: The GNN model performed the best with an accuracy of 75.8% compared to the XGBoost and CNN model with ResNet backbone, each with an accuracy of 63%. Using an ensemble strategy outperformed the individual models, and resulted in an accuracy of 81.2%. Our solution additionally highlights the sub-structure in a molecule that is likely responsible for skin sensitization. This helps in determining the mechanism by which the molecule causes sensitization.

Conclusion: We propose an ensemble technique to predict the skin sensitization potential of small molecules. We show that integrating multiple modalities in an ensemble technique helps improve the prediction performance. Further refinement and integration of these approaches will be an essential step toward strengthening our ability to predict the toxicity of chemicals.

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P04 – *In vitro* methodologies & screening

P04-01

***In vitro* skin sensitization test methods: applicability to plant protection products**

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Substantial efforts towards non-animal methods have been made on skin sensitization for years. Mechanistic understanding of skin sensitization has resulted into the set-up of an adverse outcome pathway (AOP)^[1] and enabled the development as well as the validation of *in silico/chemico/vitro* assays, each relative to a specific key event (KE). Used in an integrated testing strategy, these non-animal assays demonstrated a superior performance to the gold standard mouse Local Lymph Node Assay when compared to both animal and human data^[2–3]. However, *in vitro* alternatives were originally validated for cosmetics and single chemicals. Therefore, we decided to verify the applicability of these tests to agrochemicals.

For this purpose, we selected a set of skin sensitizer and non-sensitizer plant protection molecules based on *in vivo* studies performed for hazard classification. We followed the defined approach described in the OECD draft guidance document recently published^[2], where the concordance of 2 out of 3 *in vitro* assays results leads to the hazard identification to skin sensitization potential of a chemical. The DPRA, LuSens and h-CLAT assays (corresponding to KE 1, 2 and 3 respectively of the AOP) were conducted. As described in the defined approach, the DPRA and h-CLAT assays in addition to a Derek Nexus *in silico* prediction were used in an integrated testing strategy for the potency subcategorization of each test item.

Several problems were encountered when testing our active substances. First, a solubilization issue was identified in the DPRA assay, which led to an inconclusive result being met for most of the test items. In addition, a negative result in the h-CLAT assay obtained for a test item with a log P > 3.5 is also considered as inconclusive. Because of these limitations, the potency categorization of several active substances could not be determined. Furthermore, Derek Nexus *in silico* prediction seems not to be sufficient for the skin sensitization potential assessment when applied to the agrochemicals tested. Addressing KE 2 of the AOP using the LuSens assay did, however, allow us to conclude on the *in vitro*-based hazard identification of the selected test items, at the exception of one test item. In order to overcome these limitations, other *in silico* tools are being evaluated such as the OECD QSAR Toolbox, which is recommended in the guidance document. Furthermore, as an inconclusive result from any *in vitro* assay may unbalance the 2-out-of-3 hazard identification method in case of non-concordant results from the two remaining *in vitro* assays, we consider that an additional *in vitro* assay could help to support inconclusive results in a weight of evidence approach. From a more challenging perspective, we are also exploring the applicability of this non-animal method to agrochemical formulations.

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P04-02

Discerning between cellular protection vs. toxicological impact of topical ingredients, during environmental exposure to UV-A, by multifactorial processes

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UV radiation, even being part of our natural environment, is involved in a variety of skin diseases: inflammation, degenerative aging and cancer. UVA penetrates the epidermis and activates melanin at dermal level, also affecting connective tissue and blood vessels. UVA generates reactive oxygen species that damage DNA via indirect photosensitizing reactions in cells, inducing skin cancers. UVA is connected to the "broad-spectrum protection" seen on sunscreen products' labels. In this context, it is crucial to distinguish between protective action and potential hidden toxicity of topical ingredients. We focused on multifactorial processes targeted on cellular mechanisms involving metabolic activity disruption, plasmatic membrane permeabilization, apoptosis, as well as irritability and inflammation mediators (IL1 α , IL6 and IL8). The screening was based on keratinocytes (HaCaT cell line), the first layer of UV aggression, relevant cells for skin environmental impact. The study design involved a complete methodological approach (MTS/LDH cytotoxicity assay, flow cytometry – CBA technique for soluble proteins) to compare the cellular behavior in two experimental series: the basal unstimulated status vs. UVA stimulated cells, both treated with vegetal extracts (*Aesculus hippocastanum*, main active principle: aescine; *Medicago sativa*, *Viola tricolor*), ingredients of topical products. The irradiation was accomplished in reproducible conditions (Bio-Sun system Vilber-Lourmet) with the experimental setup of 9J/cm². Phytoextracts from lucerne, wheat, chestnut and pansies have a specific structural and percentage profile in classes of compounds: triterpenic saponins, polyphenols, flavones, isoflavones and proanthocyanidins, and they present different, dose-related actions *in vitro*. Thus, cellular apoptosis is inhibited by wheat extract, pansies extract and by purified aescin from chestnut, while total chestnut extract does not act at this level, underlining the importance of using purified compounds. A toxic effect is noticed for lucerne extract, which amplifies apoptosis initiated by UVA. Inflammation, coordinated by IL6 and IL8 signaling, is reduced by the action of lucerne and chestnut extracts, as well as by aescin (mainly on IL6). Wheat phytochemicals do not influence these mechanisms. The irritative effect expressed by IL1 α is significantly attenuated by the bioactive complex of wheat and by aescin. The total chestnut extract and the lucerne extract lead to an exacerbation of this effect under irradiation conditions, indicating a sensitization potential for epidermis, even if they present anti-inflammatory efficacy in the IL6 and IL8 pathways. These results highlight once more the importance of an appropriate screening to discriminate the toxicity/efficacy ratio of different active principles with natural origin when specialists decide over a topical product design, assuring consumers' safety.

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P04-03

Improving Quality of *In vitro* Methods: A GIVIMP Certification Program

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Recently the Organization for Economic Cooperation and Development (OECD) issued a Guidance Document on Good *In vitro* method Practices (GIVIMP). Intended to reduce the uncertainties in cell and tissue based methods used in the prediction of human safety, GIVIMP provides the field with a set of standards to improve both the quality and accuracy of newly developed, and routinely executed, *in vitro* methods. Currently organizations are struggling to define the best approach for practical implementation of the guidance within their programs. As GIVIMP is broad in scope and covers a wide range of scientific and quality topics, there is the potential for varying interpretations of the guidance and thus significant differences in implementation. A business to business certification program is one solution to harmonize GIVIMP interpretation, standardize "claims" of compliance with the document, and provide a uniform roadmap for incorporation of GIVIMP principles within routine laboratory operations and method development activities. A certification program would be beneficial to the full audience for which GIVIMP was intended including academic laboratories developing new methods, established laboratories participating in validations and/or performing routine *in vitro* studies, and industry laboratories intending to submit *in vitro* data to regulatory agencies. A pilot certification between the Institute for *In vitro* Sciences (IIVS) and BASF SE laboratories (Ludwigshafen, DE) has been launched to provide proof-of-concept for the program. This poster discusses the need for the GIVIMP certification program and provides details on its structure and administration.

P04-04

Towards a better investigation of occupational exposures: *In vitro* macrophage systems for nanomaterial exposure studies

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Nanomaterials (NM) are largely used in various applications such as electronics, medical devices, antimicrobial agent, food additive, cosmetics etc. leading to human exposure and thus also to potential risks related to short- and long-term toxicity for both consumers and workers. From January 2020, the REACH regulation has been amended with specific additional requirements tailored for nanomaterials or nanoforms. Considering the uses in daily life, an acute, single exposure mode, is not the most relevant mode to assess the relevant biological effects of NM exposure. In the context of reducing animal experimentation according to the 3R principles (Reduce, refine and replace), the development of *in vitro* systems is key, especially for the initial phases of the toxicological assessment of substances. There-

fore, we have developed *in vitro* systems for investigating exposure to particulates, including nanomaterials, keeping in mind two key elements which are dosing rate (bolus or repeated exposure) and the persistence of effects. In particular, we investigated the effects of repeated exposure of macrophages to SAS (Synthetic Amorphous Silica) and colloidal silica (Torres *et al.*, 2020^[1]) as well as the persistence of the effect of the pyrogenic SAS exposure (Torres *et al.*, 2020^[2]).

We have developed a protocol evaluating a single high dose versus repeated fractionated dosing on macrophages and have also included a post exposure period to evaluate their recovery. The RAW 264.7 Macrophage cell line yielded some interesting initial results, as exemplified by silver nanomaterial exposure which showed a good correlation with the events observed in *in vivo* mouse models (Dalzon *et al.*, 2020^[3]; Toybou *et al.*, 2019^[4]). A repeated exposure study (4 dosings) with SAS has shown a high intracellular accumulation of the particles and different inflammatory responses (TNF secretion and phagocytic function) as compared to bolus dosing.

The recovery protocol showed a significant persistence of effects by pyrogenic SAS with an increase of the pro-inflammatory response (TNF and IL-6) and changes in protein abundances (cell adhesion, carbon metabolism or mitochondrion), even after 72h recovery period.

Our protocol allows testing the toxicity of nanomaterials and particles by assessing the metabolism and the functions of the macrophages (phagocytosis, nitric oxide production, cytokine secretion and mitochondrial activity) using flow cytometry and spectrophotometer, and the cytoskeleton changes by using confocal microscopy. Physico-chemical characteristics (among them size, shape, stability, density, and coating) of the particles as potential drivers of the effects in macrophages are also being investigated. Our protocol could be a great tool to reduce the use/need for *in vivo* rodent models.

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P04-05

In vitro investigation of self-assembled amphiphilic poly-N-vinylpyrrolidone nanoparticles hematotoxicity

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Nano-scaled drug carriers can be nowadays defined as a new type of drug delivery systems having unique chemical, physical and pharmacological properties. There is growing interest in applying nanocarriers for more effective and safe delivery of various biologically

active or diagnostic agents in different fields of pharmaceuticals, medicine, biotechnology and cosmetics. The potential benefit of such novel nanoparticles in drug delivery can provide new products and methods for diagnostics and treatment of complicated oncological, neurodegenerative, immunodeficiency and other diseases.

Any novel nano-scaled carries potentially applied as diagnostics or therapeutics in medicine must be first of all assessed for potential risks for patients. That is why examination of newly-designed nanoparticles interaction with different blood components is a part of preliminary risk assessment, especially for nano-pharmaceuticals which are administrated intravenously^[1,2].

Previously, we reported the development of a novel drug delivery system on the basis of nanoparticles prepared from amphiphilic derivatives of poly-N-vinylpyrrolidone (Amph-PVP). Amph-PVP nanoparticles have further demonstrated the ability to effectively deliver different model drugs to the cells^[3,4], while presenting good stability under various biologically relevant conditions^[5], and low *in vitro* cytotoxicity and *in vivo* acute toxicity^[6,7].

The present study focuses on evaluation of the *in vitro* effects of new amphiphilic poly-N-vinylpyrrolidone nanoparticles on the whole blood, blood plasma and blood cells in order to assess such nanoparticles hematotoxicity. The Amph-PVP nanoparticles were prepared the basis of PVP amphiphilic derivatives via self-assembling in aqueous media and composed of a hydrophobic alkyl core and a hydrophilic PVP outer shell. Their hematotoxicity was evaluated by studying their effect on erythrocytes (red blood cells (RBCs)), leukocytes (white blood cells (WBCs)), platelets (PLTs) and on complement system activation *in vitro*.

The results obtained show that Amph-PVP nanoparticles are stable in presence of blood serum, have no significant effects on the function of RBCs, WBCs, PLTs and complement system activation. Also Amph-PVP nanoparticles did not show any significant inflammatory or hemolytic effect, neither influence on platelet aggregation, coagulation process, or complement activation at the tested polymer concentration range of 0.05–0.5 mg/ml. The preliminary identified low hematotoxicity of the Amph-PVP nanoparticles represent a crucial feature dictating their further investigations^[8]. Thus, our study provided specific conditions of hemocompatibility and safety of Amph-PVP nanoparticles utilization as an option for their further application as drug delivery systems for various biomedical areas.

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P04-06

Direct detection of organophosphate pesticides in water by a fluorescence-based biosensor

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Organophosphate (OP) pesticides are a group of phosphoric acid ester compounds. They are broad-spectrum compounds, characterized by high water solubility. This group of pesticides is highly applied worldwide in agriculture, but also in other activities (e.g., hydraulic fluids, plasticizers, etc.), and they are generally regarded as safe for use on crops and animals due to their relatively fast degradation rates. However, they present high toxicity, as they act by irreversibly inhibiting the enzyme acetylcholinesterase (AChE), a key protein in the transmission of nervous signals at synaptic level. Their toxicological action includes parasympathetic and sympathetic overstimulation, skeletal muscle paralysis, and sometimes respiratory failure. For this reason, AChE-based enzymatic biosensors have been widely applied and are reported in literature as the most promising tool for OP detection and for evaluating their toxicity. Nevertheless, AChE-based biosensors present some limitations, due to their relatively low selectivity in complex matrices, low stability over time, and susceptibility to react with a wide number of compounds other than OPs^[1]. Hence, the need to search for new and more efficient enzymes to be used as bioreceptors in biosensors.

In the present work, we addressed our studies using the thermostable esterase-2 from *Alicyclobacillus acidocaldarius* (EST2) as bioreceptor for OP pesticides. The use of EST2 involves some significant advantages compared to the use of enzymes such as AChE, showing high specificity and affinity towards OP pesticides, and high stability over time in a wide range of temperatures and pH, and in aqueous and organic solutions. An EST2 mutant, with the serine 35 replaced by a cysteine residue (EST2-S35C), has been produced for the binding of a fluorescent probe to the protein, for its use in visible fluorescence-based measurements for a direct detection of OP pesticides. IAEDANS probe was chosen for the specific binding to cysteine residues considering its relatively long fluorescence lifetime, and high thermal and photo-stability. EST2-S35C labeling was carried out incubating the enzyme with IAEDANS in a ratio 1:100 overnight at 4°C. The excess of IAEDANS was removed by dialysis. We carried out fluorescence spectroscopy measurements in the emission wavelength range from 400 to 550 nm exciting at 340 nm. Results showed that the addition of paraoxon aliquots (in the range from 100 to 400 pmol) to 300 pmol of EST2-S35C-IAEDANS, quenched the fluorescence of the complex, reaching a plateau at 1:1 ratio. The decrease of enzymatic activity of the EST2-S35C-IAEDANS complex in presence of these paraoxon concentrations correlated the inhibition of the labeled enzyme with the decrease in fluorescence. These results support the use of the EST2-S35C-IAEDANS complex as bioreceptors in biosensors for the monitoring and detection of organophosphate pesticides in water and environmental samples using fluorescence approaches.

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P04-07**A FRET approach to detect organophosphate pesticides using a fluorescent biosensor**

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Organophosphate (OP) pesticides are widely used in agricultural and urban areas due to their efficacy against target organisms. OPs act by inhibiting the neurotransmission in insects, binding with high affinity to the activity site of acetylcholinesterases. Nevertheless, the similarities in the biochemical mechanisms underlying the neurotransmission between insects and humans pose at risk also human health. This neurotoxic action is of special concern to children as their brains are in development, the ratio of pesticide dose to body weight is higher than that in adults, and children have lower detoxification rates. Despite the advocated low persistence of OPs, their wide and continuous use leads to their massive presence in the environment (water, soil, foods). The detection of OPs in the different environmental samples can be made by powerful analytical techniques such as gas and liquid chromatography combined with mass spectrometry measurements. However, these are high-cost techniques, time-consuming and require qualified laboratory workers. Therefore, it is of utmost importance to move to faster, sensitive and cheaper methods that also allow real-time and *in situ* detection and monitoring of these compounds. Biosensors are promising tools to achieve such objectives, and several biosensors for OP detection based on enzymatic or microbiological bioreceptors have been proposed [1]. However, some limitations have also been observed, such as thermo-dependent enzymatic activities. So, there is the need to search for new, more efficient and stable enzymatic activities to be used as bioreceptors. In this sense, this study focused on esterase-2 from *Alicyclobacillus acidocaldarius* (EST2) as this enzyme is a thermostable, single-chain protein of 34 kDa molecular weight, with a lipase-like Ser-His-Asp catalytic triad with a known high affinity to OPs. Thus, the main aim of this study was to evaluate the use of EST2 labelled with a fluorescent probe as a sensitive and reliable method to quantify OPs at environmentally relevant concentrations. We prepared an EST2 mutant (S35C-EST2), replacing the serine 35 by a cysteine residue, transformed the *Escherichia coli* strain BL21 (DE3) with the pET41b(+)-K430 plasmid containing the S35C-EST2 gene, and over-expressed the protein by IPTG induction. The protein was purified by thermo-precipitation and gel filtration steps, and its concentration determined by the Bradford method. A fluorescent probe, such as IAEDANS, was bound to the cysteine 35, near the catalytic site. Fluorescence resonance energy transfer (FRET) [2], using tryptophans as a donor to the receptor thiol-reactive IAEDANS, was evaluated. Fluorometric measurements showed significant inhibition of the enzyme after the addition of OPs at concentrations as low as 100 pmol. These results pave the way to further studies to validate the use of the fluorescent biosensor in different conditions, such as more complex matrices (foods, water, etc.).

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P04-08**PFAS & Pesticides effect on cell Survival, Permeability and Tight Junction in an in-vitro model of the Human Blood-Brain Barrier***D. Deepika¹, M. Capodiferro², R. Esplugas^{1,3}, J. Blanco³, R. P. Sharma¹, M. Schuhmacher¹, J. Grimalt², V. Kumar^{1,4}¹ Universitat Rovira i Virgili, Tarragona, Spain, Environmental Engineering Laboratory, Departament d'Enginyeria Química, Tarragona, Spain;² Institute of Environmental Assessment and Water Research (IDAEA) – Spanish Council for Scientific Research (CSIC), Department of Environmental Chemistry, Barcelona, Spain;³ Universitat Rovira i Virgili, Laboratory of Toxicology and Environmental Health, School of Medicine, IISPV, Reus, Spain;⁴ Universitat Rovira i Virgili, IISPV, Hospital Universitari Sant Joan de Reus, Reus, Spain

Widespread detection of perfluoroalkyl substances (PFAS) and pesticides in the human raises the concern about the entry of these chemicals in the brain and their ability to act as neurotoxicants. Homeostasis of the brain is required for normal functioning of the human body which is maintained by the blood-brain-barrier (BBB). Disruption of functionality and integrity of BBB by potential neurotoxicants may lead to adverse effects on different parts of the brain. The objective of this study was to investigate the role of PFAS and pesticides in disrupting tight junctions of BBB using an in-vitro model of immortalized human brain microvascular endothelial cells (hCMEC/D3). hCMEC/D3 was exposed to neurotoxicants (PFOA and chlorpyrifos) at different doses. Cell viability was determined by MTT assay and tight junction formation was assessed by transendothelial electrical resistance (TEER). Moreover, for screening the potential of these chemicals to pass BBB, permeability assay of cells was performed and apparent permeability coefficient (P_{app}) value was calculated from liquid chromatography/tandem mass spectroscopy (LC-MS) analysis. Moreover, changes induced on the mRNA expression profile of some genes that codifies for tight junctions proteins was also determined by RT-qPCR. Results demonstrated the potential role of these neurotoxicants by disrupting the BBB. Obtained data can be further applied in the physiologically based pharmacokinetic model (PBPK) for the brain using in-vitro to in-vivo extrapolation (IVIVE).

P04-09**Donor-to-donor variability of human bronchial epithelial cells in response to cigarette smoke***S. Muratani¹, A. Mori¹, T. Suzuki², K. Erami¹, S. Ito¹¹ Japan Tobacco, Inc., Scientific Product Assessment Center, R&D Group, Yokohama, Japan;² Japan Tobacco, Inc., Scientific and Regulatory Affairs Division, Minato, Japan

Development of a measurement method for *in vitro* toxicity assessment has recently gained attention because of legislative changes and animal welfare. Primary cells are widely used for *in vitro* assessment as an alternative to *in vivo* tests because they often retain similar characteristics to the tissue origin compared with cell lines. However, the response of primary cells to stimuli varies from donor to donor, which reflects the unique characteristics of the individual donor. Therefore, it is important to understand and interpret such variability biologically when evaluating toxicity. This study investigated donor-to-donor variability of the acute response to cigarette smoke extract (CSE) using normal human bronchial epithelial cells from five donors. The cells were exposed to six-to-nine concentrations of CSE for 1 hour. Then, production of intracellular reactive oxygen species (ROS) and depletion of glutathione were measured as

indexes of the oxidative stress response. We also evaluated secretion of growth factors and phosphorylation of epidermal growth factor receptor (EGFR), which are causal events of the oxidative stress response. All primary cells showed dose-dependent responses in all tested events, but their amplitudes varied. We also investigated the response-response relationship between ROS production and EGFR phosphorylation and found a marked difference between donors, which ranged from 0.8 to 2.0 in slope values ($R^2 = 0.78\text{--}0.93$). Therefore, not only the amplitude of each individual endpoint, but also their extents of causalities between the consecutive events varied among donors. In conclusion, using only single donor primary cells for an *in vitro* test could mislead interpretation of *in vivo* toxicity prediction because of a varied toxicological appearance. Therefore, the use of multiple donors and considering donor-to-donor variability are expected.

P04-10

Applicability domain of the GARD[®] skin Medical Device test for *in vitro* skin sensitization testing of medical devices

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Medical device toxicology is undergoing an exciting evolution; transitioning from a process that largely relied on the results of animal testing to evaluate the biological safety of medical devices in patients to one which is increasingly focused on the use of *in vitro* methods for the safety assessment of medical device materials.

Recently, *in vitro* methods to assess endpoints such as skin irritation and pyrogenicity have been validated and proposed for medical device testing, but a method to assess the potential for medical device-related skin sensitization to occur has not been sufficiently qualified. A number of *in vitro* skin sensitization test methods have been shown to have acceptable predictive ability for known skin sensitizers with structures that span a broad range of chemical classes, but the predictive ability of these methods has not been specifically evaluated using compounds typically found in materials used to manufacture medical devices. As a result, the need exists to qualify *in vitro* methods to assess the skin sensitization of compounds that may be released from medical devices during use, taking into account the applicability domain of known or potential skin sensitizers, including metals.

To address this challenge, the chemical space and the predictive ability of the GARD assay has been evaluated using a dataset of compounds known to be released from materials found in medical devices. Against this data set, the assay correctly predicted 19 out of 21 lipophilic and pre-/pro-hapten compounds with a total of 90.5% accuracy, with one false positive (95.2% sensitivity) and one false negative (95.2% specificity) being predicted, thus increasing the confidence in use of this *in vitro* assay to assess the skin sensitization potential of medical devices. Furthermore, we have also demonstrated that the GARD assay correctly predicts the skin sensitization response of nickel, cobalt, and chrome salts as skin sensitizers and a zinc salt as a non-sensitizer. Overall, our data support the use of the GARDskin Medical Device assay as an *in vitro* alternative for the *in vivo* methods (e.g., GPMT, LLNA) that today are typically used to assess skin sensitization as part of the biological safety assessment of medical devices.

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P04-11

In vitro strategy for biocompatibility testing of medical devices: ISO's perspective on irritation and sensitization

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According to the ISO 10993-1:2018 biocompatibility standard, irritation and skin sensitization must be evaluated for all medical devices^[1]. For years ISO Technical Committee 194's Working Group 8 (ISO/TC 194/WG8) has been pursuing a strategy of developing regulatory-accepted standards for the medical device industry that include *in vitro* alternatives for these two *in vivo* endpoints. Our presentation will provide a summary of ISO's effort.

For skin irritation, rabbit testing has been used for decades. In 2007, during the ISO/TC 194 meeting, the experts of the WG8 began discussing the possibility of replacing the *in vivo* irritation assay with *in vitro* assays. This led to an international round robin study in 2017^[2] and the January 2021 publication of the ISO 10993-23:2021 standard for irritation testing^[3]. This new standard replaces *in vivo* topical and intracutaneous rabbit tests with *in vitro* assays comprised of the SkinEthic[™] RHE^[4] and Epiderm[™]^[5] reconstructed human epidermis models. Since compliance with ISO 10993 standards enables regulatory requirements to be met in most countries, the ISO 10993-23:2021 standard represents a milestone in the transition from *in vivo* to *in vitro* for the biocompatibility testing of medical devices. Work continues on this endpoint to extend the applicability domain of the *in vitro* methods to non-extractable medical devices. In addition, Annex D of the new standard lists irritation tests for specific tissues such as ocular, oral, and vaginal that are still performed on animals. In the future, it is anticipated that these *in vivo* tests will also be replaced by *in vitro* methods.

For skin sensitization, even though significant advances have been made with *in vitro* testing of chemicals, the gold standards for medical devices remain the animal-based Buehler test, Guinea pig maximization test, and murine local lymph node assay. Nevertheless, pilot studies showed promising results with *in vitro* sensitization assays for identifying human sensitizers extracted from medical device materials^[6,7,8,9,10]. With this in mind the informative Annex C of the new ISO 10993-10:2021^[11] standard provides an overview of *in vitro* methods and general considerations for validation of *in vitro* methods for medical device testing. WG8 is currently designing a round robin study to evaluate the most promising assays already validated or under validation review by OECD for chemicals. A successful outcome of this study will lead to an updated version of ISO 10993-10 that

includes validated *in vitro* methods as partial or complete replacements for the historical animal tests.

The ultimate goal of ISO/TC 194/WG8 is to provide the medical device industry with non-animal methods-based replacements for the required *in vivo* irritation and sensitization tests.

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P04-12

Chlorpyrifos and Azamethiphos induced oxidative stress mediates cell death in human progenitor neuronal cells

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Chlorpyrifos and Azamethiphos are widely used organophosphate insecticides in agriculture sector for decades. There have been a huge body of literature regarding their association between neurodegenerative and neurodevelopmental disorders. However, the mechanisms that underpin neurodevelopment disorders associated with pesticide exposure are still in debate. Therefore, undifferentiated and differentiated human progenitor cortical neuronal cells were acutely exposed to the active metabolites (oxons) of chlorpyrifos and azamethiphos to mimic the effect of pesticide exposure, and the underlying mechanisms of toxicity to developing neurones investigated. Undifferentiated and differentiated human progenitor neuronal cells were exposed to pesticides for 24 hours over a concentration range of 0.3–200 µM and cell viability measured using Thiazolyl Blue Tetrazolium Bromide (MTT) and lactate dehydrogenase (LDH) assays. Low and high inhibition concentrations of each pesticides interpolated from viability curves were further assessed by ATP assay to evaluate the impact of pesticides upon cellular bioenergetics. Cellular reactive oxygen species (ROS) generation was assessed using 2',7'-dichlorofluorescein diacetate (DCFDA) assays while cellular oxidative stress

markers such as protein carbonylation, glutathione, and lipid peroxidation levels were also quantified. Specific targets of cytosolic oxidative damage were characterized by gel electrophoresis and oxyblots. Both cell phenotypes experienced pesticide-induced cell viability loss, ATP depletion and ROS generation that was pesticide concentration dependent. There was a pesticide-concentration dependent increase of protein carbonylation levels, and lipid peroxidation associated with the oxidative stress and GSH depletion. A protein of ~50 kDa was a major target for oxidative stress damage after exposure to either pesticide and in both undifferentiated and differentiated HNPCs. This study concluded that cytotoxic responses are dependent not only on the concentration and duration of pesticide exposure, but also on the neuronal developmental stage.

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P04-13

Borderline ranges for *in chemico* and *in vitro* skin sensitization methods determined from ring trial data – acknowledging experimental uncertainty in defined approaches

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Typically, the (continuous) results of toxicological assays are into a binary classifications (“positive” or “negative”) using cut-off values. Until now, the underlying prediction models do not consider data variability close to the cut-off. Some OECD test guidelines, however, provide a range close to the cut-off for which a repetition of the test is proposed. However, these ranges are based on few data (if on any) and not statistically derived. In this present study we determined the borderline ranges from multi-laboratory ring trial studies for five non-animal methods addressing skin sensitization: Direct Peptide Reactivity Assay (DPRA, OECD TG 442C), KeratinoSens® and LuSens (OECD TG 442D), and human cell line activation test (h-CLAT, OECD TG 442E) as well as the kinetic direct peptide reactivity assay (kD-PRA; draft update of OECD TG 442C). We used a statistical approach based on the log median absolute deviation (MAD) described in [1]. Implementing the proposed borderline ranges helps to assess (un)certainty of both individual test result and of combinations of multiple data sources in a defined approach (DA) or an integrated approach. The draft OECD guideline on DAs for skin sensitization provides the first regulatory application of borderline ranges to the “2 out of 3” DA.

P04-14

Exploring potential structure-activity relationships of graphene-based materials.

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Graphene-based nanomaterials (GBMs) show multiple remarkable properties and can be integrated in various innovations. Overall, the use of nanomaterials such as graphene-based materials is increasing extremely fast and is urging us to rethink our case-by-case method-

ology for toxicity assessment. Studying the structure-activity relationships (SARs) is one of the promising tools lastly proposed in toxicology in a context where alternative approaches such as read-across and grouping are increasingly employed. Study of SAR could allow industries to prioritize a certain group of materials by investigating their toxicity from the earliest steps of the development process, without unnecessarily having to go through a complete toxicity testing.

Some studies focused on few GBMs varying by one physicochemical characteristic and highlighted the impact of this specific characteristic on a toxicity endpoint. However, to our knowledge, no paper presented the result of a large pool of various GBMs, with the totality of their relevant physicochemical characterization assessed and various toxicity endpoints explored, allowing a thorough search of potential SARs.

Hence, this work proposes a grouping approach through a screening study of GBMs in order to discuss their potential structure-activity relationships.

We studied a total of 27 GBMs. Firstly, we characterized the physicochemical properties of these nanomaterials including lateral size, surface chemistry, metallic impurities, or specific surface area. Secondly, we assessed the toxicity impact of each of these nanomaterials on the RAW264.7 murine macrophages using various *in vitro* endpoints: cytotoxicity (LDH release), oxidative stress (ROS production, measured with the DCF-DA assay), and inflammation (TNF α production) as well as an acellular endpoint being the biological oxidative damage (FRAS assay). Finally, we explored the potential structure-activity relationships.

We highlighted a correlation between the GBMs specific surface area (SSA) and the oxidative stress they induced. We indeed observed that below a threshold of 200 m²/g, the oxidative stress is moderate while GBMs having a specific surface area higher than 200 m²/g showed a high oxidative stress impact. Moreover, the type of GBM can impact the nature of the SAR: reduced graphene oxides (RGOs) showed slightly different type of responses than graphene nanoplatelets (GNPs).

This work benefits from having both a satisfactory quantity (a total of 27 GBMs) and quality (complete physicochemical characterization and toxicity assessment done by the same team) of data, allowing to draw reliable conclusions. Further analyses are in progress to build a predictive model of toxicity using our dataset.

P04-15

Modulation of P-glycoprotein expression/activity in HK2 cells

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Introduction: P-glycoprotein (P-gp), expressed by the *MDR1/ABCB1* gene in humans, is an efflux membrane transporter, included in the "ATP-binding cassette" (ABC) superfamily, widely studied for its ability to eliminate toxic and drugs at the cellular level, tissues or even organism [1]. In this scope, it has recently been proposed that the modulation of P-gp may constitute a therapeutic strategy, since its activation/induction reduces the intracellular accumulation of its substrates and, consequently, may lead to a decrease in cytotoxicity caused by possible toxic agents [2]. In this context, the work developed aimed to evaluate whether it is possible to reduce the nephrotoxicity of P-gp substrates, modeling its expression and/or activity in the tubular cells.

Material & Methods: P-gp activity in HK2 cells was evaluated after a short (30 min) or long (24, 48 and 72h) period of exposure to differ-

ent P-gp modulators, namely doxorubicin (DOX; 2 μ M), rifampicin (RIF; 20 μ M) and thioxanthone 5 (TX5; 20 μ M) as potential activators/inducers of P-gp; and using rhodamine 123 (RHO 123; 10 μ M) as its fluorescent substrate. Finally, HK2 cells were exposed to cisplatin (CP; 10, 30 and 300 μ M), a nephrotoxic drug that is P-gp substrate, in the presence or absence of DOX, RIF, TX5 and ZOS, and cell viability (% of control) was determined by MTT assays.

Results: P-gp activation (significant increase in P-gp activity after a short incubation period) by TX5 was observed, for the first time, in HK2 cells, demonstrated by the decrease in the intracellular accumulation of RHO 123. Additionally, in the RHO 123 assays with long periods of incubation, all studied compounds were able to significantly increase P-gp activity at all endpoints studied, possibly due to induction phenomena. Cytotoxicity tests performed after CP incubation for 48h, in the presence or absence of P-gp activators/inducers, demonstrated that RIF played a potential role in protecting HK2 cells against CP-induced nephrotoxicity. However, results with the P-gp inducers/activators TX5 and DOX were inconclusive, as they demonstrated to be toxic to HK2 cells after 48h of incubation.

Conclusions: In this way, it is possible to observe an increase in P-gp activity and, consequently, a potential nephroprotective effect induced by P-gp inducers/activators. However, some additional studies, with different concentrations and incubation times for P-gp modulators, including P-gp inhibitors, and including ZOS, should be performed to confirm the role of P-gp in decreasing CP-induced nephrotoxicity in HK2 cells.

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P04-16

Toxic response in human adrenocortical cells exposed to zirconium dioxide nanoparticles

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Introduction: Zirconium dioxide nanoparticles (ZrO₂-NPs) are used in many industrial fields, such as solid oxide fuel cells, lithium-ion batteries, catalysts and ceramic membranes. Also, the unique properties of ZrO₂-NPs cause that are suitable for biomedical applications (biosensors, implants, cancer therapy). ZrO₂-NPs are also applied in structural and electronic ceramics, dental fillings, prostheses, fluorescent lighting and as well as lubricants and polishing agent. Such a wide application of ZrO₂-NPs increases the potential for their release in the environment and causes subsequent negative effects on human health (work environment) as well as ecosystem health. The information on toxicological effects of ZrO₂-NPs on human health is inconclusive, especially in terms of its potential endocrine disruption. The aim of this study was to assess the cytotoxic activity of ZrO₂-NPs in human adrenocortical cells, as the first step in the evaluation of their endocrine effects.

Methods: The cytotoxic activity of ZrO₂-NPs (<100 nm, Sigma) was assessed on human adrenocortical cells (NCI-H295R) after 24 and 48-h of exposure. Nanoparticles were characterized using the SEM, DLS and NTA methods. Toxicity studies included the effect of ZrO₂-

NPs on cell viability, cell membrane integrity (NRU assay) and mitochondrial metabolic activity (MTT assay). The morphological alterations in the cells were observed using holo-tomography technology (3D Cell Explorer, Nanolive).

Results: The cytotoxicity tests demonstrated that ZrO₂-NPs exhibited dose and time dependent low cytotoxic effects. The direction of these effects included cellular metabolism disturbances and the changes in the permeability of the cell membranes at concentration above 200 µg/mL. However, live-cell imaging demonstrated that ZrO₂-NPs induce morphological changes in cells and indicate the cytotoxic effect of ZrO₂-NPs at much lower concentrations, i.e. 4 µg/mL. Alteration of cellular morphology was observed, particularly shrunken cell size and changes in the shape, loss of cell-cell contact, cell surface folding and cytoplasmic vacuolization, as well as an increased number of lysosomes in the cytoplasm, and changes in the intracellular location of mitochondria. The toxicity evaluation of ZrO₂-NPs in adrenocortical cells may be of particular significance in the assessment of health risks and long-term consequences of exposure. It is important especially at work environment where is possible the long-term exposure to dusts of ZrO₂-NPs.

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P04-17

Medaka eggs tests, a screening tool to evaluate the Endocrine Disruption* potential of new ingredients?

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Introduction: Exposure to some chemicals can result in disruption of the endocrine system in Humans, this hot topic has interested scientists for several years and more recently has fueled public debate. In Europe, identification criteria of ED* were proposed in 2016 and were included in two sectoral regulations (i.e Protection Plant Products and biocidal products). The new Chemicals Strategy for Sustainability sets the stage of a harmonization for the whole concerned regulations, including cosmetic regulation. For cosmetics, the main difficulty comes from the context of the ban on animal testing and the demonstration of proof of the link between hormonal mode of action and adverse effects. In this perspective, the objective of this work was to evaluate the ability of two experimental models, based on whole organisms, currently undergoing OECD validation, to **predict disruption on estrogenic and androgenic pathways**, in order to put safe products on the market. From an ethical point of view, early non-autonomous stages of aquatic vertebrates have been used successfully for regulatory assessment of chemicals in line with the European Directive 2010/63/EU.

Methods: Endocrine activity was assessed on the two sexual axes: androgen and estrogen. **REACTIV** and **RADAR** tests were performed on transgenic larvae from Japanese medaka bearing fluorescent reporter constructs integrating hormonal responsive elements. These embryonic models allow *in vivo* detection and quantification of endocrine activities measuring the fluorescence levels. Assays were conducted on selected chemicals considered as suspected or proven endocrine disruptors according to ECHA in presence or absence of corresponding axis hormones to reveal different modes of action.

Results: An endocrine activity was demonstrated for all the reference substances tested without however having an exact concordance with the *in vivo* literature data (physiological threshold of the models not always reached, difference on the regulated pathway). For some chemicals, prior stimulation of the biological pathway of interest was necessary to reveal the endocrine activity. Due to intrinsic properties of some chemicals such as water solubility, toxicity, low suitable doses were tested.

Discussion and Conclusion: Even if these *in vitro* aquatic models are not necessarily predictive of the effects in humans, they make it possible to **detect endocrine disruption alert** upstream and constitute a **predictive screening tool**. An integrated approach to testing and assessment for detecting early endocrine effects, including these models, will be implemented systematically into our research projects and will contribute to guarantee the safety of our ingredients. Some challenges remain to be explored in order to enlarge the applicability domain of these experimental models, in particular for more complex structures such as plant extracts.

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P04-18

Miniaturization of *in vitro* liver metabolomics – a screening approach to predict the mode of action of liver toxicants in HepG2 cells

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in vitro Metabolomics (MIV) enables to predict the mode of action (MoA) of liver toxicants in HepG2 cells. The currently used method at BASF (LUMOX-MIV) is robust, reproducible, and predictive for different MoA^[1]. The LUMOX-MIV is appropriate for testing chemicals in the regulatory context but is not yet applicable for a fast and low-cost screening approach. The aim of this study was to miniaturize the method on a 96-well plate (µMIV) and compare the data with LUMOX-MIV. As a proof of concept, bezafibrate, a well-established quality control compound, and seven other substances known to cause liver toxicity through different MoA (aroclor, pendimethalin, b-naphthoflavone, WY-14643, acifluorfen, fluoroglycofen-ethyl, and ketoconazole) were tested with both methods.

Cytotoxicity testing was optimized for the µMIV dose selection. After testing seven different endpoints, the optimal cytotoxicity assay was defined as a multiplexing approach combining ATP measurement with membrane integrity assessment. For the testing of the substances, HepG2 cells were cultivated on Lumox dishes (35mm, Sarstedt) by seeding 200 000 cells/dish for LUMOX-MIV, and 15 000 cells/well in a 96 well plate (TPP, 10 000 cells/well) for µMIV. After 24 hours, each substance was added. Then, 48 hours later, the assay was stopped by harvesting the foil of the lumox dishes, freezing, and quenching the foil with dichloromethane/EtOH (LUMOX-MIV). For µMIV, isopropanol 80% was added to the wells, and the plates were

frozen subsequently. The foils/plates were finally extracted and analyzed by LC-MS/MS for metabolic profiling.

Bezafibrate treatment showed a clear metabolic change compared to vehicle treated cells. In both systems consistently, the lipid and energy metabolism were particularly altered, in line with the *in vivo* MoA of bezafibrate which acts as a lipid-lowering agent. The metabolome analysis for the other seven substances is ongoing and will be presented. The first results postulate that the μ MIV method is a promising *in vitro* tool for screening approach in toxicology and pharmacology.

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P04-19

***In vitro* effects of pyrrolizidine alkaloids in the lung adenocarcinoma cell line A549**

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The phytotoxins pyrrolizidine alkaloids (PAs) occur worldwide with more than 600 structurally different representatives. PAs share a common structure consisting of a necine base esterified with one or two necine acids. PAs are synthesized by plants as defense mechanisms against herbivores. Due to their toxicity and worldwide occurrence in more than 6000 plants PAs are a possible threat to humans and animals. Humans are mainly exposed via traditional medicine or recipes like bush tea or via contamination of tea, herbs and other food. Animals are at risk by uptake of contaminated hay or other feed. PAs have hepatotoxic, pneumotoxic and genotoxic properties. Consequences of PA exposure can be hepatic sinusoidal obstruction syndrome (HSOS) or pulmonary arterial hypertension (PAH). PAs are metabolized in the body by cytochrome P450 monooxygenases (CYP) enzymes into a highly reactive nucleophilic metabolite which is responsible for cytotoxic effects. However, there is still a huge knowledge gap regarding the cellular effects induced in the lung. In our study, we wanted to close this gap and characterize how different PA structures correlate with differences in the detected effects in cell viability, induction of apoptosis or DNA damage.

The cell line A549 was used for *in vitro* experiments with PAs. We performed cytotoxicity assays with an external metabolizing system of lung or liver microsomes. Flow cytometry was used to examine the induction of apoptosis by PAs and a possible influence of PAs on the cell cycle. Moreover, genotoxic effects were analyzed by the comet assay.

Activated PAs reduced cell viability with both external metabolizing systems. Although the induction of cytotoxicity and apoptosis was stronger for liver microsomes, lung microsomes were also able to bioactivate the PAs and induce cytotoxic and apoptotic effects, as seen in the flow cytometry measurements and verified by caspase activity assays. Genotoxic effects detected by the comet assay were only seen after activation with liver microsomes.

The results of these *in vitro* studies in lung cells show that the effects of PAs in the lung should not be disregarded when assessing the toxicity of PAs in humans.

P04-20

Mechanisms underlying new psychoactive phenethylamines cytotoxicity

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The emergence of New Psychoactive Substances (NPS) represents significant risks for health care and legal systems. Up to December 2020, more than 1000 NPS have been reported to the United Nations Office on Drugs and Crime (UNODC), with the majority being classified as synthetic cathinones, synthetic cannabinoids and phenethylamines^[1]. However, contrary to most classic illicit drugs, little information is available regarding the pharmacotoxicological profile of new psychoactive phenethylamine derivatives. Thus, we synthesized a selected group of phenethylamine and their corresponding *N*-benzylphenethylamine (NBOMe) derivatives, evaluated their cytotoxic profile, and assessed the putative mechanistic pathways involved in their cytotoxicity.

Mescaline, 2,5-dimethoxy-4-bromophenethylamine (2C-B), 2,5-dimethoxy-4-nitrophenethylamine (2C-N), and their corresponding NBOMe derivatives (mescaline-NBOMe, 2C-B-NBOMe and 2C-N-NBOMe) were synthesized and structurally characterized by nuclear magnetic resonance and mass spectrometry techniques. All the *in vitro* experiments were performed in human neuronal SH-SY5Y cells, differentiated into a dopaminergic phenotype using retinoic acid and 12-O-tetradecanoylphorbol-13-acetate (TPA). Phenethylamine derivatives cytotoxicity was evaluated 24 h after exposure by the neutral red uptake and the resazurin reduction assays. The potential of phenethylamine derivatives to generate free radicals was estimated after 4, 6 and 24 h of exposure by using the DCFH-DA fluorescent probe, and the intracellular reduced glutathione (GSH) levels were quantified, 24 h after exposure, by the DTNB-reductase-recycling assay to evaluate the cellular antioxidant condition. The metabolic and energetic states were evaluated, 24 h after exposure, by cytochrome P450 (CYP) isoforms inhibition (neutral red uptake assay) and by ATP intracellular levels quantification (ATP bioluminescence assay).

A concentration-dependent cytotoxic effect was observed for all the tested phenethylamines, with the NBOMe derivatives being more cytotoxic than their corresponding counterparts. Despite no significant effects on ROS/RNS production were observed, a significant depletion of intracellular GSH and a significant decrease of intracellular ATP levels were detected for all tested phenethylamines, except mescaline, with this depletion being concentration-dependent and more pronounced for the NBOMe derivatives. Lastly, the inhibition of CYP450 (with ABT), and specifically CYP3A4 (with ketoconazole) and CYP2D6 (with quinidine), significantly increased the cytotoxicity of some of the tested derivatives.

In conclusion, the addition of a NBOMe moiety to the parent drug significantly increases its cytotoxic effects, with phenethylamines-mediated intracellular GSH depletion and ATP decline. Moreover, the inhibition of CYP3A4 and CYP2D6 highlights CYP-mediated metabolism as a potential detoxification pathway for these phenethylamine derivatives.

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P04-21

SkinEthic™ HCE Time-to-Toxicity: The first method formally considered by the OECD for discriminating on its own the three UN GHS ocular hazard categories

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For more than two decades, scientists have been trying to replace *in vivo* rabbit eye irritation test with non-animal methods. So far, several *in vitro* methods have been implemented into regulations, however none of them is able to replace the test completely due to the complexity of the endpoint and the classification schemes applied by the regulation under the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS). Taking into account our expertise on the SkinEthic™ HCE model (test system of OECD TG 492) and our knowledge on associated protocols, the SkinEthic™ HCE Time-to-Toxicity test method was established for both liquids^[1] and solid chemicals.

The test method evaluates the hazard potential of a chemical based on its ability to induce cytotoxicity. Based on the viability observed for the different exposure periods (from 5 to 120-min) a classification was assigned.

In the current study, the method was developed with 74 training chemicals (32 liquids, 42 solids) and challenged with 52 test chemicals (24+28) selected on the basis of important *in vivo* drivers of classification (i.e. corneal, conjunctival and persistence effects).

The within laboratory reproducibility (concordance within 3 runs), based on 67 training and 30 test chemicals was 87% independently of the testing sets. Application to 74 training chemicals, accuracy value was above 72%, with 75% Cat.1 (N=25), 68% Cat.2 (N=20) and 74.9% No Cat. (N=29) correctly identified. Furthermore, 80% Cat. 1, 71% Cat. 2 and 70% No Cat. were well classified with the test set, confirming the robustness of the test method. In conclusion, this study provides evidence that the SkinEthic™ HCE Time-to-Toxicity method is capable of i) correctly identifying chemicals not requiring classification AND ii) distinguishing between Cat.1 (serious eye damage) and Cat.2 (eye irritation), therefore providing added value for the classification and labelling of chemicals according to the three UN GHS.

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P04-22

Batch-to-batch variation of Ames test results for *S. typhimurium* TA 98 and TA 100 correlates with growth kinetics

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The Ames test is one of the most commonly applied tools in mutagenicity testing of chemicals since the first paper by Ames *et al.* in the 1970s. Histidine auxotrophic *Salmonella typhimurium* strains grow on mineral medium supplemented with a small amount of histidine. During the test, reverse mutations occur in the cells – either spontaneously or induced via a mutagenic chemical – and enable the

bacteria to grow without further histidine supplementation. The difference in cell growth of a culture where only spontaneous mutants occurred and a culture where additionally induced mutants occurred indicates the mutagenic properties of the chemical. Distinct variations of Ames test results for different batches of the test strains *S. typhimurium* TA 98 and TA 100 have been observed in many toxicology labs but are so far not understood. This study aims to link the Ames test performance of *S. typhimurium* TA 98 und TA 100 to growth characteristics and genetic factors.

Bacterial growth of *S. typhimurium* TA 98 and TA 100 was surveyed using the Respiration Activity Monitoring System (RAMOS). The RAMOS technique allows to online-monitor the oxygen transfer rate (OTR) of the bacteria, which correlates to their growth. With this technology, maximum growth rates were easily determined and found to differ in between batches of the same strains. A correlation between maximum growth rate and Ames test results was developed. In addition, the biomass yield on histidine and glucose turned out to be another varying characteristic. Finally, several test strain batches were sequenced to investigate genetic differences and correlate those to the earlier obtained growth characteristics and Ames test results.

This study revealed differences in between batches of the Ames test strains *S. typhimurium* TA 98 and TA 100 and showed that the maximum growth rate is an indicating factor for varying Ames test results. Genomic evaluation was performed for understanding the differences in growth. In future, better quality control measures for evaluating the Ames test strains should be developed and implemented to ensure accurate and reproducible Ames test results.

P04-23

Prediction of skin sensitization on pesticides through the Direct Peptide Reactivity Assay

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Upon skin contact, skin sensitizers are chemical substances that can elicit allergic responses or diseases. Due to the animal welfare, European Union (EU) legislation on testing of chemicals has developed various alternatives to animal test. *In chemico* skin sensitization: Direct Peptide Reactivity Assay (DPRA, OECD Test Guideline 442C) is composed of applying a test substance on synthetic peptides containing cysteine or lysine and determining skin sensitization measured by reactivity of pesticides. In this study, 10 pesticides of concern for potential skin sensitization were subjected to evaluated using Direct Peptide Reactivity Assay (DPRA). These results showed that fluzinam (55.56%), benomyl (50.00%), carbosulfan (50.00%), pretilachlor (43.88%), butachlor (27.23%) and terbufos (20.94%) were reported positive, while oxadiazon (1.71%), diazinon (1.23%), glufosinate ammonium (1.31%) and tebuconazole (0.48%) were found negative in the DPRA method. Compared with *in vivo* tests (LLNA and GP data), 6 pesticides were predicted correctly with sensitizer and 4 pesticides also correctly predicted them to be non-sensitizers. These findings demonstrate DPRA method could be an important part of assessment of new ingredients in pesticides to predict the skin sensitization.

P04-24**Assessment of skin sensitization in pesticides using alternatives to animal testing**

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Skin sensitizers are chemical substances that can elicit allergic responses or diseases in skin upon contact. The assessment of skin sensitization has generally been performed in animal test. However, for animal protection, the concepts of 3R (Reduction, Replacement, Refinement) were introduced and several animal-free alternatives were developed based on adverse outcome pathway of the skin sensitization. The Direct Peptide Reactivity Assay (DPRA, OECD TG 442C, 2015) and the Human-cell Line Activation Test (h-CLAT, OECD TG 442E, 2018) are alternatives to animal testing used to distinguish between allergens and non-allergens within Integrated Approaches to Testing and Assessment (IATA). In the current study, we conducted DPRA and h-CLAT in accordance with OECD guidelines to compare these two assays and combine their results to predict the skin sensitization of 5 pesticides: fluazinam, benomyl, carbosulfan, glufosinate ammonium, tebuconazole. Analysis using two *in vitro* tests showed good prediction of sensitizer and non-sensitizers. This study showed that both DPRA and h-CLAT assay can serve as non-animal *in vitro* methods for the assessment of skin sensitization in pesticides.

P04-25**Cadmium induced temporal alterations measured in iPSC-derived proximal tubular cells***P. Singh^{1,2}, V. Chandrasekaran³, A. Wilmes³, B. Hardy¹, P. Jennings³, T.E. Exner^{4,1}¹ Edelweiss Connect GmbH, Basel, Switzerland;² University of Basel, Division of Molecular and Systems Toxicology, Department of Pharmaceutical Sciences, Basel, Switzerland;³ AIMMS, Vrije Universiteit Amsterdam, Division of Molecular and Computational Toxicology, Chemistry and Pharmaceutical Sciences, Amsterdam, Netherlands;⁴ Seven Past Nine d.o.o., Cerkljica, Slovenia

Proximal tubules are the most abundant segments of nephrons in kidney responsible for maintaining whole body homeostasis. They are also considered to be the most susceptible to xenobiotics like cadmium. Through this study, we have investigated the robustness of the human induced Pluripotent Stem Cells (iPSCs) derived renal proximal tubule-like (PTL) model for chemical testing and explored the time-related alterations caused in the common pathways affected by cadmium exposure. iPSC derived PTLs were treated with 5 μ M cadmium chloride (a non-cytotoxic concentration) for 1h, 2h, 4h, 6h, 8h, 12h, 16h, 20h, 24h, 72h and 168h.

An early activation of metal response and oxidative stress pathways was seen within the first 4h. The metal response pathway continued to maintain a stable expression level throughout the exposure period of 168h while the oxidative stress pathway returned to base level within 24h. This study shows that iPSC derived PTLs are able to characterise the toxicological response of these two pathways and can potentially be used as human-relevant alternatives *in vitro* model.

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P04-26**Role of quercetin metabolites in the flavonoid's protective effect against cisplatin nephrotoxicity**D. Muñoz-Reyes^{1,2,3}, A.G. Casanova^{1,2,3}, J. Tascón^{1,2,3}, L. Vicente-Vicente^{1,2,3}, F.J. López-Hernández^{2,3}, A.I. Morales^{1,2,3}, *M. Prieto Vicente^{1,2,3}¹ University of Salamanca, Toxicology Unit, Faculty of Pharmacy, Salamanca, Spain;² University Hospital of Salamanca, University of Salamanca, Spanish National Research Council (CSIC), Institute for Biomedical Research of Salamanca (IBSAL), Salamanca, Spain;³ University of Salamanca, Institute for Biomedical Research of Salamanca (IBSAL), Translational Research on Renal and Cardiovascular Diseases (TRECARD), Salamanca, Spain

Nephrotoxicity is the main limitation to the use of cisplatin in oncology. On the other hand, quercetin is a flavonoid with a wide range of biological properties, including a strong antioxidant capacity.

Although quercetin administered intraperitoneally has shown to have a protective effect against cisplatin nephrotoxicity, these results have not been reproduced when quercetin has been administered orally. Our hypothesis is that quercetin *in vivo* protection may be related to the activity of some of its metabolite(s). The aim of this study was to evaluate the cytoprotective capacity of quercetin and its main metabolites against cisplatin toxicity in renal tubular cells. Quercetin and five of its metabolites (quercetin 3-O-glucoside, rutin, tamarixetin, isorhamnetin and quercetin 3-O-glucuronide) were evaluated in two tubular cell lines, HK2 and MCT. The MTT technique was used as an index of cytotoxicity. First, cisplatin toxicity was tested after 18 hours of treatment, and antiproliferative, antiapoptotic and necrotic effects were observed at concentrations of 10, 30 and 300 μ M, respectively. Subsequently, safety experiments of quercetin and its metabolites were performed after 24 and 48 hours of treatment. Rutin and quercetin 3-O-glucuronide showed a higher safety range, while quercetin 3-O-glucoside was the most toxic metabolite of those tested. Finally, the cytoprotective capacity of the compounds was evaluated after 24 hours of co-treatment with cisplatin. In the case of quercetin, quercetin-3-O-glucoside, rutin, tamarixetin and isorhamnetin, there were no differences with respect to the control at the doses tested. In contrast, co-treatment with quercetin-3-O-glucuronide appears to protect against cisplatin cytotoxicity in renal tubular cells, making it a promising candidate for future *in vivo* nephroprotection studies.

P04-27**Performance of the SENS-IS *in vitro* skin sensitization assay applied to multiple agrochemical formulations.***W. Masinja^{1,3}, H. Groux², F. Cottrez², M. Cronin³, S. Enoch³, R. Currie¹¹ Syngenta, Product Safety, Bracknell, UK;² Immunosearch, Grasse, France;³ Liverpool John Moores University, Pharmacy and Biomedical Science, Liverpool, UK

At present there are no industry or OECD accepted *in vitro* methods for a complete skin sensitization hazard assessment of complex agrochemical formulations. Since 2011 *in vivo* testing (i.e. the Local Lymph Node Assay or the Buehler guinea pig assay) to assess the skin sensitization of complex formulations has not been permitted in the European Union. The aim of this work was to address the current absence of a single, *in vitro* method for the accurate assessment of the skin sensitization potential of agrochemical formulations. The SENS-IS assay is a 3D skin model based on the analysis by RT-PCR (reverse transcription polymerase chain reaction) of two sets of genes. One set of genes is associated with irritation potential, i.e. the "IRRITA-

TION" gene set. The second set of genes is divided into two subsets i.e. the "SENS-IS" and "REDOX" gene sets and these are associated with skin sensitization. Ten Syngenta agrochemical formulations which had previous, historic, *in vivo*, local lymph node assay study data were chosen for this evaluation. The SENS-IS assay measured the level of expression of the two separate gene sets at a given time point after application of the formulation as compared to an internal negative, irritant (sodium lauryl sulphate) and positive sensitizer (2,4,6-trinitrobenzene sulfonic acid) controls. The SENS-IS assay displayed good agreement with the *in vivo* skin sensitization results, correctly predicting five out of six of the previously identified formulations with skin sensitizing potential. In addition, the assay demonstrated agreement with the previous potency evaluations for these formulations. The SENS-IS assay also correctly identified three out of four formulations previously shown to be non-skin sensitizers. In conclusion, this study indicates that the SENS-IS assay clearly provides an *in vitro* testing option for accurate hazard assessment of skin sensitization potential for agrochemical formulations.

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P04-28

Performance statistics of defined approaches for eye hazard identification of liquids to distinguish between the three UN GHS categories

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Within Cosmetics Europe, two defined approaches (DAs) for eye hazard identification of non-surfactant liquid test substances have been developed. The DAs address the 3 UN GHS categories, i.e. serious eye damage and eye irritation or the absence thereof. DAL-1 combines four physicochemical properties, a Reconstructed human Cornea-like Epithelium [RhCE: EpiOcular™ EIT (VRM1)/SkinEthic™ HCE EIT (VRM2)] test method (OECD TG 492), and the Bovine Corneal Opacity and Permeability laser light-based opacimeter (BCOP LLBO) test method (OECD TG 437)^[1,2,3]. DAL-2 combines the Short Time Exposure (STE) test method (OECD TG 491) and BCOP LLBO test method (OECD TG 437)^[3,4,5]. In both DAs, only the opacity measurement of the BCOP LLBO is used to identify liquids that cause serious eye damage. Predefined minimum performance values proposed by Cosmetics Europe to the OECD Expert Group on Eye/Skin Irritation/Corrosion and Phototoxicity and agreed upon discussion by the experts were

used to compare the performance of the DAs to distinguish between the three UN GHS categories. In order to gain further insight in explaining the performance in the context of the applicability domain of the individual DAs, the predictivity was also assessed based on the drivers of classification and by the organic functional group (OFG).

Note that 16 Cat. 1, 21 Cat. 2, and 31 No Cat. reference chemicals were tested in common by the different DAs. The balanced accuracy (average of the % of correct predictions of the three categories) of DAL-1 with VRM1, DAL-1 with VRM2, and DAL-2 was 69.2%, 75.2%, and 74.3%, respectively. DAL-1 with VRM1 identified 76.5% of Cat. 1 (N=17), 59.1% of Cat. 2 (N=22) and 72.1% of No Cat. (N=55) correctly (69.2). DAL-1 with VRM2 identified 76.5% of Cat. 1 (N=17), 68.7% of Cat. 2 (N=23) and 80.4% of No Cat. (N=46) correctly. DAL-2 identified 81.2% of Cat. 1 (N=17), 56.3% of Cat. 2 (N=24) and 85.3% of No Cat. (N=123) correctly. These values were greater than the proposed minimum performance of 75% for Cat. 1, 50% for Cat. 2 and 70% for No Cat agreed by the OECD experts. Regarding the drivers of Cat. 1 and Cat. 2 classification, liquids that were classified *in vivo* based on CO severity (Cat. 1: mean CO ≥ 3 or CO = 4; Cat. 2: mean CO ≥ 1) were mostly correctly predicted with the DAs. It was noted that Cat. 1 and Cat. 2 liquids with an alcohol function were more often mispredicted with both DAs. Furthermore, No Cat. liquids from the subgroup CO=0 had the highest predictivity and no specific relationship between the mispredicted liquids and OFGs was found.

In conclusion, DAL-1 and DAL-2 have shown to successfully distinguish between the three UN GHS categories for eye hazard identification. Both DAs were submitted to support OECD acceptance and currently an OECD draft test guideline is under review to support regulatory acceptance.

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P04-29

A multiplexed *in vitro* approach for the dual detection of functional and structural cardiotoxicity

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Cardiotoxicity is a major cause of drug attrition during pre-clinical and clinical drug development. Drugs can exhibit functional changes

defined as an acute alteration in the mechanical function of the myocardium or structural in nature as defined by morphological damage to cardiomyocytes and/or loss of viability. In recent years *in vitro* strategies have been developed to allow the high throughput assessment of functional cardiomyocyte changes through kinetic monitoring of calcium transients (Pointon *et al.*, 2015) while structural morphology can be monitored in a high throughput manner using high content imaging (HCI) (Pointon *et al.*, 2013). Here we describe a multiplexed method to allow the morphological analysis of cardiomyocytes alongside calcium transient assessment in order to better define functional and structural cardiotoxicity events. Human induced pluripotent cardiomyocytes (hiPS-CMs) were seeded in 384 well plates for a minimum of 10 days before compound exposure. Compound exposure was performed acutely or combined with a 24hr pre-incubation. Prior to compound exposure or following 24hr pre-incubation, cells were incubated with EarlyTox Cardiotoxicity dye for 2 hours. Compound is then applied at 8 concentrations in triplicate utilising a compound set comprising structural and functional cardiotoxins alongside dual toxicity compounds and non-cardiotoxins (total of 42 compounds). Fast kinetic fluorescent reading is then performed on a Cytation 3 Cell Imaging Multi-Mode Reader (BioTek®). High content imaging of cell count, calcium homeostasis (EarlyTox™) and mitochondrial function (TMRE) is then performed using an ArrayScan™ XTI HCI reader (ThermoScientific). Finally, cells are lysed for the quantification of intracellular ATP using CellTiter-Glo® (Promega). This combined time-point approach allows the detection of short-term functional cardiotoxicity; epinephrine displayed increased calcium transient peak frequency (MEC; 0.03 µM) following only acute exposure, while dobutamine a longer-acting functional cardiotoxin exhibits peak frequency changes with both acute and pre-incubation exposures (MEC; 0.1 µM and 54.4 µM, respectively). A combined assay approach allows the detection of morphological changes exhibited by structural cardiotoxins; sunitinib is a dual toxicity compound (functional & structural) which exhibits a decrease in peak calcium transient amplitude (MEC; 88 µM) alongside cell morphology changes (MEC; 144 µM). This study shows a multi-parameter cellular assessment strategy can improve the *in vitro* to *in vivo* translation and risk assessment of the potential for novel compounds to elicit functional and structural cardiotoxic events on a high throughput scale.

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P04-30

***In vitro* assessment of the skin sensitizing potential and potency of epoxy resin monomers and pre-polymer mixtures using the GARD assay**

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Allergic skin diseases caused by occupational exposure to sensitizing substances are problematic in preventive occupational safety. Ingredients of epoxy resin products have sensitizing effects. Recently, so-called prepolymers have been used in epoxy resin systems, which are expected to have a lower potency than monomers.

In this project, the sensitizing potential and potency of selected epoxy resin monomers and pre-polymers were investigated using the GARD assay. The test materials were tetraethylenepentamine (TEPA), a monomeric amine hardener in epoxy resin systems, and four amine functional prepolymers*.

An important finding was that all selected test items could be tested without problems despite their different solubility and complex composition.

As expected, the tested substances were identified positive for the response of skin sensitization.

Monomeric TEPA was correctly classified as skin sens. Cat 1B. However, the TEPA prepolymer was initially categorized as Cat. 1A, which is inconsistent with the expected lower reactivity. The conventional GARD potency prediction model used takes into account the molecular weight of the test item, which is difficult to determine in prepolymers and thus might lead to an overestimation of skin sensitization potency. To mitigate such overestimations, further evaluations based on weighted molecular weights were carried out using an alternative prediction model, which ignores the molecular weight of the test substance for potency classification. The use of these alternative assessments resulted in a different classification (i.e., 1B), indicating their importance in borderline cases. Furthermore, all prepolymers with high-potency monomeric amines, were correctly classified as Cat. 1A, both with the conventional and alternative prediction model, thus indicating that the monomeric amine fraction still determines the sensitizing potency despite the lower overall reactivity of the prepolymer mixtures. This is supported by the result of the polyamidoamine with a low monomeric amine fraction, which shows the lower sensitizing potency of a prepolymer, although the monomeric amine used, i.e. TETA, is of high sensitizing potency.

In summary, the GARD Assay is a reliable method for the determination of skin sensitizing potential of not well-defined substances and complex mixtures. Regarding the potency classification, it shows that both the calculation of weighted molecular weight and the use of an alternative prediction model are useful for not well-defined substances and seem to provide a reliable statement. For occupational safety, the prepolymer approach still needs refinement, but remains a promising step towards epoxy resin systems with lower skin sensitizing potency.

* three amine functional Bisphenol A diglycidyl ether adducts in benzyl alcohol (each around 30% polymer, 30% monomeric amine and 40% benzyl alcohol), and one polyamidoamine (low monomeric amine portion, <2% triethylenetetramine (TETA) and TEPA)

P04-31

High-content multi-frequency impedance cell monitoring for label-free and time-resolved cell toxicity analysis of various cell types

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Impedance readouts from cell-covered, planar gold-film electrodes give profound insights into cell morphology, contractility, proliferation and cytotoxicity of various cell phenotypes, in a label-free fashion. The method offers a crucial advantage over standard, mostly

endpoint, cytotoxicity assays as cells can be continuously monitored, even over prolonged periods of time. Advanced information content is obtained by using multi-frequency impedance readouts as they allow zooming in on changes in membrane topography, cell-cell or cell-matrix junctions deconvoluting the complex whole cell response, such as to G-protein-coupled receptor (GPCRs) activation. The multi-frequency readout allows selecting the most sensitive frequency for a particular application or cell type. Here, impedance spectra were calculated and data acquired with CardioExcyte96 impedance recording system, for HeLa, HEK293 and CHO cells to demonstrate cell-type specific differences under stationary conditions. Single frequency recordings have been performed to investigate drug induced hepatic toxicity as well as monitoring *in vitro* breast cancer recurrence and cardiotoxic effects of anticancer drugs on pluripotent stem cell-derived cardiomyocytes.

Frequency-dependent impedance data reveal individual drifts of the most sensitive frequency, i.e. of the maximal responses indicating cell type specific changes in cell junctions and proliferation dynamics. The expected concentration dependent toxicity was observed for DMSO, Escin and Triton-X showing decreasing impedance values with exposure time and drifting of the maximal responses to lower frequencies. GPCR-mediated signal transduction was investigated by applying the endogenous agonist in dose-response studies or receptor-independent agents.

Single-frequency impedance data were performed on hepatocytes and liver sinusoidal endothelial cells (LSECs) from two donors. The initial results showed that LSECs and hepatocytes respond differently to typical hepatic compounds (acetaminophen and imipramine). Moreover, these drugs had similar pharmacological IC₅₀ values in impedance recordings compared to the intracellular ATP measurements. Additionally, we have used single-frequency impedance measurements to monitor chronic changes (500h) in confluency and viability of murine mammary carcinoma cells. Intrinsic dose dependent effects of anticancer drug mix CAF were identified and consistent with other methods, such as image-based live-cell analysis. The implication of same anticancer drug mix was tested on pluripotent stem cell-derived cardiomyocytes. CAF or doxorubicin long- and short-term implications on cell viability were observed.

In summary, single and multi-frequency impedance data allow for a non-invasive continuous monitoring of cells with the option to zoom in on certain cell aspects or to tailor the readout to individual cell types for improved data interpretation.

P04-32

Evaluation of larval zebrafish as an alternative whole-animal model for nephrotoxicity testing

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The increasing demand for testing of new and existing chemicals and legal restrictions for the use of animals creates a strong need for alternative approaches to assess systemic toxicity. Larval zebrafish (*Danio rerio*) are increasingly recognized as a promising alternative whole-animal model that might be able to overcome limitations of cell-based *in vitro* assays and bridge the gap between high-throughput *in vitro* screening and low-throughput *in vivo* tests in animals. Despite the relatively simple anatomical structure of the zebrafish larval kidney (pronephros) – composed of only two nephrons – the

pronephros presents major homologies with mammalian nephrons regarding cell types and functions. The aim of the present study was to investigate if early zebrafish larvae might be a suitable model for identification of nephrotoxic potential. At day 3 post fertilization, zebrafish larvae were treated for 48 h with model compounds (aristolochic acid, cadmium chloride, potassium bromate, ochratoxin A, gentamicin), known for their nephrotoxic effects in mammals. Histopathological analysis of nephrotoxin exposed larval zebrafish revealed tubule injury as evidenced by dilated tubules with loss of the brush border, tubule cell necrosis and disorganization of the tubular epithelium. Most severe tubular changes were observed after treatment with gentamicin, which also impaired pronephros function as evidenced by reduced clearance of FITC-dextran. Site-specific injury to the proximal tubule was evidenced by loss of *cdh17* expression at sites representing the proximal tubule by whole mount in situ hybridization and in transparent zebrafish larvae derived from transgenicTg(*cdh17:mCherry*). Injury to the proximal tubule segment was also visualized by α 6F immunofluorescence. Analysis of genes previously identified as novel biomarkers of kidney injury in mammals showed upregulation of the marker genes *heme oxygenase 1* (*hmo1*), *clusterin* (*clu*), *secreted phosphoprotein/osteopontin* (*spp1*), *connective tissue growth factor* (*ctgf*) and *kim-1* (*havr-1*) in response to nephrotoxin treatment, although the response of individual genes varied across compounds. In agreement with the severity of lesions and impaired kidney function, the most prominent gene expression changes occurred in gentamicin exposed larvae. Overall, our results suggest that larval zebrafish may be a suitable alternative model organism for nephrotoxicity screening.

P04-33

An investigative study to evaluate differences between responses in human, cynomolgus monkey and minipig in whole blood and peripheral blood mononuclear cells (PBMCs) using an *in vitro* assay to detect SEB-induced IL-2, IFN γ and TNF α

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As a result of the CD28 superagonist TGN 1412 monoclonal antibody (mAb) cytokine storm incident in 2006^[1,2], cytokine release assays (CRAs) have become more commonly used as hazard identification and risk assessment tools for therapeutic candidates, particularly mAbs with the potential to elicit adverse pro-inflammatory cytokine responses in patients. Although cytokine release syndrome (CRS) is a relatively rare event in the clinic, evaluating the potential of certain novel therapeutic mAbs to cause CRS is increasingly becoming part of preclinical safety testing^[3–6].

Here we present our first approach using cynomolgus monkey and minipig as immunotoxicology species for cytokine release assay (CRA) compared to and in addition to human as an *in vitro* test system of reference.

The objective of this study was to evaluate cytokine production in cynomolgus monkeys and minipigs after Staphylococcal Enterotoxin B (SEB) stimulation compared to responses seen in human donors. Briefly, whole blood and PBMCs from each species were stimulated for 72 hours with SEB at 1 μ g/mL alongside pembrolizumab and nivolumab in 5 dilutions and an IgG4 isotype control. Concentrations of IL-2, IFN- γ and TNF- α in cell supernatants were measured by Luminescence.

Staphylococcal Enterotoxin B (SEB), a well characterized superantigen produced by *Staphylococcus aureus*, is a potent activator of the immune system. The toxins bind to major histocompatibility complex (MHC) class II molecules and T-cell receptors (TCRs), resulting in the activation of both monocytes/macrophages and T lymphocytes.

The bridging of TCRs with MHC class II molecules by superantigens triggers an early “cytokine storm” and massive polyclonal T-cell proliferation^[7]. The use of SEB is preferential over other immunostimulants because it can provoke an innate immune response and also launch an adaptive immune response compared to other stimulants^[8].

Pembrolizumab and nivolumab are both potent human IgG4 anti-PD-1 monoclonal antibodies that directly block the interaction between PD-1 and its ligands, PD-L1 and PD-L2. This blockade enhances the functional activity of T-cells. *In vitro* assays demonstrated the ability of pembrolizumab and nivolumab to potently enhance T-cell response and cytokine production in SEB stimulation assays^[9,10].

Our results showed an increase in IL-2, IFN- γ and TNF- α in both human and cynomolgus monkey whole blood and PBMCs after SEB stimulation. The immune checkpoint inhibitors pembrolizumab and nivolumab induced a higher release of the cytokines. Minipig data showed greater variability in measurable cytokines in both whole blood and PBMCs.

In conclusion, our results demonstrated that cynomolgus monkeys, when compared to human results, could be used as a predictive model for the assessment of therapeutic immunostimulatory potential when measuring cytokines IL-2, IFN γ and TNF α .

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P04-34

Coupling H295R with U2OS ER α and AR CALUX bioassays increases steroidogenesis test sensitivity

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Over the last years, increased regulatory and public attention has been put on endocrine active substances, including those with effects on gonadal and adrenal steroid hormone synthesis (steroidogenesis). To screen and test chemicals that potentially affect steroidogenesis, the *in vitro* H295R steroidogenesis assay (OECD TG 456) utilizes a human adenocarcinoma cell line (H295R) to detect interference with 17 β -estradiol and testosterone production, but potentially other steroid hormones also. However, the detection methods currently in use (immunoassays or mass spectrometry) lack the required sensitivity for the detection of minimal changes in the hormone levels. This can

be a major drawback for the analysis of mixtures originating from food or food contact materials, which may contain only small amounts of steroidogenic chemicals and are thus expected to influence hormone levels to a small extent. To overcome this drawback, the present study trialed the use of the U2OS ER α and AR CALUX receptor activity bioassays as a detection system for the original H295R assay, as these bioassays exhibit a substantial change in activity in response to a small change in hormone concentration. To accommodate the CALUX detection system, the following refinements were made: i. perfluorooctanesulfonic acid and forskolin were both used as positive controls for induction and ii. parallel CALUX assays were performed on the same plate without prior exposure to H295R cells to control for any direct effects of the chemicals on the androgen and estrogen receptors. The performance of the assay was evaluated in a proof of concept study with chemicals from the original OECD validation study; generally similar conclusions were drawn. The respective lowest observed effect concentrations (LOECs) were at least equivalent to, and often lower than, those described by the OECD. For some substances, direct estrogenic and anti-androgenic activities were identified, complicating H295R assay interpretation. These results suggest that some steroidogenesis-mediated endocrine activities could theoretically be masked by direct (ant)agonism of the receptors by test substances. Nonetheless, these modalities are also relevant for the characterization of endocrine active substances; thus, these findings can provide additional valuable mechanistic information. Taken together, the results from this proof of concept study indicate a solid overall performance with significant additional gains in the detection of small changes in steroidogenesis, suggesting that the linked H295R-CALUX assay might have promise for the analysis of mixtures originating from food or food contact materials.

P04-35

In vitro studies on cytotoxicity, internalization and genotoxicity of graphene oxide

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Graphene-based materials have attracted much attention and have been used for a myriad of practical applications in various industries. This is also the case of the food sector, including packaging. But before its potential application as food contact material, *in vitro* studies are required to show their safety. According to the European Food Safety Authority and its guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain, we aimed to determine *in vitro* the toxicity profile of graphene oxide (GO) by cytotoxicity, internalization and genotoxicity assays. Caco-2 cell line, an experimental model representative of the human intestinal barrier, was exposed to 0–250 $\mu\text{g ml}^{-1}$ GO for 24 and 48 h to evaluate the cytotoxicity by MTS salt reduction and protein content (PC) as endpoints. The interaction between GO and Caco-2 cells was investigated by transmission electron microscopy (TEM). Genotoxicity was assessed by the Comet assay on Caco-2 cells and by the micronucleus test (OECD 487 2016) on L5178YTk +/- mouse lymphoma cell line.

The results showed no significant changes in the cell viability of Caco-2 cells exposed to GO compared to untreated cells after both exposure times at any concentration assayed. However, TEM images illustrated the internalization of GO after 24 h of exposure. No genotoxic effects on Caco-2 cells and L5178Y/Tk +/- cells were observed at the concentration range selected.

In conclusion, our results evidence that GO has a good toxicity profile for its potential applications in the food packaging industry, but further toxicological tests are required.

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P04-36

Optimization of the Thyroid Receptor, TR β CALUX, Assay: Specific Control Compounds and Appropriate Cell Viability Assays

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Thyroid hormones play a crucial role in metabolism, growth, and neurodevelopment. Their action is mediated by thyroid hormone receptors (THR), a receptor family consisting of different isoforms with tissue-specific expression. Interferences of substances with thyroid hormone binding to the THR is one target for substance-induced thyroid disruption. An established *in vitro* reporter gene assay (TR β CALUX assay system, BDS, Netherlands) was used to screen for potential substances which might affect the THR binding.

The TR β CALUX assay uses human osteoblast (U2OS) cells transfected with a firefly luciferase gene that is under control of a thyroid hormone response element containing promotor. The THR β is constitutively expressed, so the agonist activates luciferase expression via binding to the responsive element. The assay can be used to analyze the agonistic or antagonistic (in the presence of the agonist triiodothyronine, T3) potential of test items. The aim of this study was 1) the evaluation of the reliability and specificity of reference items used in the assay and 2) optimization of the viability assay considering the effects of T3 on cell proliferation.

We identified three compounds as suitable reference items: Diclazuril, a veterinary drug, and tetrabromobisphenol A, a flame retardant, as THR β -antagonist with IC₅₀ values of 3.8x10⁻⁷ and 2.7x10⁻⁷, respectively. Deoxynivalenol, a mycotoxin, that was classified as a potential THR β antagonist (Demaegdt *et al.* 2016), was considered inappropriate based on nonspecific response in the assay. As negative control for the assay system 6-Propylthiouracil, a known inhibitor of deiodinase 1 and thyroid peroxidase, was defined appropriate for the assay.

Moreover, the influence of T3 on different viability readouts was investigated. Two assays that are used to assess cell viability (Promega Realtime-Glo and MTT assay) showed increasing signal intensity in response to higher T3 concentrations. Whether this effect is caused by increased expression of metabolizing enzymes or induction of proliferation is subject of further analysis. But the CellTiter-Glo (Promega) assay system which quantifies the ATP content in the cell as a readout for cell viability was unaffected by addition of T3.

Beyond the existing operation procedure of the TR β CALUX assay, specific antagonistic control items and an appropriate cell viability assay were identified. This improves the quality of the assay and allows a robust assessment of the data.

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P04-37

U-SENSTM: APC can be used as an alternative in case of strong chemical-induced autofluorescence at the FITC-specific wavelengths

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Decades of intensive research have provided Adverse Outcome pathways and New Approach Methodologies (NAM) for animal-free assessment of skin sensitization. The U-SENSTM (OECD Test Guideline 442E), addressing the key event 3 of the skin sensitization has been foreseen to be combined with complementary information in defined approaches in the Next Generation Risk Assessment.

The U-SENSTM is modelling the dendritic cell activation upon exposure to chemicals. Upon contact with sensitizers, U937 human histiocytic lymphoma cells are activated and increased the CD86 expression measured by flow cytometry using a fluorescein isothiocyanate (FITC) CD86 monoclonal antibody. The U-SENSTM assay has been shown to be applicable to a broad range of chemicals (fragrances, dyes, preservatives, UV filter, and non-cosmetics ingredients...). However, strong fluorescent chemicals emitting at the same wavelength as FITC might interfered with the flow cytometric detection and thus cannot be correctly evaluated using FITC-conjugated antibodies.

Therefore, the aim of the present study was to 1) identify other fluorochrome-tagged antibodies, 2) demonstrate similar results as the FITC-tagged antibodies by testing the proficiency substances identified in the OECD TG 442E, and 3) determine the outcome of an interfering FITC-chemical.

As an alternative of the fluorophore FITC (excited at 488 nm), the APC (excited at 633 nm) has been evaluated. The results showed that all acceptance criteria of the test method were fulfilled by using the APC fluorochrome. The 10 OECD proficiency chemicals provided similar results (EC150 values and classification) as the FITC-tagged antibody. An enlarged set of 30 non-to-extreme sensitizers confirmed the relevance of this APC fluorochrome. In addition, the applicability domain of the APC was demonstrated with a flavonoid interfering FITC- chemical.

In conclusion, the use of the APC coupled antibody could be seen as an alternative fluorochrome of the FITC-tagged antibodies enlarging the applicability domain of the U-SENSTM test method.

P04-38

Comparison of the effects of flavone cirsimarin on the proliferation, migration, and invasion of MCF-7 cells grown in 2D monolayer and 3D multicellular tumor spheroids

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Cirsimarin (CIR) (synonym of cirsitakaoside) is a flavone extracted from the aerial parts of different plants, including the medicinal herb *Scoparia dulcis* L. (Plantaginaceae), popularly known in Brazil as "tu-piçaba" and "vassourinha". New ideas on the anticancer activities of flavones and their potential uses in breast cancer therapy suggest cell cycle arrest in the G2/M phase, and apoptosis and autophagy in different breast tumor cells. In the current work we aim to investigate the mechanisms underlying the *in vitro* antitumoral activity of CIR (10 to 320 μ M) on MCF-7 cells cultured in monolayer (2D) and three-dimensional (3D) multicellular tumor spheroids. Resazurin was used to study the effect of CIR on cell viability in MCF-7 cells (2D and 3D).

Antiproliferative effects were evaluated using Clonogenic assay (2D) and growth, morphology, and integrity of 3D spheroids. In 3D culture, reactive oxygen species (ROS) were determined by CM-H₂DCFDA probe and the mechanism of CIR's decreased cell proliferation was investigated using gene real time quantitative polymerase chain reaction (RT-qPCR). CIR (from 40 μM) decreased cell viability and colony formation in MCF-7 cells in 2D models. In the same way, CIR showed cytotoxic effects and decreased spheroid integrity after 16 days with no induction of intracellular reactive species. Through this research, we demonstrated that, when cultured in 3D, MCF-7 cells show greater resistance to cytotoxicity induced by the CIR flavone, when compared to the 2D monolayer cultures. CIR mediated antitumoral effects in 3D spheroids by negative modulation of genes associated with cell proliferation (*CCND1*, *CCNA2*, *CDK2*, *CDK4*, and *TNF*) and death (*BCL-XL*, *BAX*, *CASP9*, and *BIRC5*). *BIRC5* inhibitors have been proposed as versatile anticancer drugs, which makes our results quite interesting. High *CCND1* expression is associated with high proliferation and a threefold higher risk of death in ER-positive breast cancer. CDK inhibitors targeting CDK4 and CDK6 have been approved in hormone receptor-positive breast cancer, and inhibitors targeting other cell-cycle CDKs are currently in clinical trials. Our results of downregulation of the cell cycle regulators and *TNF* after treatment with CIR (40 and 80 μM) show that this flavone can act to decrease the proliferation of MCF-7 cells in the MCTSS and research into the role of cell-cycle dysregulation in breast cancer led to identification of their potential as attractive targets for cancer therapy. The current study provides a valuable overview of the low cytotoxicity and antiproliferative effects of CIR on MCF-7 breast cancer cells grown in 2D and 3D models, and these are relevant properties for long-term strategies to improve the prognosis in breast cancer.

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P04-39

Iterative Data Mining of a Cell Painting Multiparametric Dataset Allows Identification of 'Pre-toxic' Phenotypes

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Many high profile drug withdrawals have been due to the limitations of traditional toxicology assays, which, based on cytotoxic readouts, were not sufficiently predictive of certain forms of toxicity. The advent of high content screening (HCS) created opportunities to measure diverse phenotypic endpoints for toxicology in biological models. HCS allows for the multiplexing of multiple readouts in an *in vitro* format that is amenable to laboratory automation. Such assays have been described for all the commonly investigated forms of toxicity, including, but not limited to, hepatotoxicity, genotoxicity, and nephrotoxicity. HCS yields multifaceted data that provides critical insights into mechanisms of action, as well as compound pharmacology and toxicology. However, these large datasets are often analytically overwhelming. This makes HCS assays challenging for biologists, with little coding or data science experience, to develop and validate. Here we demonstrate how StratoMineR™, our online data analytics platform, when combined with Iterative Data Mining, can be used to rapidly gain greater insights into the performance of a HCS toxicology platform. We used the publicly available LINC Cell Painting data set from a small molecule screen, in which A549 cells were treated with ~1,500 compounds across 26 plates in dose-response. Our goal was to identify pre-cytotoxic phenotypes in compounds with known mechanisms of toxicity. We demonstrate that StratoMineR™ can be used for iterative data mining to evaluate the utility of Cell Painting for generating these 'pre-cytotoxic' phenotypic profiles for hepatotoxic compounds. Removal of low cell count from the hit picking method, based on cell count feature, allowed us to enrich our analysis on 'pre-cytotoxic' samples. In addition, compounds were further annotated for Drug Induced Liver Injury (DILI), which allows us to discover 144 DILI compounds that gave a significant phenotype. Phenotypic distance scores were calculated, and dose-response curves could be generated. We further used the clustering function of StratoMineR™ to carry out hierarchical clustering based on nine principal component scores. Clustering analysis revealed two important insights: 1) substantial numbers of DILI compounds gave 'pre-cytotoxic' phenotypes that are quite diverse, and 2) there are two distinct 'super-clusters', of which one of the clusters is more likely to have low cell count. This process can be repeated by splitting clusters into sub-clusters until a desired resolution is reached. Thus, iterative data mining using StratoMineR™ allows external annotations and/or generation of new knowledge in hit selection and clustering steps, which can further refine DILI compound phenotypic analysis from a Cell Painting data set.

P04-40

Defined approaches to differentiate between the three UN GHS categories for eye hazard identification – overall and class-specific performance metrics

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Over the last decades, significant improvements have been achieved in the context of alternatives to animal testing to assess the safety of

chemicals. To evaluate the performance of these methods, the results are compared with reference data (animal or human) based on a confusion matrix, a cross table reporting predicted against reference classes. In case of a binary response, sensitivity and specificity are widely used statistical measures of performance. In case of a multi-class response (three or more categories), sensitivity and specificity can be calculated based on a one-vs-all approach and provide information on the class-specific performance. While the focus is on the performance of alternatives methods/defined approaches for eye hazard identification, the computations shown here with a numerical example can be easily extended to other domains of hazard identification. Furthermore, they can also be expanded to address class-specific performance metrics in case there are more than 3 hazard categories. Several *in vitro* test methods have been adopted as OECD Test Guidelines (TG) to identify chemicals that cause serious eye damage (Cat.1), or to identify chemicals not requiring classification and labelling for eye irritation or serious eye damage (No Cat.). Usually, overall accuracy, sensitivity, false negatives (FN), specificity, and false positives (FP) of the test methods are calculated based on a 2x2 confusion matrix. Of note, when the distribution of the reference chemicals over the UN GHS categories is imbalanced, accuracy can be misleading whereas balanced accuracy takes into account the imbalances of the dataset. Recently, Cosmetics Europe developed two defined approaches (DAs) for eye hazard identification that distinguish between the three UN GHS categories, including Cat. 2 (eye irritation) [1,2]. The overall performance of the DAs was assessed based on 3x3 confusion matrices. To evaluate the class-specific performance, the 3x3 matrix is converted into three 2x2 matrices and sensitivity (proportion correctly predicted within the class), specificity (proportion correctly predicted outside the class), and balanced accuracy are calculated. This is the first time that this computational approach is illustrated, step by step, for eye hazard identification considering the three UN GHS categories. Both DAs were submitted to support the OECD acceptance.

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P04-41

ALT4EI: Determination of eye irritating potential of 59 chemicals using EpiOcular™ time-to-toxicity neat and dilution protocols

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Determination of the acute eye irritation potential is part of the international regulatory requirements for testing of chemicals. The objective of the ALT4EI (Alternatives for Eye Irritation) project was to confirm the testing strategy developed in the CON4EI (CONsortium for *in vitro* Eye Irritation testing strategy) project. These projects focussed on the development of tiered testing strategies for eye irritation assessment for all drivers of classification and evaluation of

whether the test methods can discriminate chemicals not requiring classification for serious eye damage/eye irritancy (No Category, No Cat) from chemicals requiring classification and labelling for Category 1 (Cat 1) and Category 2 (Cat 2). In the CON4EI project, a new testing strategy for EpiOcular ET-50 was developed, the sensitivity for predicting GHS Cat 1 and GHS Cat 2 chemicals was 73% and 64%, respectively and the very high specificity of 97% was maintained. None of the Cat 1 chemicals was underpredicted as GHS No Cat. The goal of the ALT4EI project was to fill remaining data gaps and strengthen the data set.

A new set of 59 chemicals (41 liquids: (un)diluted, and 18 solids) was tested using the reconstructed human cornea-like epithelium (RhCE), EpiOcular, in two EpiOcular time-to-toxicity tests (Neat and Dilution ET-50 protocols). The set of chemicals contained 32 chemicals not requiring classification (No Cat) and 27 chemicals requiring classification (16 Cat 2 and 11 Cat 1). The chemicals were tested blinded in two independent runs by MatTek *In vitro* Life Science Laboratories. In this study, a testing strategy to achieve optimal prediction for all three classes (No Cat, Cat 1 and Cat 2) that was developed in CON4EI project (which combines the most predictive time-points of both protocols and which tests liquids and solids separately) was used.

Using the CON4EI testing strategy and CON4EI developed prediction models for liquids and solids, we were able to identify correctly 63.6% of the Cat 1 chemicals, 56.6% of the Cat 2, and 76.6% of No Cat chemicals. Reproducibility between both runs was 88.7%. The combination of the EpiOcular time-to-toxicity neat and dilution protocols seem to be promising in an integrated testing strategy (ITS) for eye irritation assessment.

P04-42

Validation and specificity testing of an *in vitro* method assessing substance-induced DIO1 inhibition in human liver microsomes

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Regulatory requirements are increasing to identify endocrine disruptors impacting the thyroid hormone (TH) system [1]. Testing for potential endocrine disruption is predominantly done *in vivo*. To enable broad and reliable *in vitro* testing, predictive, robust, routine non-animal mechanistic methods for the identification of substances affecting the TH system are needed. Multiple *in vitro* methods covering different TH-related mode of actions are in the process of validation by the network of EU laboratories (EU-NETVAL), coordinated by the European Commission Joint Research Centre (JRC) [2].

Deiodinases (DIO) are important, local regulators of TH action by enzymatic activation or inactivation via iodide-release from the hormonal structure. DIO1, one of the three DIO isoforms, serves as one main source for circulating T3 via deiodination of T4 and plays a role in recycling of iodide via deiodination of inactive TH metabolites.

The DIO1-SK *in vitro* method uses pooled human liver microsomes as a globally easily accessible enzyme source for DIO1 and determines substance-induced iodide release inhibition [3]. Released iodide is quantified via colorimetric change in the Sandell-Kolthoff reaction. The method was transferred from the method developer to our laboratory and standardised resulting in a robust Standard Operation Procedure (SOP) compliant with the Guidance Document on Good *In vitro* Method Practices (GIVIMP) [4].

An initial relevance assessment (Part 1) showed robust and consistent dose-dependent decrease of iodide release activity in human liver microsomes for six different known DIO1 inhibitors in five independent assay runs (e.g. IC₅₀ values for the known DIO1 inhibitors: 6-propyl-2-thiouracil = 3.8 µM and aurothioglucose = 0.5 µM). Based on the results, acceptance criteria were derived for an intra-lab validation study (Part 2) to assess predictivity with ≥ 30 blinded substances, selected by an international thyroid and chemical expert group. For the assessment of the specificity of measured DIO1 inhibition, different approaches like secondary enzyme activity testing or increase of substrate concentration are tested for their applicability in the method.

The DIO1-SK inhibition *in vitro* method using human liver microsomes is a robust *in vitro* assay to assess potential DIO1 inhibition of chemicals. Consistency and reproducibility were shown in the relevance assessment (part 1) and reliability and relevance assessment (part 2). The final relevance assessment (part 2) as well as specificity testing will further strengthen the trust in the generated results and the method itself.

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P04-43

Research on the induction of cellular differentiation of osteoblast-like cells using bioceramic culture carriers

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Purpose: International Osteoporosis Foundation estimates that around 200 million people globally suffer from osteoporosis disease, and many preventive and therapeutic measures have been developed for osteoporosis. Safety evaluation of these therapeutic agents *in vitro* is expected to improve the safety of their use. Our research group has developed originally hydroxyapatite (HAp) – β-tricalcium phosphate (TCP) composite carrier as a scaffold for cultured bone. Effect of the scaffold on the cell proliferation and cell differentiation to osteocytes was investigated using human osteoblast-like cells, MG-63. It was investigated whether our cultured bone could be used to evaluate the secureness of the drugs for osteoporosis or not.

Method: Bioceramic carriers with a composition of HAp: β-TCP = 1:1.5 in weight ratio were pressed into disc-shape with a diameter of about φ14 mm under load of 20 kN, followed by sintering at 1200°C for 2h. The carriers were placed in a plate with 24 wells, and MG-63 cells were seeded on the carriers at 5.0×10³ cells/well. After four days from the seeding, they were cultured for 28 days in MEM medium with

and without differentiation factors. After that, it was evaluated the effects of the ceramic carriers and differentiation factors on cell proliferation, cell morphology and bone formation marker expression.

Results: MG-63 cultured for differentiation on the ceramic carriers showed precociously production of collagen and non-collagen proteins. It was also confirmed that an obvious production of fibrous type-I collagen, which elongated along with cellular orientations, and osteocalcin. In addition, scanning electron microscopy revealed that cellular protrusions found in living bone cells were formed on the bioceramics the cultured cells. It indicates that differentiated MG-63 produce similar structures to living bone. These results suggest that the osteoblast culture system using the bioceramic carriers seemed to be useful for investigating the effect of matters on the bone differentiation, and formation. In the near future, we will try to develop the disease model of osteoporosis by using this culture system.

P04-44

Enzymatic defense of HepG2 cells against cytotoxicity induced by T-2 and its modified forms

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The T-2 is a mycotoxin produced by several species of *Fusarium*, which belongs to group A of trichothecenes. This mycotoxin is the most cytotoxic of this group. It is distributed worldwide. It is found in cereals, mainly in oats. The T-2 is rapidly metabolized, and its main metabolites are HT-2, Neosolaniol (Neo), T2-triol and T2-tetraol. All of them have shown high production of oxidative stress in cell cultures. In this work, the enzymatic defense of HepG2 cells against cytotoxicity induced by T-2 and its metabolites was evaluated. The concentration of T-2 and its metabolites selected were IC₅₀/2, IC₅₀/4 and IC₅₀/8 of each mycotoxin. These concentrations were selected in order to stimulate the antioxidant defense system of the HepG2 cells. The cytotoxicity of these mycotoxins was previously determined by the MTT assays. Moreover, cells were pre-treated before T-2 exposure with D-L-buthionine-(S, R)-sulfoximine (BSO) a glutamate-cysteine ligase inhibitor, and N-acetyl-cysteine (NAC) a glutathione (GSH) precursor. The results obtained demonstrated that there is an increase of 21.36% of intracellular GSH at the lowest T-2 concentration (IC₅₀/8), however after pre-treatment with NAC, the GSH increases up to 117.56%. Regarding to its metabolites, the GSH levels are the same as the control, although after NAC pre-treatment they increase up to 11.60%, 19.57% and 48.66% for Neo, T2-triol and T2-Tetraol, respectively. Whereas, as expected, after BSO pre-treatment, GSH levels decreased in a dose dependent manner for all mycotoxins. On the other hand, a decrease of oxidized glutathione (GSSG) level, proportional to the increase in GSH was observed; and the GSH:GSSG ratio was significantly increased in the following order T-2 > T2-tetraol > T2-triol > Neo. Regarding cellular enzymatic defense, the glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase contribute as antioxidant defence mechanisms in HepG2 cells following exposure to T-2 and its metabolites. Alteration of GSH levels was accompanied by induction of GPx and GST activity for all mycotoxins. The highest increase of catalase activity was observed at the highest concentrations of T-2, Neo, T2-Triol and T2-tetraol (IC₅₀/2). Some differences between the metabolites in SOD activity were observed at all the concentrations tested. SOD activity was increased only after T-2, and T2-tetraol exposure. Results suggested that GSH and related enzymes play an important role in antioxidant defense system in HepG2 cells exposed to T-2 and its modified forms.

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P04-45***In vitro* volatile exometabolome signature of clear cell renal cell carcinoma**

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Clear cell renal cell carcinoma (ccRCC) represents the most common and lethal subtype of kidney cancer^[1]. Diagnostic techniques currently used impair its early detection, compromising treatment effectiveness and life expectancy^[2]. The development of more accurate and non-invasive diagnostic strategies based on the detection of ccRCC biomarkers may, therefore, constitute an important tool to overcome these issues. Since the metabolism of cancer cells markedly differs from that of normal cells, the use of metabolomics constitutes one of the most promising approaches for biomarker discovery. Indeed, recent studies have demonstrated the potential of the volatile profile in the discrimination between urine from ccRCC and control individuals^[3–5]. However, urine is a complex matrix that may also reflect alterations not related with the disease state^[6]. Hence, this work aimed to investigate, for the first time to our knowledge, the performance of the volatile profile to discriminate the exometabolome (extracellular culture medium) of non-metastatic (786-O and 769-P) and metastatic (Caki-1) ccRCC cell lines from a non-tumoral cell line (HK-2), by headspace solid-phase microextraction coupled to gas chromatography-mass spectrometry (HS-SPME-GC-MS). Multivariate and univariate statistical analyses disclosed a panel of metabolites that was able to discriminate ccRCC from non-tumoral cells. Similar changes were found in the levels of acetone, 2-ethyl-1-hexanol, formaldehyde and tetradecane (upregulated) and cyclohexanone and acetaldehyde (downregulated) in all ccRCC cell lines compared with the non-tumoral cell line. Regarding the comparison between ccRCC cell lines in different metastatic stages, dysregulations in the levels of alkanes, alkenes and benzene derivatives were the most important for stage differentiation. This study showed that ccRCC cellular volatile profile is a very promising approach to unveil potential candidate biomarkers for ccRCC diagnosis and staging.

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P04-46**Interaction of recombinant human acetylcholinesterase with demeton-S-methyl**

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Some organophosphate compounds are active substances in numerous insecticidal and biocidal products where the main target of these compounds is acetylcholinesterase (AChE). Demeton-S-methyl (DSM) is an organophosphate compound (OP) considered as a good substitute for studying the dermal exposure of other OPs such as VX gas, however, it has presented difficulties in the interpretation of its interaction with AChE when it has been tested in the presence of ethanol.

This work kinetically characterizes successfully the interaction of the DSM compound with recombinant human acetylcholinesterase (hAChE), providing information on the inhibition, spontaneous reactivation and aging reactions. The kinetic parameters estimated were very similar in all the different kind of experiments performed, obtaining an inhibition constant between 0.0325–0.0422 $\mu\text{M}^{-1} \cdot \text{min}^{-1}$, a spontaneous reactivation constant between 0.0107–0.0328 min^{-1} , an aging constant between 0.0043–0.0053 min^{-1} and an I_{50} (30 min) between 0.55–0.71 μM . In the presence of ethanol some alterations of the kinetic constants were found that had to be considered in the interpretation of previously published data.

P04-47**Effects of mycotoxins in Caco-2 cells and HepG2 cells are alleviated by bioactive compounds from goji berries**

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Bioactive compounds (BACs) in natural matrices are compartmented in specific anatomical structures resulting in low bioavailability. Here, the study of bioaccessibility and bioavailability of carotenoids presents in *Lycium barbarum* L. goji berries (GB): zeaxanthine dipalmitate (ZDP), lutein (LUT) and zeaxanthine (ZEAX) using a trans-epithelial model with Caco-2 cells is presented. Among that the potential cytoprotection ZDP against beauvericin (BEA) mycotoxin in HepG2 cells was also carried out. Results revealed that bioaccessibility of carotenoids from digested GB oscillated between 40% and 98% from 1 h to 4 h in differentiated Caco-2 cells for the most bioavailable carotenoids: LUT, ZEAX and ZDP. Bioavailability of standard carotenoids at different concentrations was lower than those from natural digested GB. The order of carotenoids according their bioavailability correspond ZDP>ZEAX>LUT. Cytoprotection of ZDP against beauvericin (BEA) in pretreatment strategy in HepG2 cells increased with the exposure time (from 24h to 48 h) and the dose (BEA 1.25 μM to 2.5 μM); while in simultaneous treatment, this was only maintained for the dose and the opposite was observed for the time.

P04-48**A cautionary note on the utility of MTT in toxicity screening***C. Buckley^{1,2}, I. Major¹, T.R. Montgomery²¹ *Athlone Institute of Technology, Materials Research Institute, Westmeath, Ireland;*² *Athlone Institute of Technology, Biosciences Research Institute, Westmeath, Ireland*

Introduction: The MTT (3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide) assay has long been considered the gold standard cytotoxicity assay due to its sensitivity (Carreño *et al.*, 2021) and noted reliability of cellular metabolic activity in a variety of adherent cell lines. The activity of MTT is reliant on the reduction of MTT to its insoluble formazan by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes (Larramendy and Soloneski, 2018). However, the rate of formation of these crystals is dependent on the metabolic rate and quantity of mitochondria. This study shows that the suitability of MTT must be considered on an individual project by project basis with regard to cell type and potential synergistic drug interactions.

Methods: The effect of low molecular weight (LMW) (30–50kDa) hyaluronic acid (HLA) on neuronal (SH-SY5Y) and glial (RT4 D6P2T) cell lines was investigated using the MTT assay, resazurin reduction (RR) assay and the trypan blue exclusion (TBE) assay. The results were then compared to elucidate the accuracy of each of the bioassays used.

Statistical approach: Statistical analysis was conducted using one-way analysis of variance for statistical significance between a single factor, with Tukey post-hoc tests. A two-way analysis of variance with Sidak's post-hoc test was used where more than one independent factor existed. $P < 0.05$ was considered to represent a statistically significant difference.

Results and conclusions: LMW HLA was significantly cytotoxic when assayed using the MTT assay in both cell lines. These results could not be replicated using RR, which works on a similar method of action to the MTT, or the live/dead TBE bioassays. Through investigation, it was determined that the apparent overestimation of cytotoxicity by the MTT assay was due to enhanced cellular detachment of live cells due to the MTT reagent itself and not mechanical disruption through assay procedure.

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P04-49**Using a comprehensive *in vitro* approach to gain insights into mechanism of mitochondrial toxicity**

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Mitochondrial dysfunction is implicated in numerous drug-induced adverse events e.g. liver/cardiac toxicity. A number of *in vitro* approaches are available to identify compounds with mitochondrial liability, e.g. the Glu/Gal assay. Here we describe assays which can identify potential mechanisms of toxicity. The mitochondrial stress test (XFe assay) measures cellular oxygen consumption rate (OCR), reserve capacity (RC) and extracellular acidification rate (ECAR). This assay can identify mitochondrial uncouplers and inhibitors of the electron transport chain (ETC) or ATP synthase. Additionally, utilising

permeabilised cells and the XFe analyser, individual complexes of the ETC can be identified. Another approach utilises high content imaging to assess changes in mitochondrial membrane potential (MMP) or mitochondrial specific reactive oxygen species (mitoROS). Using these approaches we assessed seventy compounds using the Glu/Gal, the MMP/cytotoxicity, the MitoROS, the XFe and the permeabilised cell assays in HepG2 cells. The XFe assay showed a sensitivity, specificity and accuracy of 78, 100 and 90% respectively, the Glu/Gal assay showed 41, 100 and 73%, the MMP assay was 83, 76 and 79%. Rosiglitazone flagged as positive in the XFe, the MMP/cytotoxicity and the MitoROS assays but not in the Glu/Gal assay. Assessment of positive compounds in permeabilised cells identified compounds with differing mechanisms of action, e.g. rotenone and sodium azide as complex I and IV inhibitors respectively. In summary, determining cellular OCR, RC and ECAR provides a predictive and sensitive measure of mitochondrial toxicity. The MMP assay provides a more sensitive alternative to the Glu/Gal assay, with higher throughput than the XFe assay. The MitoSOX assay provides some mechanistic information although more detailed insight into the mode of action of mitochondrial toxicants was provided using permeabilised cells.

P04-50**An *in vitro* Cell Model for Toxicity Studies and Pigmentation Regulation Utilizing hTERT Immortalized Neonatal Dermal Melanocytes***N. Perera¹, M. Spencer², L. G. Rodriguez², J. Huuskonen²¹ *LGC, Teddington, UK;*² *ATCC Cell Systems, Gaithersburg, USA*

Skin pigmentation is a complex process; melanocytes produce melanin and package it into melanosomes that are in turn exocytosed, then endocytosed by neighboring keratinocytes. Numerous genes play roles in controlling pigmentation at various levels of melanin production. Mutations in these genes are characteristic of multiple skin disorders, including hyperpigmentation, hypopigmentation, and mixed hyper-/hypopigmentation. Additionally, extrinsic factors secreted by the surrounding resident cell types also regulate the melanin expression in melanocytes. Human primary cells can be a useful model for elucidating melanocyte biology and their toxicity to dermal agents. However, primary cells have their limitations such as donor variability and limited lifespan. Consequently, a need exists for a more robust human cell model system for the study of skin pigmentation and agents which damage melanocytes.

In this study, we immortalized primary epidermal neonatal melanocytes by expressing human telomerase reverse transcriptase (hTERT) in cells that were isolated from a neonatal donor. The immortalized primary melanocytes were cultured continuously for more than 35 population doublings without any signs of replicative senescence, yet retained melanin production. The immortalized primary melanocytes maintained a consistent expression of the melanocyte-specific marker TRP-1, and lacked expression of the fibroblast-specific marker TE7. In addition, we demonstrate the capability of these immortalized primary neonatal melanocytes to transfer melanosomes to keratinocytes utilizing a 3D human dermal organotypic culture, and the ability to modulate melanogenesis with depigmentation agents and these agents' effects on cell viability. Taken together, the hTERT immortalized primary neonatal melanocytes described here provide a versatile *in vitro* cell model for the study of toxicity and melanogenesis regulation.

P04-51

Cytotoxic and Antiproliferative Effects of 'Brachydin C' in DU-145 Cells Grown in 3D Multicellular Tumor Spheroids

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The increase in the number of cancer cases in recent years, associated with the need for additional therapeutic strategies, reinforces the importance of studying new compounds for the prevention and treatment of this disease. In this context, phytochemicals are good candidates for cancer therapy because they are low cost and easy to obtain. The compound Brachydin C is a flavonoid extracted from the roots of the species *Fridericia platyphylla* (Cham.) L.G. Lohmann, popularly known as "Cervejinha-do-campo". In the current work, Brachydin C (BrC) was evaluated for cytotoxicity, antiproliferative, and oxidative potential in the human prostate cancer cell line DU145, grown in 3D multicellular tumor spheroids. The spheroids were treated with different concentrations of brachyidin C (5 to 60 µM). After 24, 48, 72, or 96 h of treatment, the cell viability was analyzed (resazurin and LDH assays), and the highest concentrations of BrC significantly reduced cell viability. BrC at 50 µM reduced cell viability in 24, 48, and 96 h, while BrC at 60 µM was cytotoxic in all treatment times. After 96 h of treatment 60 µM of BrC reduced 53% of viable cells. A similar effect was observed in the two assays used, the resazurin and LDH assays. The release of LDH, caused by damage to the plasma membrane, was observed after 72 h of treatment with 60 µM of BrC. Subsequent mechanistic studies revealed that the cytotoxic effect observed after treatment with 60 µM of BrC might be due to the induction of apoptosis through the intrinsic pathway by positive regulation of caspase-7 and BAX. To evaluate a possible effect of the redox state on the decrease in cell viability induced by BrC, the CM-H₂CFDA probe was used, which allows quantification of reactive species through the emission of a fluorescent signal. The results show that none of the tested concentrations was able to change the redox status after 24, 48, or 72 h of treatment. This demonstrates that, probably, cell death is occurring regardless of the induction of reactive species. The effect of BrC on integrity and spheroid size was also observed for 15 days at 48-hour intervals. After day 11, BrC (5, 10, and 20 µM) decreased spheroid growth. A different effect was observed after treatment with BrC (30, 40, 50, and 60 µM) that induced spheroidal disruption, causing an increase in its area before the disintegration of the spheroid. The resazurin assay demonstrated that all BrC concentrations which induced spheroid disruption (30, 40, 50, and 60 µM) reduced cell viability by 42, 53, 86, and 89% respectively. The reduction in viability does not seem to be associated with the induction of reactive oxygen species. Altogether, these results indicate that Brachydin C can induce cell death and interfere with the size and integrity of the tumor spheroids, substantiating further investigations that aim to develop prostate cancer treatments.

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P04-52

Deciphering the influence of physicochemical and microbial parameters of the human digestive tract on orally-ingested microplastics using *in vitro* gut models

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Purpose: The human gastro-intestinal tract is daily exposed to various contaminants, such as microplastics, mainly brought by food items and beverages. These microplastics can be a vector for pollutants, pathogens and/or antibiotics. Up to now, there is scarce data on the fate of virgin or contaminated microplastics in the human digestive tract and how this fate can be impacted by both physicochemical and microbial factors of the gut. Due to obvious ethical issues, such studies cannot be easily performed in humans. Therefore, *in vitro* systems mimicking the human digestive environment represent a powerful platform to decipher the mechanisms of physicochemical and/or microbial transformations and degradation of microplastics, as well as their impact on human gut microbiota and interactions with host epithelial cells.

Methods: Multi-compartmental and dynamic *in vitro* gut models such as the TNO Gastrointestinal model (TIM) and Mucus-Artificial Colon (M-ARCOL) are among the most complete and relevant models compared to the human *in vivo* situation. Based on *in vivo* data, such models reproduce the main physicochemical and microbial parameters of the human digestive tract, such as pH, temperature, transit time, digestive secretions, passive absorption of nutrients and water, oxygenation status, and complex and/or metabolically active lumen and mucus-associated microbiota. These models can be advantageously associated with human intestinal epithelial and/or goblet cells to integrate host-related parameters.

Results: These *in vitro* systems can allow a better understanding of how the successive niches of the gut with various pH, bile, enzymes

and microbial conditions influence the degradation/physico-chemical transformations of microplastics and/or the release/survival of associated contaminants. The models can also give valuable information on the metabolism by the gut microbiota of microplastics and their impact, as well as that of associated contaminants (pollutants, pathogens, antibiotics) on gut microbiota structure and fermentation activity (by measuring gas or fermentation end-products such as short chain fatty acids). By coupling *in vitro* gut models with intestinal epithelial cells in culture, we could also elucidate how gastrointestinal passage influences microplastics and/or associated contaminants toxicity and effect on intestinal barrier integrity. Ultimately, the potential of *in vitro* gut models allows to study the effect of various parameters related to the food matrix ingested with microplastics (water, dairy products, normal or western-diet), age conditions (child, adult or elderly) or pathological situations (e.g. obesity or irritable bowel syndrome). All these data will be helpful to anticipate key outcomes for a better human health risk assessment, especially in at-risk populations.

P04-53

***In vitro* Acute Toxicity of Metal-Based Nanoparticles in Human Trophoblast BeWo b30 Cells**

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Metal nanoparticles (M-NP) are among the most widely used nanomaterials in consumer products available in the market. Thus, human exposure to these nanosized materials is increasing, which raises serious concerns regarding their environmental and human safety. Biological barriers are important lines of defence to xenobiotics, thus expected targets for M-NP. In this regard, special consideration must be given to the placenta that acts as barrier between maternal and the developing fetus. The present study aimed at evaluating *in vitro* toxicity of different M-NP in a human cell model of placental barrier: trophoblastic (BeWo clone b30) epithelial cells. BeWo b30 cells were exposed for 24 h to varied concentrations (0.8–48 µg/cm²) of M-NP of different chemical composition (Au, Ag and TiO₂), primary size (10, 30 and 60 nm), capping (citrate and PEG) and crystal structure (rutile and anatase). *In vitro* toxicity was assessed by determining changes in cell morphology, metabolic activity, plasma membrane integrity, generation of intracellular reactive oxygen species (ROS) and ATP intracellular levels. The tested M-NP can be ranked for their *in vitro* toxicity potential with AgNPs > AuNPs > TiO₂ NPs, being the effects more visible at higher concentrations. No significant changes in cell morphology following exposure to all tested M-NP were detected. The influence of the size in the cytotoxic-induced effects was more evident for AgNP than for AuNP, with the smaller NP causing more toxicity. In addition, PEG-capping effectively attenuated AuNP-induced toxicity. Only cells exposed to AgNP exhibited a significant increase in ROS levels. All tested M-NP significantly increased intracellular ATP levels compared to control cells, with the exception of AuNP 10 nm. Thus, our data support that the physicochemical properties of M-NP are an important determinant of their cytotoxicity in BeWo b30 cells. Future studies will be useful to further explore the effects of M-NP in the placental barrier.

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P04-54

Endocrine disruption of thyroid hormone system – *in vitro* assessment of several modes of action of chemicals and environmental samples

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While endocrine disruption associated with steroid hormones is a relatively well-described phenomenon nowadays, there is less info related to interference of chemicals with thyroid hormone (TH) system. Nevertheless, TH play a crucial role in many vital processes such as metabolic activity or neurodevelopment. Xenobiotics that can interfere with the thyroid hormone (TH) system (Thyroid hormone Disrupting Chemicals; TDCs), have been linked with neurodevelopmental disorders such as attention-deficit hyperactivity disorder (ADHD), learning disabilities and mental retardation in children. This poses an urgent need for the development and implementation of suitable methods for the screening of chemicals as well as environmental pollutant mixtures for their TH-disrupting properties. Unlike in steroid hormone systems, receptor-mediated effects are probably not the most important mode of action of TDCs. TH synthesis, transport, and metabolism steps in TH metabolism are likely more important targets for TDCs. We have developed a set of bioassays focusing on prioritized endpoints in thyroid hormone disruption. The assay for the assessment of iodide uptake by thyroid cells mediated by Na⁺/I⁻ symporter (NIS) is based on stably transfected human cell line overexpressing NIS and the detection of uptaken iodide levels by the cells used a non-radioactive assay based on Sandell-Kolthoff reaction. Transport of TH is addressed by fluorimetric assessment of displacement of TH from its plasma transporter transthyretin (TTR). The TH metabolism step is addressed by a cell model with a reporter gene under transcription control by aryl hydrocarbon receptor (AhR) that plays an important role in triggering the metabolism systems. Interaction with thyroid receptor signaling is assessed using reporter-gene assay. These assays have been used for screening a set of prioritized chemicals and environmental samples from the aquatic environment for their potential to disrupt the thyroid hormone system. The results demonstrate the utility of the newly developed bioassays for high-throughput screening of chemicals as well as environmentally relevant complex pollutant mixtures for the characterization of their thyroid hormone-disrupting potential.

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P04-55

Towards the development of potential dual GSK-3β/BACE-1 inhibitors: a strategy to fight Alzheimer's disease

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Alzheimer's disease (AD) is the most common type of dementia, which affects mainly elder people, a population characterized by the uptake of multiple medications. Several pathological pathways are associated with AD, being the formation of amyloid plaques, as consequence of the aggregation of β -amyloid ($A\beta$) peptide formed by β -secretase (BACE-1), and neurofibrillary tangles, resultant from protein tau hyperphosphorylation by glycogen synthase kinase 3 (GSK-3 β), considered the major hallmarks. Therefore, the creation of dual GSK-3 β /BACE-1 inhibitors will be beneficial once it will act in two pathological pathways, simplifying the regimen treatment and reducing the probability of drug-drug interactions [1,2]. P-glycoprotein (P-gp), an efflux transporter present in blood-brain barrier where plays a major role in brain protection against xenobiotics, is also associated with drug bioavailability reduction, decreasing treatment efficacy. Furthermore, once $A\beta$ peptide is a P-gp substrate, a positive modulation of the pump can also be beneficial for AD treatment, increasing its efflux out of the brain [3]. Herein, we present the synthesis of potential dual GSK-3 β /BACE-1 inhibitors, and *in vitro* evaluation of their putative neuroprotective properties and their direct effect on P-gp efflux activity.

Ten small molecules were obtained through the conjugation of a tricyclic chemical substrate with aliphatic amines resulting in amides which later were reduced to the respective amines, leading to a small library of compounds. Regarding the neuroprotective properties, differentiated SH-SY5Y cells were submitted to chemical-induced cytotoxicity using MPP⁺ and iron (II), in the presence of the referred compounds at the 10 μ M and 25 μ M non-cytotoxic concentrations. Two compounds demonstrated slight but significant neuroprotective effect against MPP⁺-induced cytotoxicity, and the presence of halogens in the structures of the tested compounds proved to be important for the neuroprotection against iron (II)-induced cytotoxicity. Several compounds significantly increased P-gp activity after a short incubation period of 90 minutes and none caused its inhibition. Therefore, $A\beta$ clearance should not be compromised and can ultimately be enhanced using the P-gp activators.

In the future, neuroprotection assays using $A\beta$ peptide as chemical-induced cytotoxicity agent, and the evaluation of the effect of the compounds on GSK-3 β and BACE-1 enzymes will be conducted, expecting to discover a new efficacious treatment to fight AD.

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P04-56

Prevention of allergic contact dermatitis, myth or reality? Avoiding contact allergens: from basic research to development of a new medical device

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Purpose: Allergic contact dermatitis (ACD) is one of the most common skin diseases with a prevalence rate of 15–20% over the general population. This prevalence results in a large cost for the healthcare system, but also greatly affects the quality of life of the affected patients, highlighting the relevance for the development of preventive strategies. These assumptions were the starting point, which aim to uncover the potential of selected molecules to rescue key events involved in the elicitation of the adverse outcome pathway (AOP) for skin sensitization.

Methods: The THP-1 cell line was used and in accordance with the OECD guidelines for skin sensitization assessment, 3 non-animal approaches were performed (maturation of THP-1 cells through up-regulation of the CD54 and CD86 proteins, reactivity of allergens towards cysteine and lysine residues using in-house developed approaches and increase on Nrf2-dependent genes in keratinocytes through RT-PCR) aiming to uncover whether the selected molecules could prevent key molecular events evoked by 2 allergens: 1-Fluoro-2,4-dinitrobenzene (DNFB) and Methylisothiazolinone (MI). A hydrogel was formulated with the most promising molecules (TOX1–TOX4), under a quality by design perspective, taking into consideration its mechanical and rheological profile, safety and effectiveness.

Results: One of the molecules tested (TOX1) inhibited the up-regulation of CD54 evoked by DNFB and MI in 70 and 95%, respectively. The dendritic cell maturation marker CD86 was also inhibited in 49 and 21% after cell culture with the skin allergens DNFB and MI, respectively. *In chemico* studies also reveal that TOX1, TOX2, TOX3 decreased lysine and glutathione depletion evoked by strong allergens. Tested molecules decreased the expression of Nrf2-dependent genes in human keratinocytes. Preliminary results performed in both keratinocytes and *in vitro* reconstructed human epidermis support the absence of cytotoxicity and skin irritation for the formulated hydrogel. We optimize the hydrogel pursuing a quality by design approach, the quality target product profile (QTPP) was firstly defined, this preliminary evaluation allowed to identify potential critical quality attributes (CQAs). Two sequential factorial plannings were then outlined envisioning the design of definition of the hydrogel base, and devoted to optimizing the microstructure after incorporating TOX1 and TOX4. Fenistil gel 1mg/g was considered as reference.

The results obtained highlight two molecules able to chemically sequester skin allergens with the potential to prevent the development of key events involved in the AOP for ACD. We started to conduct some studies in a *in vivo* model of the disease. This is the first step in proof-of-concept that will generate important insights into the preventive value of new medical devices, acting as skin barriers. The successful development of this work might allow the translation of this technology into a marketable/clinical application.

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P04-57

Amphetamine-like stimulants reduce cell viability of human neuroblastoma SH-SY5Y cell line

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Amphetamine-type stimulants (ATS) are designer drugs that stimulate the release of neurotransmitters such as dopamine, noradrenaline, and serotonin and/or inhibit their reuptake^[1]. While "old stimulants", including amphetamine, methamphetamine, and 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) continue to be used, novel designer drugs with similar psychotropic effects (e.g. mephedrone) have been placed on the market^[2]. Research on ATS usually focuses on the mode of action, with limited attention to mechanisms concerning cytotoxic effects.

The aim of our research was to determine viability of human neuroblastoma SH-SY5Y cells exposed to selected ATS. SH-SY5Y cells were exposed to a wide concentration range of ATS (0.039–100 µM) for 24 h and cell viability was determined by the colorimetric MTS assay^[3].

As results indicate, tested ATS caused a significant concentration-dependent decrease in cell viability up to 25% compared to the control. It was observed that MDMA already influenced the viability at the concentration of 0.078 µM. After 24 h exposure, the order of toxic potencies was observed as following: MDMA > methamphetamine > amphetamine > mephedrone.

In conclusion, mephedrone's cytotoxicity appeared to be lower than that of classical ATS. However, further studies are needed to investigate mechanisms of cytotoxicity of tested psychoactive substances in detail.

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P05 – *In vitro* to *in vivo* extrapolation (QIVIVE)

P05-01

This abstract has been withdrawn.

P05-02

The integration of *in vitro* chemical transplacental passage into a generic PBK model for pregnancy

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With the increasing application of cell culture models as primary tools for predicting chemical safety, the quantitative extrapolation of the effective dose from *in vitro* to *in vivo* (QIVIVE) has become increasingly important. For developmental toxicity this requires scaling the *in vitro* observed concentration effect levels to *in vivo* fetal exposure, by integration of *in vivo* kinetics, including information on the transplacental transfer. This transport of substances across the placenta barrier, has been studied here with the use of the BeWo cell system, an *in vitro* model mimicking *in vivo* passage of a chemical from maternal blood across a one-cell barrier into the embryo. Six model compounds with known embryotoxic potential have been applied. The *in vitro* BeWo assay results were thereafter, incorporated as generic QIVIVE of transplacental transport of xenobiotics in Physiologically Based Kinetic (PBK) modelling. For this purpose, an existing PBK model was extended specifically for the rat pregnancy. This included the adaptation of the model to account for maternal body changes during pregnancy, as well as the addition of a specific fetoplacental sub-compartment. The obtained PBK-predictions were compared with *in vitro* developmental toxicity data of the respective chemicals, as taken from the open literature. The BeWo results illustrated different transport profiles of the chemicals across the BeWo monolayer, allocating the substances into two distinct groups: the 'quickly-transported' and the 'slowly-transported'. Exposure PBK-simulations during gestation demonstrated satisfactory kinetic predictions, at least for some of the compounds, when compared to experimentally measured maternal blood and fetal concentrations. Comparisons of the PBK-predicted concentration-response curves with *in vitro* concentration-response curves, advocate either the C_{max} or the $C_{average}$ exposure as the best dose metric for developmental toxicity, depending each time on the chemical. Overall the *in vitro* to *in vivo* comparisons suggest a promising future for the application of such approaches in chemical risk assessment.

P05-03**Study on the effects of 19 perfluoroalkyl substances on gene expression and biokinetics of PFOS and PFOA in human HepaRG liver cells**

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Poly- and perfluoroalkyl substances (PFASs), like perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) have been reported to cause liver toxicity in experimental animals and to disturb lipid homeostasis in experimental animals and humans. To obtain more insight into the cellular effects of PFASs on the human liver, we assessed the effects on gene expression of 19 PFASs in HepaRG cells by performing microarray studies for PFOS and RT-PCR analyses of selected genes for all PFASs. We also assessed the biokinetics of PFOS and PFOA in the *in vitro* model, determining time- and concentration-dependent cell-associated PFAS levels. BMDEExpress analysis of the PFOS microarray data point to various affected processes, with cholesterol biosynthesis (downregulated) and ATF4-related signaling (upregulated) being among the processes with the lowest BMC values. Results from RT-PCR analyses for genes related to ATF4 signaling, cholesterol biosynthesis, PPAR signaling and other sensitive genes point to differences in potencies for the tested PFASs. The shorter chain PFASs (PFPeA, PFHxA, PFBS, HPFO-DA) only impacted on the expression of PPAR-regulated genes. Interestingly, BMC values for different PFASs related to ATF4 activation were correlated with those for decreased expression of the cholesterol genes, suggesting a possible relation between these processes. Of the tested PFASs, HFPO-TA was shown to be the most potent modulator of gene expression. The *in vitro* biokinetic data indicate that at the applied culture conditions maximum cell-associated PFOS and PFOA levels are obtained around 1 hour after exposure, remaining stable up to the end of exposure (24 hours), being up to 10-fold higher for PFOS compared to PFOA, depending on the nominal concentrations applied. Altogether, the study provides mechanistic insights into the effects and relative potencies of PFASs on the human liver, pointing to a possible association between ATF4 signaling and the PFAS-induced decrease in cholesterol gene expression. In combination with *in vitro* biokinetic data, as obtained for PFOS and PFOA in the present study, these results will be used as a basis for quantitative *in vitro* to *in vivo* extrapolations (QIVIVE), with the application of physiologically-based kinetic (PBK) modelling, to assess whether effects found *in vitro* can be expected at relevant *in vivo* exposure scenarios.

P05-04**Including the intestinal microbiome incubations in physiologically based kinetic modeling of pyrrolizidine alkaloid N-oxides**

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The intestinal microbiome is able to affect the susceptibility to a wide range of pharmaceutical and foodborne chemicals through a broad range of reactions. An example is the reduction of food borne pyrrolizidine alkaloid N-oxides to the parent pyrrolizidine alkaloids (PAs) enabling their further bioactivation in the liver to reactive toxic pyrrole metabolites. To include the reactions by the intestinal microbiome in physiologically based kinetic (PBK) models requires

not only definition of a conceptual model that includes an intestinal compartment containing the intestinal microbiota, but also a way to determine the related kinetic constants in an *in vitro* model and to scale the resulting kinetic constants to the whole organism. Using pyrrolizidine N-oxides as the model compounds it was shown that anaerobic fecal incubations provide a way to define kinetic constants for pyrrolizidine alkaloid N-oxidation by the intestinal microbiota of both rats and human. The Vmax values thus obtained require subsequent scaling to the whole organism. This can be done in various ways including i) fitting of PBK model predicted data to available *in vivo* data, ii) based on the fecal fraction of body weight and iii) using the bacterial counts and volumes of the various intestinal compartments. Using the PBK models thus obtained the role of the intestinal microbiota in the bioactivation of pyrrolizidine N-oxides to the parent PAs can be taken into account. The PBK models enable definition of relative potency factors for the N-oxides relative to their parent PAs by comparison of the area under the concentration time curves for the parent PA upon dosing either the N-oxide or an equimolar amount of the parent PA. This reveals that the relative potency of PAs is predicted to be lower than that of the corresponding PA.

P05-05**Identification of Monotonic Trends in the Dose-response Relationship of Nanomaterial Toxicity Data using the R package NMTox**

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Nanomaterials, present in many products nowadays, have different physico-chemical, biological and toxicological properties compared to the same material in the bulk form. Therefore, their potential toxicity needs to be analyzed to ensure their safety for human, animals and the environment. *In vivo* studies are commonly used to assess the risk of chemicals. However, due to the large amounts of nanomaterials that can be produced and for reducing animal testing and financial and time costs, there is a growing interest in incorporating *in vitro* and *in silico* models in the risk assessment process and increasing the focus on *in vitro-in vivo* extrapolation (IVIVE) methods.

To support research on nanomaterial toxicity, including IVIVE, several project initiatives aim to enhance the usability of nanomaterial data by developing databases containing information from various available experimental nanomaterial datasets. The H2020 NanoInformaTIX project is one of these initiatives. Based on data collected over the last decades, this project aims to build a user-friendly platform for risk management of engineered nanomaterials. Our aim is to utilize the data gathered within the NanoInformaTIX project to develop an approach to perform *in vitro-in vivo* extrapolation analyses for nanomaterial risk assessment. In order to select nanomaterials within the vast amounts of data within the NanoInformaTIX database, which have to be analyzed for dose-response trends, we developed a software tool (the R package NMTox) to explore the database and to identify monotonic trends in the dose-response relationship of nanomaterials for toxicity endpoints. In the second stage, dose-response models are fitted (using a software tool that is currently being developed) on the nanomaterials for which a monotonic trend is identified. These nanomaterials/toxicity endpoint combinations will be the focus for *in vitro* to *in vivo* extrapolation.

As a case study, we focus on the cell viability data available within the NanoInformaTIX database. Inference was performed by testing the significance of monotonic dose-response relationships using Likelihood ratio tests. Since a high number of data subsets were test-

ed, a method to adjust for the multiplicity was implemented. The analysis was performed on 14 nanomaterials, across different study providers, cell types, methods and exposure time. A significant monotonic dose-response relationship was found for 20 subsets of the data. Dose-response curves for the cell viability endpoints are fitted and we identify toxic concentrations that will be implemented in the risk assessment process.

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P06 – New approach methodologies: 3D models, stem cells, organ-on-a-chip, microfluidics

P06-01

Development of multiparametric kinetic measurements and analytics workflows for the generation of an iPSC derived cardiomyocyte based cardiovascular model

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Purpose: Unexpected cardiotoxicity underlies high levels of late-stage attrition and post-market withdrawals, accounting for up to 45% of the liability. Typically, cardiotoxic effects of new drugs are predicted using *in vitro* expression of single cardiac ion channels, *in vivo* and *ex vivo* animal studies. However, these models fail to reflect the complexity of the human cardiac microenvironment and are contrary to the principles of the 3Rs (Replacement, Reduction, and Refinement) in animal research. Human pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) have now become an attractive platform for capturing the effects of chronic modulators or toxicants and could complement existing assays to improve cardiac safety assessments. We profiled a panel of 42 compounds with known cardiotoxic effects on monolayer cardiomyocytes however, despite good predictivity a few compounds were missed in this assay. Our research aims to improve this model by taking into consideration the role of non-cardiomyocytes to better recapitulate the *in vivo* myocardial microenvironment. This would lead to a more physiologically relevant model and therefore a more predictive functional and structural cardiovascular toxicity assay.

Methods: Cardiomyocytes, cardiac endothelial cells, and fibroblasts cells were generated from human pluripotent stem cells using established differentiation protocols. The baseline function and structure of hiPSC-CMs were assessed using calcium flux, kinetic live-cell imaging, and fixed cell imaging for sarcomere organisation and cellular morphology. These assays were conducted for both in-house differentiated cardiomyocytes and commercially available iCell cardiomyocytes provided by GSK. 2D co-cultures of cardiomyocytes with fibroblast and endothelial cells were assessed against monoculture cardiomyocytes with regards to cardiac electrophysiology, ion channel expression & function, cellular metabolism, and sarcomere organisation.

Results: Monolayer cardiac differentiation of hiPSC produced high-purity beating cardiomyocytes (>85% alpha-actinin positive). We used CelloPTIQ-based optical imaging to assess contractility and intracellular Ca²⁺ transients. For cardiac nonmyocytes, differential expression of pluripotency, endothelial and fibroblast markers were

seen throughout endothelial and fibroblast lineage specialisation. We further tested and characterised these three cell types individually (e.g. endothelial tubulogenesis assay and cellular metabolism) and examined different conditions for co-cultures. The presence of each cell type within the co-culture models was confirmed at the protein and gene expression level. These co-culture models differed in viability, morphology and pharmacological responses when compared to monolayer cardiomyocytes. These results indicate that co-culture hiPSC-CM models may increase the scope, maturity, and predictivity of this assay.

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P06-02

This abstract has been withdrawn.

P06-03

Kidney-on-a-Chip – Integrating glomerular filtration and tubular reabsorption models

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The kidney's excretory function is crucial in drug development, as it dictates drug clearance and reabsorption. Furthermore, nephrotoxic-

ity of candidate drugs is one of the major reasons for drug attrition. Therefore, an accurate kidney model for Multi-Organ-Chip applications could revolutionize drug trials by providing a relevant *in vitro* platform. Emulating the kidney's distinct functions accurately, however, requires a two-fold approach combining glomerular filtration and tubular reabsorption. The HUMIMIC Chip4 harbors a glomerular and a tubular compartment, which together form the interface between the separate surrogate blood circuit and the excretory circuit. The surrogate blood circuit comprises three additional organ compartments, which could e.g. contain liver and intestine equivalents for ADME studies.

In this study, iPSC-derived glomerular and tubular organ equivalents are generated and co-cultured in the Chip4. Using iPSC-derived organ equivalents allows the creation of an autologous chip, which has major implications for future studies, as it enables the incorporation of an immune system equivalent. The employed podocytes exhibit typical podocyte morphology and express key markers. For the tubular model, kidney organoids are used. These organoids contain mostly proximal and distal tubule epithelial cells and few podocytes, endothelial and interstitial cells, as confirmed by immunohistochemistry staining and gene expression analysis. When dissociated and seeded onto a permeable membrane, the cells form a barrier. The developed glomerular and tubular models can be co-cultured within the HUMIMIC Chip4 for several weeks whilst maintaining their morphology and marker expression.

Taken all together, the developed kidney-on-a-chip constitutes a potent tool for advanced *in vitro* drug trials. It is designed to generate high-quality *in vitro* data predictive of renal drug clearance, reabsorption and nephrotoxicity in humans.

The HUMIMIC Chip4 was developed under EU-H2020 "EU-ToxRisk"-Flagship project, Grant Agreement No 681002.

P06-04

3D Human Vasculature-on-a-chip: Biological impact of combustible cigarette smoke and heated tobacco product vapor on 3D reconstituted vessels comprising coronary artery endothelial cells

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Purpose: Cigarette smoke is a known risk factor for atherosclerosis, which is associated with endothelial dysfunction. Vascular endothelial cells normally form a physical barrier separating the vessel walls from the lumen through which blood flows. However, dysfunctional changes in the vascular endothelium lead to higher penetration of low-density lipoproteins and immune cells, giving rise to atherosclerotic plaques. Although heated tobacco products hold promise to reduce the risk of atherosclerosis, the true cardiovascular impacts are not yet fully understood. To compare the biological effects of cigarette and heated tobacco products on the vascular endothelium in a physiologically relevant situation, we performed experiments on an *in vitro* microfluidic platform comprising tubular structures and mimicking blood flow through vessels. **Methods:** The total particulate matter from reference combustible cigarette 1R6F smoke (CS) and aerosol collected mass from our indirectly heated novel tobacco vapor product (NTV) vapor were collected on a Cambridge filter pad, dissolved in dimethyl sulfoxide, and added to culture medium at concentrations ranging from 100 to 400 µg/mL and 200 to 1000 µg/mL, respectively. Human primary coronary artery endothelial cells were cultured on two-lane OrganoPlates®, a three-dimensional (3D) microfluidic cell culture platform, until tube-like structures formed. Fluorescence-based oxidative stress assays, dihydroethidium (2 h exposure) and monochlorobimane (24 h exposure), were performed

to measure the increase in intracellular reactive oxygen species (ROS) and decrease in glutathione (GSH), respectively. Cytotoxicity was evaluated with a WST-8 assay, and tissue barrier integrity was assessed by monitoring the leakage of fluorescent dye from the lumen to subendothelial compartment (24 h exposure). **Results:** The tubules exposed to CS exhibited dose-dependent increases in ROS production and loss of GSH, suggesting that CS exposure induced oxidative stress. Moreover, high levels of CS exposure induced dose-dependent cytotoxicity and reduced the tissue barrier integrity, suggesting that CS disrupts endothelial barrier function. By comparison, the biological impacts associated with NTV vapor exposure at the highest concentration (1000 µg/mL) were lower than those associated with CS exposure at any concentration. **Conclusion:** The 3D vascular model recapitulated several key features of CS exposure-induced endothelial dysfunction. Notably, the biological and functional changes induced by CS were more significant than those induced by higher concentrations of NTV vapor. Since the current study attempted to assess a subset of the biological events that are associated with endothelial dysfunction, a reasonable next step for understanding the potential risk of heated tobacco products is to investigate other major key effects such as the impact on immune cell adhesion.

P06-05

Human proximal tubule kidney barrier for toxicity testing under static and perfusion conditions

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Introduction: The proximal tubule of the kidney consists of epithelial cells (RPTEC) that are involved in secretion and reabsorption of molecules, drugs, and metabolites via active transporters. These cells are common targets for toxicity. RPTECs form a tight barrier and are ciliated and constantly exposed to flow. *Vitrofluid* is a multicompartamental microphysiological system for perfused cultures which can accommodate one or several tissues. Here, we set off to characterize RPTECs maintained under static or dynamic culture conditions in terms of cell behavior and response to toxicants.

Materials and methods: Primary human RPTECs were cultured on collagen-coated polycarbonate membranes and maintained over 14 days under static (transwell) or flow (*Vitrofluid*, Philip Morris International, Neuchâtel, Switzerland; flow rate of 150 µL/min) conditions. The formation of a leak-tight barrier was demonstrated by immunostaining (zonula occludens 1: ZO-1) and the apparent permeability of Lucifer Yellow. Gene expression of specific transporters (SLC22a2, SLC22a6, LRP2, hOAT-1, CUBN, and AQP1) and albumin uptake were also determined. The morphology and orientation of cilia were investigated by immunostaining (acetylated tubulin). The effect of several nephrotoxicants (colistin, tenofovir, benzo[α]pyrene, cadmium chloride, ochratoxin A, and methylmercury II chloride) on RPTECs exposed for 48 h was evaluated by viability assays (ATP content) and cell proliferation (Ki-67 immunostaining).

Results: The human kidney barrier model developed with human primary RPTECs under both static and dynamic conditions was viable and functional for 14 days. The cells formed an integral barrier characterized by tight junctions and low permeability. The RPTEC barrier retained the ability to reabsorb albumin and expressed several proximal tubule transporters (SLC22a2, SLC22a6, CUBN, and AQP1) but did not express LRP2 or hOAT1. Shear stress did not have any

damaging effects, as assessed by barrier integrity and expression of transporters. However, continuous medium flow led to a marked change in the morphology of the cilia, which became significantly longer and aligned with the flow. RPTECs in static conditions responded to most test compounds with an expected decrease in cell viability. As an exception, tenofovir did not cause cytotoxicity, as it requires uptake via hOAT-1. Furthermore, studies with colistin under static or flow conditions showed that RPTECs grown in both systems exhibited a similar dose–response behavior to the compound.

P06-06

An *in vitro* lung-on-chip model of *Streptococcus pneumoniae* infection as a preclinical efficacy tool

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Purpose: Community Acquired Pneumonia (CAP) accounts for approximately 50–60% of all Acute Respiratory Distress Syndrome (ARDS) cases. *Streptococcus pneumoniae* is the main cause of bacterial-induced CAP. The current standard of care for ARDS is mechanical ventilation and there are no approved pharmacological therapies. Given the complexity of the disease, *in vivo* *S. pneumoniae* infection models are difficult to establish and relevant *in vitro* models to accurately mimic key features of respiratory bacterial infection are required.

We aimed to develop an advanced, physiologically relevant *in vitro* model of *S. pneumoniae* infection which could be used to screen relevant drug candidates in a preclinical setting.

Methods: A model of the alveolar barrier was developed using the AlveoliX lung-on-chip (LoC) system which reproduces two key features of the alveolus: the breathing motion (*i.e.* 3D cyclic strain) and the ultrathin basement membrane (*i.e.* porous membrane). Dynamic co-cultures of primary human alveolar epithelial cells and primary human lung microvascular endothelial cells were grown on the apical and basal side of the chip, respectively, until a tight barrier was formed. The co-cultures were infected with different dilutions of *S. pneumoniae* (ATCC #49619). Six hours post-infection, barrier integrity, cytotoxicity and proinflammatory cytokine levels were assessed.

Results: Infection with *S. pneumoniae* led to a reduction in barrier integrity in the alveolar model after six hours. This was associated with reduced tight junction staining in the epithelial cell layer and the appearance of gaps in the endothelial cell layer, as observed by laser scanning confocal microscopy. These effects were accompanied by a dose-dependent increase in cytotoxicity, as measured by LDH release into the basal supernatant, and by proinflammatory cytokine and chemokine release (IL-6, IL-8, MCP-1, RANTES).

This advanced alveolar co-culture infection model in the lung-on-chip system shows a robust response to infection with *S. pneumoniae* and may serve as a tool in preclinical efficacy studies and lead candidate selection.

P06-07

Contact-free cell-cell communication can elicit fibrotic responses in liver cell lines

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Background: The development of an *in vitro* model of the liver and its pathologies has made great progress in recent years. However, it is still difficult to transfer the *in vitro* data to humans. Here, we aim to develop a suitable model that can accurately reproduce the multi-cellular processes leading to liver fibrosis. The model is based on the three major cellular players in liver fibrosis: hepatocytes, Kupffer cells and hepatic stellate cells, identified as drivers in the liver fibrosis adverse outcome pathway (AOP). We set off to investigate if solely soluble factors or direct cell-cell contacts are involved in promoting the sequence of events that ultimately lead to liver fibrosis.

Methods: Human cell lines representing hepatocytes (HepaRG), Kupffer cells (THP-1) and hepatic stellate cells (hTERT-HSC) were cultured individually either in 2D or 3D. HepaRG were exposed to profibrotic compounds (TGF- β 1, MTX, TAA) for 72 hours, whereas THP-1 and hTERT-HSC were exposed to supernatant collected from HepaRG or conditioned-medium treated THP-1, respectively. The response of the different cell types was evaluated using several endpoints such as viability assays (ATP content), albumin production, glutathione content, secretion of TGF- β 1 and expression of profibrotic markers (*e.g.* α SMA). Dose responses were analyzed using mathematical models.

Results: The sequence of events could be reproduced using conditioned medium. Albumin expression and GSH/GSSG ratio decreased in HepaRG exposed to TGF- β 1. Activation of THP-1 through TAA- or MTX-conditioned medium (from HepaRGs) led to an increase in TGF- β 1 secretion. Increased α SMA deposition in TGF- β 1-, TAA- or MTX-conditioned medium (from conditioned medium treated THP-1) indicated activation of hTERT-HSC.

Conclusion: Our preliminary experiments demonstrate that cell-cell interactions mediated by soluble factors trigger key events of the liver fibrosis AOP: damage of hepatocytes, activation of macrophages, and activation of hepatic stellate cells did not require direct cell-cell contact. Quantification of the responses using appropriate mathematical models will be basis for a more quantitative approach for the study of AOPs.

P06-08

A microfluidic thyroid-liver platform to evaluate species-specific dysregulations of the thyroid hormone axis

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Thyroid hormones (TH) are essential regulators of the development and metabolism in mammals. In recent decades, more and more chemicals have been retrospectively identified as having endocrine-disruptive potential. However, these data are mainly based on rodent studies. Given significant differences in rat and human TH metabolism and the known sensitivity of rat liver enzymes to xenobiotic nuclear receptor activations, the relevance of these findings for human risk assessment is questioned. Accordingly, the distinction of rat and human-specific effects remains a central challenge in the development of drugs or agrochemicals. For agrochemicals, regulatory authorities increasingly request comparative *in vitro* studies. Therefore, the need for new test methods is growing. Currently, no validated *in vitro* assays exist which allow concomitant investigation into direct thyroid-related (impaired TH synthesis) and indirect liver-mediated impairments of the TH axis (influences on TH catabolism).

For this purpose, we have developed both a rat and human test platform which enables these investigations by co-cultivating thyroid and liver organoids in a perfused organ system. The three di-

mensional thyroid models derived from human and rat tissue proved *in vivo*-like follicular architecture while demonstrating high physiological functionality. They responded to the thyroid-stimulating hormone with a significantly increased TH synthesis. This ultimately enabled the reproduction of direct thyroid-toxic effects, as the treatment with methimazole significantly reduced TH secretion. The liver models were generated by the self-assembly of human HepaRG cells and primary rat hepatocytes. This complex structure showed stability of the hepatic phenotype over 21 days (maintained albumin secretion, formation of a bile canalicular network). The models demonstrated an active TH catabolism via the formation of glucuronidated and sulfated thyroxine (gT4/sT4). Applying prototypical phase I and II enzyme inducers, both liver models demonstrated species-specific nuclear receptor activations via the induction of cytochrome P450 enzymes. With regard to alterations of the TH catabolism, the HepaRG model demonstrated a much higher resistance than the rat model. Only the latter responded to nuclear receptor activations via increased gT4 formation. Having established physiologically relevant single-organ-chip cultures over 21-days, their functional reproduction was demonstrated in a co-culture over the same period.

Compared to existing *in vitro* assays, the developed two-organ models offer the advantage of covering multiple steps of the published adverse outcome pathways for chemical TH disruption while allowing the assessment of species differences. Although validation work is still required, we consider the model to be a major contribution to improved risk assessment.

P06-09

This abstract has been withdrawn.

P06-10

Investigation of the interplay between oxidative stress and antioxidant defense in 3-dimensional primary human liver microtissues

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Background: Reactive oxygen species (ROS) have been implicated in the pathogenesis of numerous diseases as well as hepatotoxicity. Menadione, a synthetic analog of vitamin K (K3), is a redox cycling quinone known for causing oxidative stress. N-acetyl cysteine (NAC), a precursor for glutathione (GSH) synthesis, plays a pivotal role in protecting cells from ROS. Here, we investigated the relationship between menadione-induced toxicity and antioxidant response in 3D primary human liver microtissues (MTs).

Methods: 3D InSight™ Human Liver Microtissues (InSphero AG, Schlieren, Switzerland) consisting of primary hepatocytes and non-parenchymal cells were used as a reliable model for hepatotoxicity studies. The MTs were pre-incubated with medium (control) or 250 µM NAC for 4 h to boost antioxidant response. MTs were subsequently treated for 1.5 h, 4 h or 20 h with 12.5 µM menadione (with or without NAC co-incubation). The response to the different treatments was evaluated using viability assay (ATP content), GSH content, expression of genes involved in antioxidant response (qPCR), and superoxide formation (MitoSOX™ staining).

Results: Menadione induced toxicity in MTs with an EC50 of approximately 11 µM; cytotoxicity was completely prevented by NAC. Time-course experiments showed that menadione alone induced a significant and time-dependent GSH depletion (1.5 h, 4 h, 20 h) while

cytotoxic effects shown by ATP depletion and superoxide formation were only detectable after 20 h of incubation. A significant upregulation of Nrf2-regulated genes (HMOX1, NQO1, SOD1, and TXN) was observed after 20 h incubation with menadione alone, but not in presence of NAC. Moreover, heme oxygenase 1 (HMOX1) was transcriptionally induced in a time-dependent manner.

Conclusion: Oxidative stress caused by menadione led to immediate cellular GSH depletion followed by superoxide formation and cell death. Effects on GSH-content were observed already at 1.5 h, whereas increased superoxide concentration was seen only after 20 h incubation. Consistent with the early onset response, menadione treatment triggered the Nrf2 antioxidant defense pathway, with first gene expression changes detectable as early as 1.5 h after treatment. Our experiments demonstrate for the first time in primary human liver MTs that menadione-induced toxicity can be completely prevented by boosting GSH content with NAC. Overall, our results show that primary human liver MTs are a suitable model to study ROS-related hepatotoxicity and to elucidate the kinetic of antioxidative responses in complex, multicellular systems.

P06-11

Human induced pluripotent stem cell (hiPSC)-derived neural progenitor cells as brain region-specific models for neurotoxicity testing

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Animal studies are the current gold standard for neurotoxicity (NT) testing. However, high costs and low testing throughput hamper their applications for a large number of chemicals. In addition, high number of animals and possible species differences pose ethical concerns and question their predictivity for humans, respectively. To overcome the data gaps produced by low-throughput *in vivo* NT evaluation, alternative *in vitro* methods are currently developed which are based on human stem cells and allow a higher testing throughput. A prerequisite for their predictive value is the thorough characterization of the cell model and a clear definition of their applicability domain.

For this purpose, we characterized three different 2D neural induction protocols for the generation of human induced neural progenitor cells (hiNPCs) from human induced pluripotent stem cells (hiPSCs) and compared two media for the subsequent neural differentiation. All protocols featured dual inhibition of the SMAD signaling pathway, yet two of them were modified from published protocols, while one was a commercially available kit (Stemcell Technologies™). After the 2D neural induction, single cells were transformed into 3D neurospheres and pre-differentiated by cultivation on a shaking platform. The emergence and functionality of neuronal networks from the pre-differentiated hiNPCs was measured using microelectrode arrays (MEAs). The cell models were characterized at different stages of the protocols for stemness and neural marker expression using FACS analysis, qPCR, and immunocytochemistry and for response to chemical exposure on MEAs.

All induction protocols generated hiNPCs expressing the proliferation marker Ki-67 and NPC markers Nestin and Pax6 while losing expression of the stemness marker Oct 3/4. However, although all hiNPCs differentiated into neuronal networks consisting of MAP2-positive neurons and S100β-positive astrocytes, they differed in expression of specific neuronal subtype markers and synaptic markers. Consistent with that, the protocols generated hiNPCs with distinct transcriptional signatures of specific brain regions, i.e. forebrain (FOXG1), midbrain (LMX1A) or midbrain (EN1)/hindbrain (HOXA2). Moreover, assessment of the electrical activity revealed differences

in the functionality of the neuronal networks depending on the induction protocol and differentiation medium. In summary, the different protocols generated hiNPCs-derived neuronal networks featuring distinct spatial brain characteristics with specific cell type compositions. Hence, our generated hiNPC models represent useful tools to study neurotoxicity in a brain region-specific manner.

P06-12

Transcriptomic approach to improve the understanding of doxorubicin induced intestinal toxicity in 3D intestinal organoid models

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Doxorubicin is a frequently used pharmaceutical for the treatment of several cancers, even though side effects in different tissues can be severe. In the intestines, symptoms such as diarrhoea and abdominal pain caused by intestinal inflammation and damage can lead to the interruption of chemotherapy. The understanding of the molecular mechanisms underlying doxorubicin-induced intestinal toxicity has been poorly explored. Therefore, this study aims at investigating the molecular mechanisms that lead to intestinal toxicity by exposing well-established 3D colon and small intestine (SI) organoids to different concentrations of doxorubicin, and duration of exposure. The selected *in vitro* concentrations of doxorubicin were based on physiologically based pharmacokinetic (PBPK) modelling of treatment regimens for cancer patients and were ranging between 1 μM and 60 μM. Following exposure, cell viability, apoptosis and cell structural features were assessed as functional endpoints, as well as gene expression profiles by performing RNA sequencing. Based on transcriptomic analysis of differentially expressed genes (cut-off values for fold change (FC) < 0.01, adjusted p-value < 0.05 and q-value < 0.05), cell cycle, p53 signalling pathway and oxidative stress were identified as the most relevant molecular pathways. Additionally, significant differences between colon and SI organoids were also evident, particularly in important metabolic pathways, including ATP synthesis and glycolysis. Short time-series expression miner (STEM) was used to further explore changes in gene expression across time, which demonstrated distinct tissue responses. In colon, pathways that stood out were p53 signalling pathway, energy generation metabolic pathways, including glycolysis and metabolism of pyruvate, and metabolism of β-alanine and histidine. In turn, one carbon metabolism mediated by folate, metabolism of pyrimidine and sphingolipids, HIF-1 and AMPK signalling pathways were observed in SI. Overall, this study provides not only new insights into doxorubicin-induced effects at the gene expression levels in the intestinal tissues, but also highlights the potential relevance of human organoid models in predicting drug-induced damage in human intestines.

P06-13

The oxygen tension during embryonic stem cell culture preceding differentiation influences chemical sensitivity in the embryonic stem cell test (EST)

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An embryo's physiological oxygen (O₂) level ranges between 2.4% and 8%. The cardiac differentiation embryonic stem cell test (EST) is an *in vitro* model for developmental toxicity predictions and is usually performed under atmospheric 20% O₂. We studied the chemical sensitivity of EST carried out under 20% O₂, using embryonic stem cells (ESCs) cultured under either 20% O₂ or 5% O₂ tension. The viability of ESCs was more sensitive to valproic acid (VPA) but less sensitive to FLU when cultured under 5% versus 20% O₂. However, during EST differentiation, lower ID₅₀ values were found for FLU and VPA using ESCs that had been cultured under 5% versus 20% O₂ tension. Differentiation effects included higher gene expression levels of the pluripotency marker *Pou5f1* using ESCs from 5% O₂ instead of 20% O₂ cultures. An effect that persisted after exposure to FLU or VPA. At differentiation day 4, gene expression values were primarily driven by the level of O₂ during ESC culture instead of exposure to FLU. In addition, using ESCs cultured under 5% O₂ tension, VPA enhanced the expression of ectodermal marker *Nes*. The mesodermal marker *Bmp4* was enhanced by VPA only when using ESCs cultured under 20% O₂. At differentiation day 10, using ESCs cultured under 5% instead of 20% O₂, differentiation showed a lower sensitivity of the cardiomyocyte differentiation markers *Nkx2.5* and *Myh6* after exposure to FLU or VPA. These results show that the O₂ tension used in ESC culture influences chemical sensitivity in subsequent use in EST. In general, these observations enhance awareness that standard culture conditions affect the outcome of *in vitro* assays, which may impact their use in quantitative hazard assessment of chemicals.

P06-14

"Worm-on-Chip" technology as a new alternative for early toxicity studies

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Purpose: Currently existing biological testing strategies still involve an extensive animal experimentation in vertebrate models, which is expensive and is associated with a number of important ethical concerns and regulatory constraints. The alternative methods to animal testing are typically based on cellular models. The main limitation of these *in vitro* techniques is that they cannot predict complex responses at the level of an organism, usually involving a multi-organ cross-talk. *Caenorhabditis elegans* (*C. elegans*) has been known for more than 60 years as a powerful model organism in fundamental research, allowing exploration of different facets of aging, development, neurosciences and genomics. This nematode gained popularity amongst the scientific community due to its small size, short life cycle, ease of cultivation and propagation and a powerful genetic toolkit. Despite their relative simplicity, nematodes present a wide range of behaviors and possess well-defined tissues. Nowadays *C. elegans* starts to get recognition as a valuable alternative for rodent and cell models in predictive toxicology studies. However, experimentation in *C. elegans* is still mainly based on manual labor and requires a specific skill-set from the person manipulating the worms. These testing strategies relying on manual handling techniques and direct observation by the operator hence lack reproducibility and standardization, overall largely limiting the potential of the worms for high-throughput and high content screenings required for toxicology studies.

Methods: Nagi Bioscience SA developed a microfluidic-based platform for automated long-term worm culture and phenotyping. By integrating a microfluidic chip within a system for automated multiplexed liquid delivery and large-scale image acquisition we are capable to perform high-content phenotypic analysis of *C. elegans*. The imaging potential is further extended by a possibility to acquire fluorescent pictures. Finally, an integrated incubator guarantees a fine-tuned temperature control for the whole duration of the ex-

periment. Overall, our platform offers the possibility of data acquisition on up to 64 conditions in parallel.

Results: Using this platform, we successfully demonstrated the execution of different types of toxicological studies on *C. elegans*, including the assessments of reproductive toxicity, developmental toxicity, acute toxicity and embryotoxicity. We moreover applied these new test methods to the characterization and safety assessment of different classes of substances, comprising small molecules, nanoparticles, anthelmintics or food additives in worms. Finally, the possibility of performing fluorescent imaging permits to profit from the large existing collection of *C. elegans* reporter strains: in this manner a user can collect additional information on the underlying molecular mechanisms by monitoring the actors of different stress pathways.

P06-15

Intestinal organoids as *in vitro* model system to assess safety and ADME properties of compounds

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Introduction: Despite advances made in drug development, safety, absorption, distribution, metabolism, and excretion (ADME) studies are often performed using traditional models such as cell lines, primary tissues, or animals. However, these models poorly mimic human physiology leading to adverse effects in clinical trials and compounds' attrition. HUB Organoids™ are derived from adult stem cells from epithelial tissues including, but not limited to, the gastrointestinal tract. These organoid cultures have long-term expansion capacity, are genetically and phenotypically stable, and retain biological and functional properties of the original tissue. The aim of this study was to evaluate human and animal intestinal organoids as an *in vitro* model for predicting pre-clinical safety.

Methods and results: Metabolic activity of human colonic and duodenal organoids was assessed by incubating HUB Organoids with substrates of Phase I and II enzymes. Metabolite formation detected by mass spectrometry showed, in both colon and duodenum organoids, metabolic activity for most Phase I enzymes, except CYP2J2 and CES2 with high activity, was low but detectable. The main phase II metabolizing enzymes were detected with high activity.

To assess permeability and transport, epithelial monolayers derived from human duodenal organoids were incubated with test compounds. Bi-directional transport from apical to the basolateral compartment and vice versa was determined. As expected by comparison to human bioavailability data, atenolol was shown to be a low and propranolol, a high permeable compound. Transport of digoxin (p-Glycoprotein (P-gp)-substrate), and estrone-3-sulfate (Breast cancer resistant protein (BCRP)-substrate), in presence and absence of selective inhibitors of their respective efflux transporters, showed functional activity of the P-gp, and BCRP transporters.

For Gastrointestinal toxicity (GIT) assays, human, rat, and dog colonic and duodenal organoids were seeded and treated with compounds having positive and negative GIT profiles followed by a cell viability assay. The toxicity data indicate that HUB Organoids can predict GIT comparable with clinical and pre-clinical *in vivo* data.

Conclusion: Pre-clinical toxicity, metabolism, transport, and permeability assessment of compounds using HUB Organoids reduce costly

traditional animal experiments and provide a quick indication of drug safety, allowing decision making at early stages of drug development.

P06-16

Human Precision-cut Lung Slices: Development of a cryopreservation strategy for “on-demand” use

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Of the most relevant 3-dimensional human lung models, precision-cut lung slices (hPCLS) offer native architecture and a large complement of cell types, including immune competent cells that are known to drive inflammation and sensitization pathways. However, the sporadic availability of donor lung tissue remains an issue and hampers experimental throughput.

We describe the sequential evaluation and improvements that have allowed the successful cryopreservation of hPCLS. Several human donor lungs were obtained sequentially and the cryopreservation technique was progressively evaluated for 1) optimal freezing buffer and day of freezing, 2) viability in culture post-thaw, 3) comparison of submerged and ALI cultures, and 4) comparison of two culture media formulations. Evaluations included the WST-8 viability assay and cytomorphological evaluation following culture (up to 21 days), post-liquid nitrogen storage and thaw.

Results indicate that overnight culture prior to freezing yields hPCLS of lower overall viability (post-thaw) for all buffer formulations tested. Both submerged and ALI cultures maintained viability, but submerged cultures showed a trend of decline after day 11-14. E199 based media groups typically showed higher WST-8 viability at earlier post-thaw days prior to a decline while DMEM/F12 groups appeared stable through day 21.

Recent success in cryopreservation of hPCLS has created the potential to overcome sporadic lung availability and makes this test system more accessible to many laboratories. The potential for access to banked cryopreserved hPCLS of different disease states, repeat testing with the same donor, and testing “on demand” has been realized with these newly developed methodologies at IIVS.

P06-17

Research on the evaluation of hepatic toxicity using hepatocytes in 3D culture with bile excretion function

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Purpose: In the research and development of pharmaceuticals, it is important to evaluate the pharmacokinetics of candidate compounds *in vitro* and *in vivo* in order to confirm their efficacy and safety. The excretion of drugs from the body is particularly important because it has a significant impact on pharmacological effects and the occurrence of side effects. Bile excretion is one of the major drug elimination pathways, and many drugs are excreted in bile. MRP2 and BSEP, which are transporters expressed in the liver, play a role in this series of processes. Based on the above, there is currently a need to develop a culture system that reflects the *in vivo* uptake and bile excretion of candidate compounds in the liver.

In this study, we used HepG2 cells (expressing MRP2) and PLC/PRF/5 cells (expressing BSEP), which were both liver cancer cell lines, as model cells for human hepatocytes. We also used Cellbed™, which

was 3D culture carrier made of high-purity silica fibers, to develop a new culture system that can serve as a model for bile excretion of drugs in the liver.

Method: HepG2 cells and PLC/PRF/5 cells were cultivated for 7 days using Cellbed (Japan Vilene Co., Ltd.) along with the cells cultured in monolayer. Immunostaining for MRP2 in HepG2 cells and BSEP in PLC/PRF/5 cells was performed, and observed by using laser confocal microscope. We also examined the function of MRP2 in HepG2 cells and BSEP in PLC/PRF/5 cells using 5(6)-Carboxy-2',7'-dichlorofluorescein diacetate (CDFDA), which is widely used as a fluorescent bile model compound for MRP2, and Tauro-nor-THCA-24-DBD, a fluorescent bile acid model compound for BSEP, respectively.

Result: It was confirmed by immunostaining that the expression of bile excretion transporters of HepG2 and PLC/PRF/5 cells cultured in Cellbed increased compared to those of monolayer culture. After uptaking the model compounds into the cells, the intracellular fluorescence of the model compounds decreased with time. These results indicated that these bile excretion transporters of both cells were functional in the 3D culture system using Cellbed. Therefore, the 3D culture system is expected to be useful as a pharmacokinetic evaluation to test the safety of candidate compounds for drug discovery.

P06-18

Evaluation of liver fibrosis by human hepatic stellate cell spheroids

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Purpose: Nonalcoholic steatohepatitis (NASH) is a cause of hepatic fibrosis and the pathogenesis of the disease becomes serious from liver cirrhosis to liver cancer. Therefore, it is important to investigate the mechanism of liver fibrosis, because it may be an indication of liver toxicity. Liver fibrosis is associated with the accumulation of extracellular matrix (ECM) proteins, which are produced by hepatic stellate cells. Hepatic stellate cells are in quiescence state in healthy conditions, but become activated during steatohepatitis and start to produce ECM. No culture method has been established to reproduce this change *in vitro*, since hepatic stellate cells quickly shift to the activated state in normal culture. We have already reported that culturing hepatic stellate cells in spheroids leads to their deactivation. So, in this study, we investigated the degree of activation of hepatic stellate cells in order to construct an evaluation system for activation in pathological changes.

Methods: Human hepatic stellate cell line LI90 or human hepatic stellate cells (HSC) isolated from human liver were cultured on a spheroid-forming culture plate EZSPHERE (AGC) to form spheroids. Spheroids were cultured in normal culture plates as adherent culture or low attachment culture plates as non-adherent culture. Activation was assessed by morphology and filamentous actin (F-actin) formation by phalloidin staining. Expressions of activation marker genes were measured by quantitative PCR. In addition, we evaluated the activation of spheroids by TGF- β stimulation in each culture condition.

Result & Discussions: Spheroid-forming LI90 cells and HSC showed reduced expressions of activation marker genes. When spheroids were cultured in adherent culture, elongated cells and F-actin were observed, and the expression of activation marker genes increased. These results indicated a change to an activation phenotype from deactivated one. Moreover, their stimulation of TGF- β showed a pro-

liferative trend and an increase of activation marker genes. On the other hand, in non-adherent cultures, the spheroid morphology was maintained and the gene expressions of activation markers remained low. These results indicate that the spheroid culture method can be used to study the shift from deactivated to activated hepatic stellate cells, and that hepatic stellate cells in culture can be used to evaluate liver toxicity.

P06-19

Liver spheroid cocultures with fresh or cryopreserved hepatocytes and endothelial cells as tool to investigate metabolism and hepatotoxicity

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Primary human and animal liver cells are the gold standard for all pharmacological-toxicological *in vitro* studies during drug development. Three-dimensional (3D) cultures became more popular in recent years since they might mimic the *in vivo* cell morphology, polarity, and cell-cell interactions better than traditional two-dimensional (2D) cultures. Due to better access and continuous availability, cryopreserved cells become more popular, but functional differences to fresh hepatocytes might occur.

Here, 3D spheroids were generated in U-bottom ULA (ultra-low attachment) plates with fresh and cryopreserved human hepatocytes as single culture, and in coculture with liver endothelial cells to display an even more physiological situation. The metabolic activity of different Cytochrome P450 activities and the acute toxicity for known substances like acetaminophen were tested and compared for fresh and cryopreserved cells. Spheroids from fresh and cryopreserved human hepatocytes were inducible for CYP activity, but a strong variability of basal CYP activity and CYP induction potential were detected between different donors especially in fresh hepatocytes. A higher CYP activity and CYP inducibility were determined in 3D-cultures compared to 2D. In total, CYP inductions demonstrated a better and more stable inducibility in spheroids from cryopreserved cells. The ATP assay displayed almost the same concentration-dependent toxicity for acetaminophen in 3D and 2D cultures. In contrast, the AlamarBlue assay didn't show this dependency for spheroids, here only the highest concentration led to a decrease in viability. Our results indicate that differences may exist between 3D-cultures with fresh and cryopreserved hepatocytes and in comparison to standard 2D-culture models. These differences may lead to different and conflicting results in the assessment of drug toxicity and drug-drug interaction.

P06-20

Nanoparticle aerosol exposition of a human lung Co-Culture on a 3D system

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Nowadays it becomes more and more important to establish physiological *in vitro* systems for the respiratory tract, because it is the main port of entrance for nanoparticles (NP). NP are used in daily-use products, e.g. suncreme. They can be emitted at different stages of

the products life-cycle from production to recycling. We are establishing a novel exposure system on special cell culture inserts termed MatriGrid (MG). MG are shaped like alveoles and harbor a co-culture of primary peripheral lung cells (PLC) on one side and an endothelial cell line (EA.hy926) on the other as a model for the blood lung barrier.

Viability of the co-culture was determined by Resazurin-assay and Lactate dehydrogenase-assay, Glutathione-content (GSH) was measured by HPLC after derivatization with Monobromobimane. The confluency of the culture was detected by TEER-measurement (trans-epithelial/transendothelial electric resistance), because confluency cannot be visualized with a standard light microscope. The size-distribution of the particles in the aerosol was determined by Scanning Mobility Particle Sizer (SMPS) and Optical Particle Sizer (OPS).

First of all we exposed co-culture to BaSO₄- and TiO₂-NP aerosols. In the Resazurin-assay exposure of co-culture to 0.9 g/l (conc. of stock suspension resulting in averagely 5x10⁴ particles/cm³ for BaSO₄ in the aerosol) BaSO₄-aerosol shows a distinct viability-decreasing effect after 24 h. The exposure of Co-culture to 0.9 g/l (conc. of stock suspension resulting in averagely 1x10⁵ particles/cm³ for TiO₂ in the aerosol) TiO₂-aerosol shows a noticeable decreasing effect after 24 h, too. The LDH-assay shows a slight toxic effect at the same concentrations. For the GSH-determination co-cultures were incubated with BaSO₄- (0.9 g/l) and TiO₂-aerosols (0.1 g/l) for 1 h and a post-incubation phase of 23 h and 71 h. The NP aerosols induce a 2-fold induction of GSH for both NP after 24h. The GSH-content is reduced to nearly normal level after 72h. The particle size of both aerosols was measured during every exposure. Based on the number size distribution most particles have a size below 100 nm. In the BaSO₄-NP and TiO₂-NP aerosols the median diameters were 57 and 76 nm, respectively. The applied NP aerosols of BaSO₄ and TiO₂ cause toxic effects in co-culture after 24 h but not after 72 h. These results show that co-cultures (PLC/EA.hy926) have the ability to regenerate after contact to NP aerosols. We also investigated the transferability of these findings to mini organ cultures (MOC) of the lung. The MOC are less sensitive to NP aerosols.

P06-21

Development of a new *in vitro* device for risk assessment of inhaled xenobiotics: Lung/Liver

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Purpose: Air pollution represents an increasing hazard to human health [1]. Improvement of global risk assessment and management regarding this issue requires further predictive toxicological data. European research methods are challenged by the implantation of the 3R rule, to accommodate these guidelines *in vitro* methods are powering through [2]. Multi-organ platforms are widespread tools used to mimic and study *vivo*-like inter-organ cross-talks [3]. Presented here is a new approach to tackle toxicological *in vitro* assessment of inhalation-like exposures based on a lung-liver-on-a-chip platform comprising a microfluidically connected bronchial tissue to a metabolically functional liver biochip.

Methods: Characterization and validation of our device was assessed through cellular responses of separate and cocultured configurations of both compartments to referenced hepatotoxic exposures of acetaminophen (APAP) [4]. 72-hour exposures to solubilized APAP were

carried out and various cell parameters were tracked. Coculture was achieved using an Integrated Insert in a Dynamic Microfluidic Platform (IIDMP box) [5]. Transit of APAP through the device was followed by mass spectrometer media analysis.

Results: Our developed multi-organ platform shows signs of maintained cell viability and tissue functionalization despite imposed stress. Both pulmonary and hepatic tissues display strong differentiation and active metabolisms: uninterrupted transepithelial electrical resistance (TEER) values and strong E-Cadherin and Claudin-1 markers indicative of good pulmonary barrier functions, continuous glucose consumption, and albumin secretion on the hepatic side and high mitochondrial activity on the pulmonary side provide evidence of sustainable metabolic functions and active passage of APAP through both compartments.

Conclusion: Exposure to a hepatotoxicant resulted in notable adverse effects on both pulmonary and hepatic compartments revealing that our device is responsive to xenobiotic exposures and is endowed with the capacity of inter-tissue communication. Therefore, our lung-liver-on-a-chip platform could lead to new physiologically relevant ways to tackle inhalation-type toxicology such as pesticide investigations.

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P06-22

Characterization and use of 3D mini organ cultures of human lung for inhalation toxicology with respect to the effects of barium sulfate and titanium dioxide nanoparticles

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In general, there is still a lack of well-characterized and predictive *in vitro* alternatives to animal experiments in the field of nanotoxicology. In order to possibly be able to make better predictions about inhalational toxicological effects of substances, especially nanoparticles, on humans in the future, the human 3D *in vitro* model of lung mini organ cultures (MOC) is characterized in this work and tested for its suitability as a long-term culture model. Additionally, the toxicological effects of barium sulfate nanoparticles (BaSO₄-NP) and titanium dioxide nanoparticles (TiO₂-NP) on human lung MOC were investigated.

MOC of human bronchus and peripheral lung tissue (B-/L-MOC) were cultivated as air liquid interface culture and were exposed to BaSO₄-NP suspension as well as BaSO₄-NP and TiO₂-NP aerosol. The viability (metabolic activity) was determined by Resazurin assay. Glutathione-content (GSH) was measured by HPLC. The size-distribution of the NP suspension was determined by dynamic light scattering (DLS) and the size-distribution of NP aerosols were determined

by Scanning mobility particle sizer (SMPS) and optical particle counter (OPC). Characterization of MOC was carried out over a period of up to three months using the Resazurin assay and immunohistochemical stains such as Haematoxylin-Eosin overview stain, cytokeratins, Desmin, Smooth muscle actin, Vimentin, E-cadherin and the mucins MUC1 and MUC5AC.

B-/L-MOC show a stable metabolic activity over a period of up to three months. However, in connection with the immunohistochemical staining, a clear cellular change can be observed after about four weeks. After short term exposition of B-/L-MOC for 24 h and 72 h with BaSO₄-NP suspension, no decrease of viability was detectable at the highest concentration (0.0417 mg/cm²). L-MOC also show no decrease of viability and no change of GSH-content after short term exposure for 1 h to BaSO₄-NP and TiO₂-NP aerosols at the highest concentration of 0.9 g/l (concentration of stock solution, resulting in averagely 4.5x10⁴ particles/cm³ for BaSO₄-NP aerosol and 8x10⁴ particles/cm³ for TiO₂-NP aerosol) and post-incubation for 23 h and 71 h. Moreover, no decrease of viability and no significant change of GSH-content could be determined after chronic exposure of L-MOC to BaSO₄-NP and TiO₂-NP aerosols at the highest concentration of 0.9 g/l (cells exposed for four times in two weeks).

For future experiments with B-/L-MOC, cultivation for more than four weeks is not recommended. However, this already represents a significant extension of the cultivation time of cell cultures and allows the toxicological evaluation of substances after prolonged and repeated exposure. MOC show no sensitivity to the concentrations of BaSO₄-NP and TiO₂-NP applied in our study. These results were also compared with a co-culture of primary peripheral lung cells and an endothelial cell line (EA.hy926), where toxic effects due to the applied nanoparticle aerosols could be shown.

P06-23

HUB Organoids™ improve pre-clinical toxicology, metabolism, and pharmacokinetic studies for drug discovery and development

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Drug safety assessment at the preclinical stage is hampered by a lack of *in vitro* model systems that recapitulate healthy tissue physiology and gene expression for the evaluation of on- and off-target effects, drug metabolism, and pharmacokinetics (PK). Available cell line models are transformed to withstand *in vitro* culture and therefore do not represent normal tissue physiology and often do not express relevant enzymes and transporters. On the other hand, primary model systems have limited availability, expansion capacity, and are not stable in culture for experimental testing. Last but not least, pre-clinical studies using animal model are not always translatable to humans. Therefore there remains a need for advanced *in vitro* models to address drug toxicology, metabolism, and PK.

HUB Organoids are innovative “mini-organs in a dish” derived from adult stem cells within epithelial organs – such as the gastrointestinal (GI) tract organs – which form tissue-like structures resembling the parental organ histology and physiology. HUB Organoids are genetically and phenotypically stable in culture and retain the biological and functional properties of the original tissue and therefore simulate their responses to external stimuli. HUB Organoids can be

scaled up as necessary for high-throughput screening and thus provide a unique and robust *in vitro* model for drug development, screening, and toxicology studies.

The application of HUB Organoids from the GI tract of human, dog, and rat to safety studies using compounds with positive and negative GI toxicity profiles was evaluated. All four positive compounds were determined to be positive using human organoids, whereas for the two negative compounds high IC₅₀'s were observed. In addition, known interspecies as well as regional (colon vs duodenum) toxicity differences were detected. Next to GI organoids, we demonstrated that human liver organoids differentiated towards hepatocytes can be used for toxicity and metabolism assessment. Functionality of multiple CYP enzymes was detected in liver organoids, with CYP3A4 activity level comparable to that of primary hepatocytes (PHs). In addition, toxicity of 12 different compounds show a similar trend compared to PHs and HepG2 cells. Moreover, hepatotoxicity of Fialuridine, which cannot be detected with other pre-clinical models, was also observed after long-term exposure.

Altogether, our data indicate that HUB Organoids can predict toxicity of test compounds and can differentiate interspecies and regional toxicity differences as expected from *in vivo* studies. In addition, hepatotoxicity and metabolism in human liver organoids indicate differentiated human liver organoids are promising models to study drug-induced liver injury and drug metabolism. Therefore, HUB Organoids can serve as a model system in drug development and improve processes such as screening, and evaluation of transport, permeability, metabolism, and toxicity resulting in reduced cost and time.

P06-24

An Advanced *In vitro* Respiratory Model for Genotoxicity Testing at the Air-Liquid Interface

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Introduction: There is a need to develop more advanced *in vitro* models for toxicity testing of engineered nanomaterials (NMs) and environmental pollutants, in compliance with the 3 R's to reduce animal experiments. Respiratory cells exposed at the air-liquid interface (ALI) comprises a more realistic inhalation model compared with standard submerged exposure of lung cells. To better simulate *in vitro* the cellular interplay in the respiratory system, different cell types should be co-cultured.

Aim: The aim of the study was to apply an advanced inhalation model for testing genotoxicity of Ag-NMs. Methods

Human lung epithelial A549 cells were co-cultured with endothelial EA.hy926 cells on transwell inserts at the ALI, with lung cells on top of the membrane and endothelial cells submerged at the bottom. The co-cultures were exposed to aerosolized spherical (NM300K) and rod shaped (NM302) silver (Ag) nanomaterials (NMs) using the Cloud system (VibroCell). Size and size distribution of the NMs in dispersion was measured by nanoparticle tracking analysis.

Cytotoxicity was tested by the alamarBlue assay, release of the cytokine Interleukin-8 (IL-8) by ELISA, and genotoxicity (strand breaks and oxidizes base lesions) by the enzyme-linked version of the comet assay, in both cell types. For validation of the ALI-model, the toxic responses were compared with results from submerged exposure of A549 cells to NM300K and NM302.

Results: Different response was seen on cytotoxicity and genotoxicity of spherical NM300K and rod-shaped NM302 exposure, with NM300K being most toxic. NM300K induced increase in DNA damage at non-cytotoxic concentrations only in the EA.hy926 cells, while no genotoxicity was detected after exposure to NM302 under the experimental conditions. Our results show that the co-culture ALI model is reliable, gives reproducible results and can be combined with the comet assay for genotoxicity assessment.

Summary: The ALI co-culture model of human epithelial lung cells A549 and endothelial EA.hy926 cells is a promising model to be applied for inhalation exposure of NMs, in compliance with 3 R's, and is compatible with genotoxicity testing by the comet assay.

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P06-25

Micronucleus assay applied to advanced *in vitro* lung models at ALI for nanotoxicity assessment

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Introduction: Human lung cells cultured at the air-liquid interphase (ALI) represent an advanced *in vitro* model of the respiratory system, which is more likely to mimic the human lungs compared to cells cultured in submerged conditions. Genotoxicity assessment is crucial in toxicity evaluation of nanomaterials; the micronucleus assay measures the critical endpoint, chromosomal damage. The micronucleus assay has to our knowledge not previously been applied to cells cultured and exposed at the ALI.

Aim: To measure chromosomal damage in human *in vitro* lung ALI models, induced by nanoparticles and chemicals.

Method: Human alveolar epithelial cells A549 and human bronchial epithelial cells Beas2B were separately cultured at the ALI on transwell inserts. The cells were exposed to an aerosol of silver nanoparticles and control chemicals in the Vitrocell Cloud system. Chromosomal damage was measured by micronucleus assay after 24h exposure, in parallel with cytotoxicity by the alamarBlue assay and DNA damage by the comet assay. Experiments with cells cultured in submerged conditions were performed in parallel.

Results: Our results show that advanced *in vitro* lung models can be successfully applied for genotoxicity testing by the micronucleus assay, in addition to the alamarBlue and comet assays. Chromosomal damage in ALI culture was compared to damage in submerged culture.

Conclusions: The ALI culture of alveolar A549 and bronchial Beas2B cells represents a promising *in vitro* model for genotoxicity assessment of nanomaterials.

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P06-26

Predicting drug-induced liver injury *in vitro* by combining human 3D liver models and organ-on-chip technology

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Drug-induced liver injury (DILI) remains an important cause for drug attrition during clinical development or post-marketing approval. Preclinical drug development requires the use of complex biological systems and mainly relies on animal studies, with a limited success to predict a patient's response.

There is a continuous improvement of the complexity of human *in vitro* models. Human 3D liver microtissues, also known as liver spheroids, have an improved predictivity to study DILI, however, the low number of cells per spheroid limits the model's metabolic power and available cell material for analysis. In attempt to increase the metabolic power of spheroids, pooling spheroids without physical barrier lead to spheroid aggregates with a reduced viability and decreased expression of multiple CYP enzymes^[1].

Organ-on-chip systems allow the biological complexity of human *in vitro* models to be increased and made more relevant, by 1) exposing organ models to flow and 2) interconnecting different organ models with each other. For both single- and multi-organ purposes, organ-on-chip technology aims to improve the predictive value of *in vitro* systems.

In this work, we set up a human liver *in vitro* system that combines 100 human 3D liver microtissues in a 96 well format. Liver microtissues are formed from a mix of human primary hepatocytes with non-parenchymal cells. When comparing the setup of the system in a plate and organ-on-chip platform, spheroids in the organ-on-chip system show a higher viability and CYP3A4 activity after 2 weeks of culturing versus the plate culture. Furthermore, the liver-chip system shows significantly higher metabolic activity versus single liver spheroid cultures, which highlights the potential of this tool for predicting the turnover of compounds with a low hepatic clearance. Nevertheless, some compounds show extensive non-specific binding to the chip housing, which limits the available drug concentration in the system. Finally, the human liver-chip model shows a correct prediction of the liver toxicity of various reference compounds, as determined by spheroid viability, functionality and morphology. In conclusion, this work contributes to the evaluation and future implementation of organ-on-chip technology to support preclinical drug development.

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P07 – Omics in toxicology

P07-01

This abstract has been withdrawn.

P07-02

Relating early cellular events to Drug-Induced Liver Injury (DILI) using time-resolved transcriptomic and histopathology data

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Adverse Outcome Pathways (AOPs) aim to formalize links from molecular initiating events to key events and adverse outcomes on different biological levels^[1]. However, AOPs are often insufficiently quantitative, both in the time- and predictivity domain, rendering their application insufficient for risk assessment in practice. To advance this aspect of the field, we quantify the strength of temporal association between cellular events and later adverse histopathological changes which we use as surrogate readout for Drug-Induced Liver Injury (DILI). To do so, we use data from the TG-GATEs database^[2] which comprises liver transcriptomics and histopathology data from single- and repeat-dose studies in rats across eight time-points, ranging from 3 hours to 4 weeks post-treatment.

After inferring dysregulated cellular processes from gene expression, we quantified how frequently and confidently these events precede adverse histopathology. We firstly evaluated known key events in DILI^[3], and found that all tested events are significantly enriched before adverse histopathology, in comparison to time-series without any observed histopathological changes. Among those, some are frequent but less specific; while others possess higher positive predictive values (PPVs), but at the expense of lower recall. For example, mitophagy is upregulated in 45% of adverse time-series, but was (to a lesser extent) also found in non-adverse time-series, resulting in a PPV of 76%. Among the events with perfect confidence (PPV of 100%), the down-regulation of bile acid-related pathways is the most frequent event, but with a relative frequency of still only 33%. Data-derived events with higher frequency point to RNA metabolism and EIF2AK4 (GCN2) response to amino acid deficiency, which was previously reported as regulator of liver fibrogenesis in mice^[4]. However, further analysis is required to evaluate the precise role of these processes in DILI.

Next, we extended our analysis to transcription factors (TFs) by inferring their activity using DoRothEA regulons which link TFs and regulated genes⁵. This identifies an increased activity of E2f2, Nr1h3 (LXRa), Atf4, and Nfe2l2, as well as decreased activity of Srebf1 and Srebf2 as most frequent TF events before adverse histopathology (Relative frequency > 30%). Out of those, gene expression changes are also significantly enriched for Atf4 and Nfe2l2, indicating that these changes are frequently induced through TF expression. In contrast, the fact that only activity but not expression is altered for the other TFs suggests posttranscriptional regulation, e.g. as a response to cellular signaling or environmental stimuli.

Overall, we in this work hence derive mechanistic information on events preceding DILI from gene expression data, including their strength of association and longitudinal order, and demonstrate how time-resolved transcriptomics can be used to contribute towards the development of quantitative AOPs.

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P07-03

R-ODAF: Omics data analysis framework for regulatory application

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While the use of omics techniques is increasing in research, their application in regulatory toxicology is still extremely limited. Omics are commonly criticized for not being sufficiently reliable for regulatory application because the output depend on the applied analysis pipeline. This reticence to trust omics data is further magnified by the lack of internationally agreed upon guidelines and protocols for both the generation and processing of omics data. One way forward would be to reach a consensus on an omics data analysis framework for regulatory application (R-ODAF) based on rigorous data analysis. The authors of this study are proposing an R-ODAF for transcriptomics data for microarrays and RNA-Sequencing. The R-ODAF will then be reviewed and evaluated by the main regulatory agencies and consensus forums such as the Organization for Economic Co-operation and Development (OECD). This work is running in parallel alongside an OECD initiative to develop a guidance document called a transcriptomics reporting framework (TRF) that will enhance the quality of reporting of omics data when generated for regulatory purposes. The presentation of the project is now published^[1], and the final proposed pipeline will be presented in the event.

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P07-04

Comparison of toxicity and transcriptomic effects of emissions from heated tobacco products, electronic cigarettes and tobacco cigarettes in human bronchial epithelial cells

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Context: Cigarette smoke exposure is responsible for nearly 90% of lung cancers^[1]. Smoking cessation is currently the only effective way to slow down the progression of cancer. Electronic cigarettes (e-cig)

and more recently heated tobacco products (HTP) provide an alternative for smokers as they are generally perceived to be less harmful than conventional cigarettes^[2,3]. These new devices can quickly gain popularity, even before there is sufficient scientific evidence to determine their effects in users.

Purpose: This work was performed to compare the toxicity of e-cig, HTP and cigarette emissions on human bronchial epithelial cells.

Methods: BEAS-2B cells cultured at air-liquid interface were exposed to HTP (IQOS), e-cig (Modbox set at low (Mb-18W) or medium (Mb-30W) power, with 0.5 Ohm resistance), and 3R4F tobacco cigarette emissions generated by a smoking machine. Cytotoxicity, DNA strand breaks and chromosomal aberrations were measured by ATP, comet and micronucleus tests, respectively. A pangenomic microarray analysis was performed to better understand the potential mechanisms of toxicity of the different devices.

Results: HTP emissions exhibited reduced cytotoxicity and DNA strand breaks, compared to cigarette smoke but higher than e-cig aerosols. No chromosomal aberrations were evidenced whatever the device tested. Sub-toxic doses (~85–95% cell viability) were chosen for the transcriptomic analyses: 120 puffs for Mb-18W, Mb-30W and HTP; 4 puffs for 3R4F cigarette. A two-dimensional hierarchical clustering showed two main clusters: one branch grouped tobacco products (HTP and 3R4F cigarette) and the second branch showed a specific signature for Mb-18W and Mb-30W. Significant deregulated genes were selected with a fold-change of 1.5. A total of 58 or 573 deregulated genes were identified after exposure to 120 puffs of Mb-18W or Mb-30W, respectively, demonstrating that increasing e-cig power impacted the transcriptome expression. By contrast, 120 puffs of HTP induced deregulation of 1641 genes, while 4 puffs of 3R4F cigarette modulated 667 genes. Among them, 542 transcripts were deregulated similarly between both tobacco products. Finally, functional analysis of deregulated transcripts was assessed using the Ingenuity Pathways Analysis software. The mRNA profiles of HTP- and cigarette-exposed cells showed similar deregulations: increase of Nrf2-mediated oxidative stress response, induction of proliferation and cell survival, increase of glycolysis and cholesterol biosynthesis. HTP had the potential to deregulate these signalling pathways, in a manner similar to that of cigarette smoke, but after a more intensive exposure (120 puffs vs 4 puffs, respectively).

Conclusion: This study provides relevant data for risk assessment by demonstrating that HTP could be less harmful than tobacco cigarette but considerably more harmful than e-cig.

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P07-05

Omics reveal different oxidative stress levels induced by nanomaterials *in vitro* and *in vivo*

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The wide application of nanomaterials (NMs) in e.g. medicine, cosmetics or packaging and the continuous development of novel NMs, raise the demand for a facilitated risk assessment. One way to achieve this is to classify the NMs according to their modes of action (MoAs). Since omics approaches result in detailed information about ongoing processes, they are highly suitable to investigate NMs MoAs. Especially proteomics and metabolomics reveal information about phenotypes and were thus used to investigate the *in vitro* and *in vivo* effects of four silica NMs (SiO₂_15_Unmod, SiO₂_15_Amino, SiO₂_7, and SiO₂_40) and one titanium dioxide-based NM (TiO₂_NM105) at different doses. The systematic variation of properties within this set of silica NMs allowed us to get insights into their MoAs as well as the impact of their properties, like size, surface modification, and production route.

Since the lung is considered the main entry portal for airborne NMs, the focus of this study was on lung toxicity. Thus, alveolar epithelial cells (cell line RLE-6TN) and alveolar macrophages (cell line NR8383) were used to assess *in vitro* toxicity, and short-term inhalation studies (STIS), as well as instillations, were performed for *in vivo* investigations of rat lungs. Importantly, both chosen cell lines originated from rats to assure *in vitro* to *in vivo* comparability.

Based on the obtained proteomes and metabolomes, we examined the NM effects with a focus on oxidative stress, using a previously described stratified oxidative stress model (Li *et al.*, 2002). Since the level of oxidative stress strongly depends on the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG), we concentrated on the GSH/GSSG signaling first and found opposite trends in the two cell lines for the “active” NMs that showed most significant changes. While in alveolar epithelial cells increased abundances were observable for GSH/GSSG signaling and thus GSH production, we observed a decrease of GSH/GSSG signaling in alveolar macrophages, in turn leading to the induction of inflammation and cytotoxicity. Interestingly, no highly significant enrichment of GSH/GSSG signaling was observable *in vivo*, but pathways like chemokine signaling and apoptosis were induced. Moreover, endocytosis and cellular remodeling were found to be enriched. Furthermore, this study allowed us to identify analytes like Ornithine and Sod2, which showed dose-dependent changes, thus indicating them to be valuable biomarker candidates.

With this multi-omics approach, we obtained insights into NMs MoAs *in vitro* and *in vivo*, and we showed that the induction of different levels of oxidative stress strongly depends on the used model system. We classified the NMs into “active” and “passive”, thus also revealing information about the impact of production routes and surface properties. Finally, we identified biomarker candidates that might facilitate future risk assessment.

P07-06**Epirubicin alters the DNA methylation in cardiac microtissue**

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DNA methylation is an epigenetic modification. It can influence the functional state of genome regions without changing the genetic sequence. Studies have determined the essential roles of DNA methylation in multiple biological processes and diseases. However, there is not much investigation of the DNA methylation under drug exposure. This study explores the changing of DNA methylation under the influence of epirubicin, chemotherapeutic agent with cardiotoxic adverse effects. Human cardiac microtissues were exposed to epirubicin with either therapeutic or toxic (IC₂₀) doses over 2 weeks. The samples were collected at 7 time points and the genome-wide DNA methylation profiles were captured by methylated DNA immunoprecipitation-sequencing (MeDIP-seq) method. The differentially methylated DNA regions (DMRs) were identified by using the quantitative sequencing enrichment analysis (QSEA) package. Generally, the DNA methylation profiles differed between EPI and control samples, especially at early time points. From all detected DMRs, we selected genes, which had DMRs in their promoter region and had at least 4 DMRs over gene regions. The DNA methylation of these genes appeared as being strongly influenced by epirubicin. Some of these genes, such as EMILIN 1 and MEIS1, have been studied in the heart disease context. Emilin1 is an elastin-binding glycoprotein. The deficiency of Emilin1 relates to early postnatal cell-matrix defects in aortic valve tissue, resulting in latent AVD and premature death in mice model. Meis1 is a critical regulator of the cardiomyocyte cell cycle. It can modulate postnatal cardiomyocyte cell cycle arrest, and cardiovascular regeneration. Thus, the alteration of DNA methylation in these genes can be candidates for epirubicin-induced cardiotoxicity investigation. Furthermore, the DNA methylation can serve as potential biomarker for drug safety, and early drug screening.

P07-07*This abstract has been withdrawn.***P07-08****Studying the systemic effects of PFOS on the Hypothalamic-Thyroid-Pituitary (HPT) axis by transcriptomics**

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The Hypothalamic-Pituitary-Thyroid (HPT) axis regulates metabolism, cardiac and reproductive function, growth and development, and more. Disruption to the HPT-axis at any life stage may adversely affect development or function of vital organs and systems, ultimately affecting overall health. Exposure to environmental chemicals with thyroid hormone disrupting properties is therefore a major concern. Many chemicals have been linked to thyroid hormone system disruption, among them the per- and polyfluoroalkyl substances (PFASs). Animal studies have linked perfluorooctanesulfonic acid (PFOS) exposure to altered levels of triiodothyronine (T₃) and thyroxine (T₄); but strangely, these effects on T₃ and T₄ occur without any compensatory change to the circulating levels of thyroid-stimulating hormone (TSH). Similar trends have also been seen in epidemiological studies. However, the molecular mechanisms driving this effect remain unknown. This study aims to start unravelling some of these

mechanisms by characterizing the molecular effects at multiple organs of the HPT-axis in an effort to better pinpoint where in the thyroid hormone signaling axis PFASs may exert its actions. We exposed adult male rats for 7 days to either vehicle (Control) or 3 mg PFOS/kg/day. Blood samples were collected for determining PFOS and thyroid hormone concentrations after 4 and 7 days of exposure. Hypothalamus, pituitary, thyroid, and liver tissues were collected and subjected to bulk-RNA barcoding and sequencing (BRB-seq), which will be followed up with analyses at the protein levels, and histopathologically, depending on what transcriptional changes occur in what tissue(s).

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P07-09**Low-cost toxicogenomic profiling of Human iPSC derived minibrain reveals key adverse outcome pathways**S. Govindan¹, L. Stoppini¹, *A. Roux¹¹ HEPIA HES-SO, Tissue Engineering Laboratory, Geneva, Switzerland;² ARIMA Lifesciences PVT Ltd, Chennai, India

Genome-wide transcriptomic interrogation of organoid models are gaining popularity in characterising drug, toxicity responses and neurodevelopmental disorders. In this study, we have adopted a novel low-cost transcriptomic pipeline to study neurotoxicity associated adverse outcome pathway. We utilise a 3D minibrain spheroid model developed in the lab called minibrain^[1], an RNA sequencing library preparation methodology called BRBSeq^[2] and low depth sequence profiling for toxicogenomic profiling. Using this pipeline, we were able to uncover the molecular changes and differentiation dynamics involved in minibrain development, illustrating the ability to identify key cellular dynamic using our pipeline. We compare the transcriptomic changes associated with the dose-time neurotoxic response of minibrain to trimethyltin chloride both using high and low depth sequence profiling. The distinct gene expression changes and molecular candidates identified with our pipeline provide insight into the key events involved in the adverse outcome pathways of trimethyltin chloride associated neurotoxicity. We identify key processes such as endoplasmic reticulum stress, dysregulation of synaptic molecules and downregulation of neuron-morphology associated molecules to chronic but not acute exposure to trimethyltin chloride. We further validate these results functionally to confirm the validity of the low cost toxicogenomic profiling pipeline.

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P07-10

Unravelling the molecular mechanisms of the adverse events after Bio-Alcamid® injection

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Bio-Alcamid® is a non-biodegradable hydrogel polymer that was initially a popular soft tissue filler due to its longevity and resistance to hydrolysis. However, through the years this non-resorbable filler has shown short and long term adverse events (e.g. inferior migration, infections, abscess formation, inflammatory reactions such as granulomas) in 4.8% of patients in the Netherlands. In this study, we investigated the gene expression changes after Bio-Alcamid® injection in human skin and in reconstructed human epidermis to unravel the molecular mechanisms of the adverse events of Bio-Alcamid®.

Facial skin biopsies of patients showing adverse events to Bio-Alcamid® injection were collected using an 18G needle. From these biopsies RNA was isolated, which subsequently was analyzed using Agilent SurePrint G3 Human GE v2 8x60K Microarrays. The obtained expression values of the Bio-Alcamid® exposed biopsies were compared to the expression values from non-exposed skin biopsies collected from ArrayExpress (E-MTAB-4382).

In addition, samples were taken from the 3D Phenion® Full-Thickness Skin Model, either injected with Bio-Alcamid® or non-exposed, after incubation at 37 °C for 24h. After RNA isolation gene expression values were obtained using the same microarray platform and a comparison between the Bio-Alcamid® exposed and non-exposed 3D skin model was made.

Pathway analyses of the differentially expressed genes (adjusted p-value <0.05) in both biopsies as well 3D skin model revealed significant changes (p-value <0.05) related to immune and inflammatory response, as expected, but also related to zinc/copper homeostasis. In the latter pathways, the expression of several metallothioneins was significantly elevated. Metallothioneins are a family of metal-binding proteins involved in cell proliferation and matrix remodeling. These proteins have also been shown to act as anti-inflammatory/anti-oxidative stress mediators. Our findings confirm the inflammatory reaction to Bio-Alcamid® injection, but also show a potential counteract to the adverse events as seen by the up-regulation of several metallothioneins.

P07-11

Potential pathways involved in Montelukast neurotoxicity: a metabolomics approach

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Montelukast (MTK), a cysteine leukotriene receptor 1 inhibitor widely used for asthma management in children and adults, is undergoing clinical trials to explore its repurposing for other therapeutic applications, namely against some neurodegenerative disorders (e.g., Alzheimer's disease). However, this possibility should be regarded with care, since the number of reported MTK-induced adverse drug reactions, including the occurrence of neuropsychiatric events among children, has been increasing over the last years.

After evaluating the *in vitro* and *in vivo* metabolism of MTK, and taking into account the unexplained neurotoxicity of MTK, as well as its potential repurposing as an anti-neuroinflammatory agent, our goal was to understand the influence of MTK on metabolic pathways using an untargeted metabolomics approach. Test mice were treated orally with a daily dose of 1 mg/kg MTK for one week, and different tissues and fluids were collected for metabolite extraction. Samples were analysed by ultra-performance liquid chromatography coupled to high-resolution electrospray ionization tandem mass spectrometry (UPLC-ESI-HRMS/MS), followed by a bioinformatics-driven data treatment approach using XCMS.^[1]

The identification of metabolites and their up/down regulation status allowed us to elucidate which cellular metabolic processes were altered by MTK. Briefly, energy pathways, as well as detoxification routes, were some of the most affected ones. Pathways involved in the turnover of biomolecular building blocks (amino acids and nucleosides) were also changed. Regarding the brain tissues, metabolomics data suggest that MTK is able to interfere with neurosteroids and neuromodulators pathways, causing changes in the levels of dopamine, serotonin and histamine, as well as adenosine and arachidonic acid. These metabolites play important roles in various brain processes (e.g., signalling and sleep regulation), which may explain the adverse neuropsychiatric effects but also provide support for the repurposing applications. Dysregulated metabolites also suggested a hyper-activation of the hypothalamic-pituitary-adrenal axis. A decreased glutathione (GSH) level was also observed, consistent with our previous identification of MTK-GSH conjugate metabolites during the drug metabolism studies. The production of these conjugates could possibly be involved in the downregulation of GSH levels, interfering with the neuromodulators pathways by means of a thiolomic-wide change.

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P07-12

The secretome of mesenchymal stem cells ameliorates the cytotoxic effects of doxorubicin in non-tumoral cells

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The chemotherapeutic drug doxorubicin (Dox) is widely used for the treatment of solid tumours, including breast cancer, being cardiotoxicity one of the most important adverse side effect. Dox displays different mechanisms of action involved in its effects on tumour and

non-tumour cells. This might create an opportunity to develop new adjuvant strategies, aiming at decreasing off-target toxicity of Dox while not interfering with the chemotherapeutic activity. It has been shown that the secretome (CM) of mesenchymal stem cells (MSCs) present immunomodulatory and regenerative properties, whose potential is improved with 3D culture conditions. In cancer, the role of MSCs is still controversial with some studies reporting both pro-tumour and anti-tumour effects *in vitro* and *in vivo*. However, no studies have explored their combinatory use with chemotherapeutic agents. As such, the objective was to evaluate the effect of co-exposure of Dox with CM from 3D (CM3D) and 2D (CM2D) MSC cultures in breast cancer cells (MDA-MB-231) and in non-tumour cells, specifically breast epithelial cells (MCF10A) and differentiated AC16 cardiomyocytes, as well as to potentially link proteins from the MSC-CM with the observed effects. CM effects on tumour cell viability, migration and invasion were evaluated, followed by a Ge-LC-MS/MS proteomic analysis. Under concomitant incubation of Dox with CM3D/CM2D, no significant differences were observed regarding MDA-MB-231 cell viability, migration and invasion when compared to Dox alone. Notably, MCF10A and AC16 cardiomyocytes viability of Dox was consistently improved upon CM3D treatment, suggesting a protective effect. Finally, the proteomic analysis of CM content allowed to identify potential protein players behind the improved effects of CM3D, which were associated with cytoprotection, migration/invasion, oxidative stress and inflammation. Overall, UC-MSC-derived CM3D consistently showed better results than CM2D in decreasing Dox-induced cytotoxic effects on non-tumour breast cells and cardiomyocytes, which warrant further mechanistic studies.

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P07-13

Transcriptome Data to Substantiate the Assessment of Similar Mechanism of Actions in a Read-Across Context – a Case Study on Volatile Diketones

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This case study explores the applicability of transcriptome data to characterize a common mechanism of action within groups of short-chain aliphatic alpha-, beta-, and gamma-diketones. Human reference *in vivo* data indicate that the alpha diketone diacetyl induces bronchiolitis obliterans in workers involved in the preparation of microwave popcorn. The other three alpha-diketones induced inflammatory responses in preclinical *in vivo* animal studies, whereas beta and gamma diketones in addition caused neuronal effects.

We investigated early transcriptional responses in primary human bronchiolar (PBEC) cell cultures after 24h and 72h of air-liquid exposure. Differentially expressed genes (DEGs) were assessed based on transcriptome data generated with the EUToxRisk gene panel of Temp-O-Seq®. For each individual substance, genes were identified displaying a consistent differential expression across dose and exposure duration. The log fold change values of the DEG profiles indicate that alpha and beta diketones are more active compared to gamma diketones. Alpha diketones in particular showed a highly concordant expression pattern, which may serve as a first indication of the shared mode of action.

In order to gain a better mechanistic understanding, the resultant DEGs were submitted to a pathway analysis using Reactome-database^[2]. The four alpha diketones showed very similar results with regard to the number of activated and shared pathways. Overall, the number of signaling pathways decreased from alpha to beta to gamma diketones. Despite these differences, all diketones were active in pathways related to inflammation, in addition alpha and beta diketones revealed pathways related to apoptosis.

Additionally, we reconstructed networks of genes that interact with one another and are associated with different adverse outcomes such as fibrosis, inflammation or apoptosis using the TRANSPATH-database. Transcription factor enrichment and upstream analyses with the geneXplain platform^[1] revealed highly interacting gene products (called masterregulators, MRs) per case study compound. The mapping of the resultant MRs on the reconstructed networks, visualized similar gene regulation with regard to fibrosis, inflammation and apoptosis.

This analysis showed that transcriptome data can strengthen the similarity assessment of compounds, which is of particular importance e.g. in read-across approaches. It is one important step towards grouping of compounds based on biological profiles.

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[1] <https://platform.genexplain.com>

[2] <https://reactome.org/>

P07-14

Cell response after exposure of chlorpyrifos and chlorpyrifos-oxon on T98G as a model of glial cells

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Chlorpyrifos (CPF) is an organophosphorus compound (OP) widely used worldwide as an insecticide. CPF has been associated with both acute and chronic toxicity in different organ systems, including the liver and nervous system, additionally to the well-known acute toxicity due to acetylcholinesterase inhibition by its activated metabolite, chlorpyrifos-oxon (CPO). A complete understanding of those effects is lacking, especially at the cellular and molecular levels. The aim of this study was to identify the transcriptomic alterations in the T98G human glioblastoma cell line after CPF and CPO exposure. Using this model of glial cells and massively parallel Illumina sequencing of mRNA molecules (RNA-seq), a set of 304 genes that were differentially expressed ($p < 0.01$) between the CPF exposed and control cells were identified. The set of differentially expressed genes was then searched for significantly overrepresented GO (Gene Ontology) terms. We observed that the protein products of the differentially expressed genes were linked with an overt inflammatory response in these cells as a first impact on the nervous system. A bioinformatic search for proteins containing relevant sequence motifs was performed using the R programming language with the IDE RStudio through Bioconductor package, which allowed us to retrieve a list of 49 proteins that contain a pattern of serine-esterases susceptible to be modified by OPs among the differentially expressed genes.

P07-15

Quantitative proteomics reveal mechanistic insights into direct and indirect drug-induced thyroid toxicity in rats

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As an endocrine organ, the thyroid regulates various physiological mechanisms in humans, such as individual development, cell proliferation and differentiation, whereby secretion disorders can cause diseases. Several clinical drugs have been described to cause thyroid toxicity (TT) by interfering with the synthesis, transport and metabolism of thyroid hormones (direct TT) or by altering the synthesis and secretion of thyrotropin (indirect TT). However, the underlying mechanism of drug-induced TT (DITT) is not fully understood yet, thus hampering the risk assessment of novel drugs.

Here, we applied a quantitative proteomics study to investigate rodent TT, which is part of a multi-omics study in the framework of the LRI-C5 XomeTox project funded by CEFIC. Therefor, male Wistar rats were exposed to a direct (6-propyl-2-thiouracil, PTU) and an indirect (phenytoin) thyroid toxicant (BASF), and proteomics was applied to thyroids and livers. Thereby, two doses were tested for each drug, and the samples were analyzed at three time points, including one after a recovery phase.

Based on the obtained data, significant changes compared to untreated controls were investigated for the different conditions, with subsequent KEGG enrichment analyses based on the significantly altered proteins (SAPs). Thus, we found that the use of phenytoin did not affect the thyroid as indicated by the lack of significant changes, while slight changes (up to 82 SAPs) were observable in the liver. These proteins were related to KEGG pathways like chemical carcinogenesis, glutathione, and xenobiotics metabolism via cytochrome P450. Notably, after the recovery period, no significant changes were observable anymore, suggesting full rehabilitation. Also, after treatment with PTU, only slight effects were induced in the liver even with the higher dose, including effects on carbon and amino acids metabolism, which were ameliorated after the recovery phase again. In contrast, such a full recovery was only achieved for the lower dose in the thyroid and not for the higher dose, which led to stable effects on carbon metabolism, oxidative phosphorylation, and lysosomal processes even after the recovery time. These results indicate that the PTU effects on the thyroid were more severe than those on the liver.

Additionally, a Weighted Gene Correlation Network Analysis was applied to identify key drivers, which are supposedly valuable biomarker candidates. Thereby, P450 family members such as CYP2B1 and CYP2C70, which are involved in hepatic metabolism and detoxification, were found highly connected with the drug-induced liver toxicity. In the thyroid, ATP5FA (mitochondrial ATP synthase subunit alpha) was identified to be a key driver for instance, which was found to be upregulated.

In summary, this study allowed mechanistic insights into direct and indirect DITT and the identification of biomarker candidates, thus potentially facilitating future risk assessment of novel drugs.

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P07-16

Characterization of chaotropic effects of detergents on lipids homeostasis in an *in vitro* model of ocular toxicity: a molecular network based-lipidomic approach

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Detergent chemicals, widely used in household products, in pharmaceutical, medical, cosmetic and industrial fields, have been linked to side effects and involved in several eye pathologies such as in toxicity-induced dry eye. The underlying biochemical mechanisms of ocular toxicity induced by these detergents remain unclear and despite a major role in numerous biological functions, lipids involvement in such toxic processes is poorly described. We have thus investigated the effects of two widely used cationic and anionic ocular irritants, namely benzalkonium chloride (BAK) and sodium lauryl sulfate (SLS), on the lipid homeostasis of human corneal epithelial (HCE) cell line. Furthermore, correlation was performed between concentrations of BAK or SLS recovered in HCE cells and abundance changes of some lipid species.

HCE cells were exposed for 24 hours at subtoxic concentrations of BAK (6.10⁻⁵% and 10⁻⁴%) and SLS (3.10⁻³% and 5.10⁻³%) leading to cell death less than 25%. Following lipid extraction, an untargeted lipidomic analysis was performed using liquid chromatography hyphenated to high resolution tandem mass spectrometry (Synapt G2 Waters). Lipid species identification was based on the molecular network strategy we previously developed.

BAK and SLS both induce a major disruption of phospholipid homeostasis in HCE cells. BAK significantly decreased phosphatidylcholine (PC) containing saturated fatty acid (SFA) side chains and BAK concentration recovered in HCE cells was negatively correlated with these PC species ($r = -0.93$, $p < 0.0001$). In contrast, the cell content of phosphatidylinositol (PI) displayed an increase and a positive correlation with BAK concentration ($r = 0.71$, $p < 0.0027$). SLS induced a decrease in PI cell level concomitant with an increase in PC species ones. A significant correlation of SLS concentrations with cell levels of PI ($r = -0.83$, $p < 0.0001$) and PC ($r = 0.88$, $p < 0.0001$) was also exhibited.

These results indicate that BAK and SLS profoundly disturb the phospholipid homeostasis of HCE cells with specific effects. BAK, which is a cationic compound, induce a shift in favor of phospholipids bearing a negative charge namely PI species. In contrast, SLS, an anionic compound, induce a shift in favor of zwitterionic phospholipids i.e. PC species. This study is the first to highlight correlation between the incorporation of detergents into cells and significant changes in specific phospholipids cell levels. These chemicals may be considered as chaotropic agents targeting cell membrane, which can be the key events of the detergent toxicity mechanism. Such results may be help-

ful to define new markers of ocular irritancy in *in vitro* models as well as to graduate detergents toxicity within a chemical family.

P07-17

Emerging substitutes of BPA cause inflammation-like state and impaired insulin sensitivity in differentiating human adipocytes

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Introduction: Bisphenol A (BPA) is commonly used in consumer-related plastic products and is suspected of causing adverse effects regarding metabolic disorders. Reported effects include the disruption of adipocyte differentiation, interference with obesity mechanisms, and impairment of insulin- and glucose homeostasis, linking BPA to obesity and related co-morbidities, e.g. the metabolic syndrome. Endocrine disruptive effects in adipocytes are suspected to be mediated through binding and activation of peroxisome proliferator-activated receptor γ (PPAR γ), which plays a pivotal role in the regulation of adipogenesis. Although substitute compounds are increasingly emerging, they are not sufficiently investigated.

Objective: We aimed to investigate the mode of action of BPA, BPS, BPB, BPF, BPAF during the differentiation of human preadipocytes and analyzing their molecular interaction with PPAR γ using global proteomics.

Methods: Human preadipocytes were exposed to BPA, its substitutes, and the adipogenesis inhibitor GW9662. After 12 days of differentiation, lipid accumulation and global protein profiles were assessed via LC-MS/MS-based proteomics. Adverse effects on insulin sensitivity were determined by quantification of insulin-sensitive pAKT. Binding and effective biological activation of PPAR γ were investigated by surface plasmon resonance (SPR) and reporter gene assay.

Results: We could show that all tested bisphenols bound to human PPAR γ with similar efficacy as the natural ligand 15d-PGJ₂ and displayed an antagonistic effect on human PPAR γ at non-cytotoxic concentrations. During differentiation of human preadipocytes, all bisphenols exhibited an inhibitory effect. Global proteomics revealed a decrease of proteins involved in adipogenesis mechanisms and classical PPAR γ targets, similar to the PPAR γ inhibitor GW9662. Interestingly, pro-inflammatory pathways were up-regulated. The release of adipokines MCP1 and leptin were increased, and adiponectin decreased, suggesting potential adverse effects on insulin homeostasis.

pAKT ratios revealed significantly reduced insulin sensitivity by BPA, BPB, and BPS upon insulin stimulation.

Conclusion: Our results show that not only BPA but also its substitutes disrupt crucial metabolic functions and insulin sensitivity in adipocytes at low, environmentally relevant concentrations. These effects, mediated by inhibition of PPAR γ , may promote hypertrophic dysfunction of adipose tissue, leading to insulin resistance and development of the metabolic syndrome.

P07-18

Early pathways of toxicity triggered by 3,4-methylenedioxypyrovalerone unveiled by *in vivo* toxicometabolomics

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Synthetic cathinones represent a worldwide popular class of recreational drugs, despite the hazard associated with their use. Currently, there is an urgent need to better understand the precise cellular mechanisms by which synthetic cathinones induce toxicity, since traditional toxicological studies frequently use high concentrations/doses that are not representative of common use or intoxication scenarios. Therefore, in this work, we used a sensitive untargeted metabolomics approach based on gas chromatography-mass spectrometry (GC-MS) to detect early adverse effects on putative target organs (brain, heart, kidneys, liver) and biofluids (urine) of mice acutely treated with 3,4-methylenedioxypyrovalerone (MDPV) at human relevant doses. Male CD-1 mice received intraperitoneal administrations of saline or MDPV (2.5 mg/kg or 5 mg/kg) every 2 hours, in a total of three injections. Twenty-four hours after the first administration, the target organs and 24h-urine samples were collected for toxicometabolomic analysis. Multivariate analysis showed that MDPV induces significant changes in the metabolic profile of all organs as well as in urine. Each organ developed a specific response to the drug, with the liver and kidneys showing the greatest metabolic changes. Metabolites related with energetic metabolism (TCA cycle and fatty acids metabolism), amino acids metabolism, antioxidant defenses and inflammatory response were significantly altered in the liver. Kidneys revealed a dysregulation in several amino acids and lactate, which may contribute to the metabolic acidosis observed in users of MDPV. The heart, upon MDPV exposure, increased fatty acid biosynthesis, while the brain activated neuroprotective pathways and used alternative sources to obtain energy. The urinary profile of treated mice suggested that antioxidant pathways may be particularly involved to counteract oxidative stress triggered by MDPV. Overall, our findings provide first insights into the early pathways of toxicity elicited by MDPV and reinforce the use of toxicometabolomics as a robust and sensitive approach to detect adaptive/toxic cellular responses after exposure to human relevant doses of a toxic agent.

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P08 – Cardiovascular diseases

P08-01

Second-generation antipsychotics alters spliceosome signaling in mouse heart: implications from proteomic and transcriptomic analyses

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Purpose: Second-generation antipsychotics (SGAs) are first-line drugs that are prescribed for mental disorders in clinic. Severe cardiotoxicity has been widely reported and thus limits their clinical application. This study aimed to identify the common mechanism underlying SGAs-induced cardiotoxicity using dual-omics analyses.

Methods: Balb/C mice were intraperitoneally injected with two representative SGAs, olanzapine (2.5mg/kg) and clozapine (25mg/kg), at clinically comparable doses for 0, 7, 14 and 21 days. The SGAs-treated hearts were then examined with histopathologic staining, transmission electron microscopy, and subject to proteomic and transcriptomic analysis, respectively.

Results: It showed that both SGAs induced cardiomyocyte degeneration, inflammation infiltration, and cardiac fibrosis, all of which worsened with time. Proteomic analysis showed that 22 differentially expressed (DE) proteins overlapped in olanzapine and clozapine-treated hearts. These proteins were significantly enriched in muscle contraction, amino acid metabolism and spliceosomal assembly by GO term analysis and spliceosome signaling was among the top enriched pathways by KEGG analysis. Among the 22 DE proteins, three spliceosome signal proteins were validated in a dynamic detection, and their expression significantly correlated with the extent of SGAs-induced cardiac fibrosis. Following the spliceosome signaling dysregulation, RNA sequencing revealed that alternative splicing events in the mouse heart were markedly enhanced by SGAs treatments, and the production of vast transcript variants resulted in dysregulation of multiple pathways that are critical for cardiomyocytes adaptation and cardiac remodeling. The use of pladienolide B, a specific inhibitor of spliceosome signaling, successfully corrected SGAs-induced alternative splicing, and significantly attenuated the secretion of pro-inflammatory factors and cell deaths induced by SGAs exposure.

Conclusions: Our study concluded that the spliceosome signaling was a common pathway driving SGAs cardiotoxicity in mice.

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P08-02

Acute phase response and inflammation in mice following exposure to metal oxide nanoparticles

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Acute phase response (APR) is a systemic reaction to inflammatory states characterized by the increase of acute phase proteins, including C reactive protein (CRP) and serum amyloid A (SAA). SAA induces plaque progression through foam cell formation, and is thus causally related to cardiovascular disease. Welding, among other processes leading to the formation of nanoparticles in the breathing air, has been related to an increased risk of cardiovascular disease. In previous studies, healthy volunteers showed a dose-dependent increase in CRP and SAA blood levels 24 hours after exposure to zinc oxide nanoparticles, while in another study, mice instilled with zinc oxide nanoparticles showed increased expression of *Saa3* mRNA in lung tissue. As zinc oxide produces metal fume fever and APR, we hypothesize that other metal oxides related to metal fume fever could also induce APR in mice. In the present study, C57BL/6 mice were intratracheally instilled once with two different doses (low and high) of nanoparticles of copper oxide, tin dioxide, manganese oxide, aluminum oxide and zinc oxide, along with titanium dioxide and carbon black, as benchmark nanoparticles. We have evaluated the resulting inflammation and APR after 1 and 28 days of exposure through a cellular bronchoalveolar lavage fluid profile, *Saa3* mRNA levels in lung tissue, *Saa1* mRNA levels in liver tissue, and SAA3 and SAA1 protein levels in plasma. Furthermore, we have investigated whether the physico-chemical characteristics of metal oxide nanoparticles are the driving factors for APR induction. The results will be presented on the poster.

P08-03

The influence of the anticancer drugs doxorubicin and mitoxantrone on cardiac metabolism and autophagy

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Doxorubicin (DOX) and mitoxantrone (MTX) are classical chemotherapeutic agents widely used to treat solid cancers, leukemia and lymphomas^[1]. However, these agents are also known for their severe adverse side effects, notably cardiotoxicity^[2]. While both drugs induce toxicity, it is not clear if heart molecular mechanisms are similarly affected^[3].

Therefore, our goal was to study the effects of DOX and MTX in molecular mechanisms of adult male CD-1 mice heart, focusing on cardiac metabolism and autophagy. All animals were injected with 6

intraperitoneal administrations twice a week for three weeks: control mice received saline solution and DOX- and MTX-treated mice received a total cumulative dose of 18 mg/kg and 6 mg/kg, respectively, as this dosing scheme is pharmacological relevant and provides biologically equivalent doses. During the entire experimental period, animal welfare was assessed daily, and mice were euthanized one week after the last administration. The experiments were performed with the approval of the Portuguese National Authority for Animal Health (General Directory of Veterinary Medicine) (reference number 0421/000/000/2016). After heart's removal, a slice contemplating the two ventricles was obtained and homogenized for analysis by Western blot. Additionally, serum was obtained by centrifugation of blood taken from the inferior vena cava of each animal.

Serum glucose decreased after treatment with DOX, while alanine and aspartate aminotransferases increased. Additionally, DOX decreased the cardiac expression of ATP synthase subunit β (ATPB), electron transfer flavoprotein-ubiquinone oxidoreductase (ETFDH) and phosphofructokinase (PFKM), while MTX exclusively decreased the expression of Beclin1. Moreover, both DOX and MTX decreased glyceraldehyde-3-phosphate dehydrogenase (GAPDH), autophagy protein 5 (ATG5) and microtubule-associated protein light chain 3 (LC3B) expression. Although both drugs decreased important autophagic proteins, this molecular process seems more affected by MTX. On the other hand, DOX administration induced a general metabolic adaptation towards downregulation of glycolysis and fatty acids oxidation. These results suggest new pathways of the heart molecular mechanisms being modulated by DOX and MTX.

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P08-04

Dibutyl phthalate acts through estrogen receptors to increase Akt phosphorylation and NO production in human endothelial cell line EA.hy926

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Endothelial dysfunction represents the basis of many cardiovascular diseases (CVDs), which account for 31% of all deaths globally. Apart from poor lifestyle, environmental chemicals are increasingly recognized as potential CVD risk factors. Dibutyl phthalate (DBP) is an environmental chemical widely used in the production of various industrial and consumer products. Although epidemiological studies suggest a possible association between DBP exposure and endothelial dysfunction and CVDs, mechanistic and functional impact DBP exerts on endothelial cells remains unclear. Here, we examined the functional and molecular events associated with endothelial dysfunction after acute exposure of human endothelial cell line EA.hy926 to three concentrations of DBP: 10^{-6} , 10^{-5} , and 10^{-4} M. The results showed that cell viability was not significantly affected after

24 h and 48 h of exposure to all concentrations of DBP. Decreased adhesion to the extracellular matrix and increased cell migration were observed after 24 h of exposure to 10^{-5} M DBP. Decreased endothelial permeability and increased expression of two tight junction proteins ZO-1 and occludin were detected after 24 h of exposure to 10^{-4} M DBP. Both 10^{-5} and 10^{-4} M DBP increased adherence of calcein AM-labeled human monocytic cells U937 to the EA.hy926 monolayer. An incline in the nitric oxide (NO) production after 24 h and 48 h, increased endothelial NO synthase (eNOS) phosphorylation after 60 min, and sustained Akt activation after 15, 30, 60, and 120 min of exposure to 10^{-4} M DBP were detected. To analyze the mechanism of DBP action in EA.hy926 cells, we applied G-15, a G-protein-coupled estrogen receptor (GPER) antagonist, and ICI 182,780, a classical estrogen receptor (ER) antagonist. G-15, but not ICI 182,780, prevented the DBP-induced increase in cell migration. Both G-15 and ICI 182,780 diminished the NO production, eNOS and Akt phosphorylation in the DBP-exposed cells. These preliminary results suggest that DBP acts through nuclear ER and GPER in EA.hy926 cells to increase Akt phosphorylation, eNOS activity and NO production. The results also revealed that DBP-induced increase in cell migration depends on the GPER activation. Further comprehensive investigation is needed in order to provide a more detailed insight into the mechanism of DBP action in endothelial cells.

P08-05

Involvement of inflammation in DOX-induced cardiotoxicity: an in vivo study

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Doxorubicin (DOX) is a topoisomerase II inhibitor commonly used in the treatment of several types of cancer^[1]. However, despite its efficacy, DOX can cause potentially fatal adverse effects, like cardiotoxicity^[1]. This work aimed to assess in more detail the role of inflammation on DOX-induced cardiotoxicity in infant and adult populations. Two differently aged (infant or adult) CD-1 male mice were administered with two biweekly doses of DOX until reaching a therapeutic relevant cumulative dose (18.0 mg/kg). The animals' welfare was assessed daily, and mice were euthanized one week after the last injection. Blood was collected and hearts were removed, weighed, and processed: segments of heart that covered both the left

and right ventricle were placed in 4% paraformaldehyde for histological and immunohistochemistry analysis; cardiac sections were placed in complete RIPA buffer for immunoblotting analysis. The experiments were performed with the approval of the Portuguese National Authority for Animal Health (General Directory of Veterinary Medicine) (reference number 0421/000/000/2016).

Significant body weight loss was observed in DOX-treated infant and adult animals, when compared to controls. Regarding relative heart weight/brain weight ratios, significant decreases in both populations were observed. Cardiotoxicity was also manifested by significant elevation of serum levels of creatine kinase-MB (CK-MB), total creatine-kinase (total-CK), aspartate aminotransferase (AST) and AST/alanine aminotransferase (ALT) ratio. Histopathology findings confirmed that DOX caused cardiotoxicity in both populations. However, the increase in fibrotic cardiac tissue was only observed in DOX-treated adults. In DOX-treated infant mice protein carbonylation decreased, while in DOX-treated adult mice those levels increased. Considering more specific inflammatory parameters, the macrophage M1 marker increased in DOX-treated infant and adult animals, while the expression of nuclear factor kappa B (NF- κ B) p65 subunit only increased in DOX-treated adult animals. No differences were found in the expression of tumour necrosis factor (TNF- α), interleukin-1 β (IL-1 β), mitogen-activated protein kinase (p38 MAPK), and NF- κ B p52 in both populations. Myeloperoxidase and IL-6 expression significantly decreased in adult mice treated with DOX. In conclusion, adult mice seemed to be more prone to DOX-induced toxicity. Inflammatory markers underwent variable modulations, but NF- κ B p65 pathway activation is consistent with the observed toxicity and deserves further analysis.

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P09 – Clinical toxicology

P09-01

Predicting blood lead levels among children 2 years old, following the fire at Notre-Dame de Paris cathedral

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Purpose: To estimate the blood lead level (BLL) among 2-year-old children exposed to lead after the Notre-Dame de Paris fire, using two previously published kinetic models. The “Agence Régionale de la Santé”(ARS)^[1] has published aggregated data on 0–6-year old children. We present a scenario for 2-year old children as they have high hand-to-mouth behaviour and might be most at risk for lead poisoning.

Methods: Predictions were based on measured dust concentrations in schools and nurseries, as reported by the ARS. Predictions were made using two kinetic models: the IEUBK model^[2] and the chelation therapy (CT) model.^[3]

Firstly, the baseline BLL was computed for 2-year-old children, with an assumed weight of 12.4 kg, with 1 μ g/kg body weight of lead intake via food, per day. Secondly, a supplementary exposure was added with values of dust concentrations varying from 70 up to 1000 μ g/m². The simulated scenario was a child playing outside for at least 2 hours per day, 5 days a week, over a period of 11 weeks. This duration was chosen because this is the time which elapsed between the fire starting and the beginning of the school holidays. The oral exposure by hand-to-mouth contact was calculated as follows: $C \times TC \times DF \times t \times 0.2 \times 0.5$ with C = the concentration of lead on the wipe sample (μ g/m²); TC = transfer coefficient (m²/hour); DF = dislodgeable fraction; t = duration (hour); 0.2 because the skin surface of both hands is 20% of the skin surface of hands, forearms, legs and feet; 0.5 because 50% of the chemical that ends up on the hands is available for hand-to-mouth contact. The two kinetic models were used to convert the external lead exposure into BLLs. A standard bioavailability of 30% was assumed for the supplementary oral exposure due to hand-to-mouth contact.

Results: With actual dust lead concentrations measured between 70 and 700 μ g/m²,^[1] the predicted BLLs ranged between 49–51 μ g/L (geometric mean) with the IEUBK model and 39–54 μ g/L (arithmetic mean) with the CT model. Between 6 and 8% of the children were estimated to have a BLL above 100 μ g/L (IEUBK model).

Discussion and conclusions: We conclude that the estimated BLLs were quite comparable with both kinetic lead models. The number of children having a BLL above 100 μ g/L seems to be overestimated by the IEUBK model. For comparison, the actual mean BLLs measured by the ARS in n = 588 0–6 years old children were 16.1 (arithmetic) and 13.5 (geometric) μ g/L, with minimum & maximum values of 1.4 and 135 μ g/L, respectively. Sixty-eight children (11.6%) had a BLL ranging between 25–49 μ g/L, 11 children (1.9%) a BLL $\geq$ 50 μ g/L & 2 children (0.3%) a BLL $\geq$ 100 μ g/L. The time elapsed between the end of exposure and the BLL measurements was not reported. It would be interesting to compare the predicted BLLs with measured individual lead blood concentrations, taken from 2-year-old children, as followed by the ARS.

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P09-02

Hemorrhagic stroke due to arteriovenous malformation and carbon monoxide toxicity

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Objective: Carbon monoxide (CO) is an odorless, colorless gas that owes its effects to its affinity for proteins (hemoglobin, myoglobin, cytochromes), nitric oxide generation, mitochondrial oxidative stress, apoptosis, lipid peroxidation and other pro-inflammatory mechanisms. We report a case of CO poisoning manifested with hemorrhag-

ic stroke in a young patient with a preexisting arteriovenous malformation (AVM).

Case report: A 13-year-old male was admitted to the emergency department (ED) due to a holocranial headache 30 minutes after a minor fire broke out at his home. Medical history was negative for neurological conditions. At ED admission he was awake, with blood pressure 108/65, pulse 70 bpm, respiratory rate 24 bpm, oxygen saturation 93% and normal neurological evaluation. Arterial blood gas analysis showed a carboxyhemoglobin (HbCO) concentration of 13.9% (hemoglobin 17.3 g/dl). Considering his clinical condition, oxygen therapy was administered with a nonrebreather mask for three hours, until the patient was asymptomatic. At this time, a new arterial blood gas analysis showed a HbCO concentration of 4.1%. One hour after, before he was discharged the patient complained of an oppressive frontotemporal headache. Initially reported an intensity 6/10, however it increased to 10/10, followed by vomit so he was not discharged. 14 hours after exposure, his vital parameters were: blood pressure 136/88, pulse 50 bpm and respiratory rate 12 bpm, presenting sudden loss of consciousness and requiring endotracheal intubation. Brain computed tomography scan showed a right occipital intraparenchymal hemorrhage, with peripheral edema and mass effect. He was treated supportively and required a decompressive craniotomy, with a partial drainage of hematoma and dural plasty. After surgery, he was successfully extubated 48 hours later (3 days after exposition). On the next day, a magnetic resonance angiography showed a residual occipital collection, right parietal encephalomalacia and a reduced flow in the parietal vascular pathways. The patient was discharged on day 5 presenting only mild weakness of his left arm.

Conclusion: AVM are congenital lesions composed of a conglomerate of arteries and veins connected by at least one fistulae, so the arteries drain directly to the veins, which develop a chronic tissue injury owing to the high velocity of blood flow. Patients with AVM may experience spontaneous intracranial hemorrhage or as a consequence of a triggering factor such as physical exertion or a head concussion. In the present case the triggering factor was CO poisoning manifested only with headache that remitted after oxygen therapy. However, intraparenchymal hemorrhage could have been due to vasodilation induced by increased nitric oxide synthesis, as well as its interference with cellular respiration and oxidative stress.

P09-03

Lack of data hamper a proper toxicological risk assessment of the offlabel use of testosterone in women with gender dysphoria

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Introduction: Off-label use of testosterone in females living as males (i.e. transmen) has become routine without adequate risk assessment of this indication. Considering the vast increase in number of patients given this treatment world-wide, this risk should be evaluated. In Sweden, new cases of diagnosed gender dysphoria among females aged 13–17 have increased by almost 1,500 percent since 2008. Long-term treatment at these high levels of testosterone may add to the overall risk. A recent meta-analysis of testosterone treatment in women concluded that testosterone supplementation was safe only up to premenopausal physiological levels, due to the lack of safety data at higher doses.

In this study, we review open scientific literature and databases in order to assess the current knowledge base regarding safe use of testosterone in females. We include data on prescribed doses of tes-

tosterone in women and diagnosis of gender dysphoria in Sweden. Also, reported off-label adverse effects of testosterone drugs in females from the Swedish MPA BiSi database, the FDA FAERS database and the EU EudraVigilance database is reviewed. In addition, FDA and EMA summaries are reviewed for preclinical and clinical safety data of marketed testosterone drugs.

Results: The normal range of serum testosterone in adult females range between 20–60 ng/dL, the upper limit for athletics is 144 ng/dL. In transmen given testosterone, reported average concentrations are 711 ± 211 ng/dL, i.e. similar to the normal range in males, corresponding to an increase of 6 to 68 times. No preclinical toxicology studies in females have been performed on testosterone drugs available in Sweden, rendering a safety assessment for use in women based on preclinical data, impossible.

During 2019, 996 females under 40 years of age received testosterone treatment. A decade ago, the corresponding number was 147, indicating a marked increase. However, only one adverse event report (swelling of feet and ankles, tingling) is available in the pharmacovigilance database BiSi.

In the US and EU, 121 and 83 reports of adverse effects from testosterone use (e.g. embolisms, cancer, cardiac arrests) in women have been reported, respectively, and the vast majority of these were reported during the last five years.

Conclusions: Off-label use of testosterone in transmen has some unique characteristics, making it inherently riskier. High dose treatment is life-long and preclinical and clinical safety assessment studies performed for testosterone drugs lack the whole female group data as testosterone use is contraindicated in females due to teratogenicity. In addition, adverse effects related to off-label testosterone use in transmen is underreported and is a possible patient safety risk. The lack of toxicology data would not be accepted for a new drug and the need for a comprehensive risk assessment is urgent.

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P09-04

This abstract has been withdrawn.

P09-05

Carbon Monoxide: The Invisible as well as Silent Killer? A Quality Improvement Project raising awareness in the Emergency Department

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No conflicts of interest.

Ethics approval was requested for this project.

Background: Carbon monoxide (CO) is an odourless gas produced by the incomplete combustion of carbon containing materials. CO poisoning causes a range of symptoms of which headache is the most common^[1-5], occurring in up to 90% of patients^[6]. Unfortunately, none of these symptoms are pathognomonic and this lack of an recognised toxidrome means diagnosis is easily overlooked. An audit in the Emergency Department (ED) at University Hospital Aintree (UHA), Liverpool, UK looked to ascertain how often ED Healthcare Professionals (HCPs) consider CO when seeing patients with non-traumatic headache. It found a lack of clinical awareness with CO exposure only being considered in 0.8% of patients. Thus, improvement in awareness is warranted. This Quality Improvement Project (QIP) aimed to increase the consideration of CO exposure in non-traumatic headache patients to 50% of presentations.

Methods: Three separate sequential Plan Do Study Act (PDSA) cycles were instigated. Interventions involved promotion of verbal reminders to frontline ED HCPs on consideration of CO as a differential (cycle 1), utilising strategically placed CO posters in ED (cycle 2) and finally designing and introducing a CO sticker education scheme (cycle 3). These stickers, which highlighted the approved CO COMA acronym^[7], were placed in patient notes to serve as a physical and targeted reminder for HCP when seeing these patients.

Rapid cycle sequencing was utilised with each cycle lasting two weeks. The notes of patients presenting with non-traumatic headache were analysed for evidence that the HCP considered a diagnosis of CO. Suitable evidence was deemed to be any mention of CO di-

rectly, or indirect evidence such as questions relating to the COMA acronym.

Results: An average of 60 patients were included in each PDSA cycle of which the median age was 49 years with more females than males presenting. Given baseline findings that CO exposure was only considered in 0.8% of patients, each PDSA cycle demonstrated positive results with CO awareness being considered in 1.7% and 10% of non-traumatic headache patients following cycle 1 and 2 respectively. The third and final cycle is currently being analysed, however the expectation of the principal researchers is that it will lead to a further increase in consideration of CO as a differential in patients presenting with non-traumatic headaches to ED at UHA.

Conclusion: This QIP has shown that even small changes in the ED can led to a significant change in awareness of CO as a differential. Implementation of a CO Sticker education scheme is a feasible way of increasing awareness regarding CO poisoning among emergency care professionals. CO stickers serve as a low-cost, easy to use, transferable and sustainable solution to address the lack of CO awareness in acute ED settings, thus only serving to promote improved patient safety.

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P09-06

A survey of health professionals' opinion about web and smartphone application in clinical toxicology training

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Purpose: Clinical toxicology is complex, given the multiplicity of situations, of products, and the scarcity of randomized trials. The Global Educational Toxicology Uniting Project (GETUP) showed the importance of an Internet-based toxicology curriculum for emergency doctors. However, managing poisoning cases remains a challenge, as the formation of new healthcare professionals (HCP) in clinical toxicology. We aimed to obtain the opinion of HCP about the potential utility of an educational and interactive tool available on the internet and smartphones for initial and continuous training in clinical toxicology.

Methods: We sent an online survey in French and English, using i) mailing lists of HCP available in our tertiary care center, ii) social network as Twitter®, Facebook®, iii) using communication relays by local associations, during 3 months (from 12,2020 to 02,2021). Analyses were descriptives, stratified by age and by professions using Excel software.

Results: A total of 317 HCP completed the survey. The majority were French (90%), in 35–50 years old (36%) and in 50–65 (27%). Pharmacists accounted for 42% of respondents, clinical physicians 39%, students in health science 13% and others 6%. They worked in hospitals (59%) or private office (40%). They had wifi at work (89%), with unlimited access for most of them (96%). 86% of them used at least one internet app per day. Most of them (85%) never looked for a clinical toxicology app. However, 67% of them thought that it would be helpful and 26% that it may help them for their everyday work. 20% of the HCP had never received any training in toxicology, 70% had a few lessons during their studies. When facing intoxication cases, the HCP would call the poison information center (65%), search for guidelines (42%), look on official health agencies websites (34%), and call emergency services (20%). One-third of the HCP used a single source of information. HCP agreed on the importance of an e-learning app and their expectations regarding its content and utility of this were classified as follows, on a 10 point scale: obtaining an answer quickly while facing poisoning cases (8), finding the information required as fast as possible (8), recognizing the common syndromes (8), assessing the seriousness (8), knowing the routine dosage and their interpretation (7) and managing the case on their own (7). One-third of the respondents did not want to pay for a clinical toxicology app (35%) and more than one-third accepted to pay only if it includes certified training for continuing medical education (40%). In this case, 24% of respondents agreed to pay more for the app (> €20). HCP under 25 years old were more interested in continuing medical education than the older HCPs (67% vs 28%). Our survey was subject to non-response bias. Our survey does not reflect the opinion of all HCP in France due to moderate sample size, but suggested the need for extending online educational tool and for recognizing training.

P09-07

COVID-19, health recovery by medicinal plants (health solutions): An overview

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Purpose: Coronavirus disease became a pandemic in 2019; and according to WHO, has not been recommended drug for its definitive treatment^[1]. Some drugs used for control of this pandemic including Lopinavir; Ritonavir, Tocilizumab, Azithromycin, Remdesivir, Chloroquine and Hydroxychloroquine may have the potential benefits, but the clinical data do not yet fully confirm these medications^[2]. And they have potential for inducing overt toxicities.

Methods: Since the Plants with high potential from secondary metabolites and compounds such as phenolic compounds, flavonoids, alkaloids^[3–5], may be able to help and improve the recovery of patients, therefore in this study we reviewed the properties of 12 herbal extracts or infusions among 50 plant species which could have improved recovery quality in COVID-19 affected patients. The method of plant selection was based on past studies, active ingredients and therapeutical properties on viruses^[6–12].

Results: Our results showed *Thymus vulgaris* L., *Hyssopus officinalis* L., *Mentha pulegium* L. and *Mentha piperita* L. from Lamiaceae family and *Ziziphus jujuba* (Rhamnaceae), *Althea officinalis*, *Malva sylvestris* L. from Malvaceae and *Viola odorata* L. (Violaceae), *Echinacea purpurea* and *Matricaria chamomilla* L. from Asteraceae, *Verbascum*

thapsus L. (Scrophulariaceae) and *Daucus carota* (Apiaceae) have capability to improve the recovery in COVID-19 affected patients.

Conclusion: The presence of terpenoids, flavonoids and the rich amounts of phenolic compounds which have anti-inflammatory effects and also up regulation of immune system are among major agents that; they can help in recovery improvement of these patients.

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P10 – Developmental neurotoxicology

P10-01

This abstract has been withdrawn.

P10-02

Perinatal exposure to diesel exhaust origin secondary organic aerosol causes autism-like behavior in rats

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Background and Aims: Autism spectrum disorder (ASD) is a neuro-developmental disorder characterized by impaired social communication, social interactions and repetitive behaviors. The etiology of autism remains unknown and its molecular basis is not well understood. Both genetic and environmental factors may contribute ASD. In this study, we used diesel exhaust origin secondary organic aerosol (DE-SOA) as environmental pollutants. The aim of present study

is to examine autism-like behaviors and related gene expressions in rats exposed to DE-SOA perinatally.

Methods: Sprague-Dawley pregnant rats were exposed to clean air, diesel exhaust (DE) and DE-SOA in the exposure chamber (Sibata) for 5 h per day (5 days per week) from gestational day 8 to postnatal day 21. At postnatal day 21, the male offspring rats were allocated into three different groups ($n=8$ per group) as follows: 1) rats exposed to clean filtered air; 2) rats exposed to DE; 3) rats exposed to DE-SOA. Social dominance behavior was investigated at 10-weeks-old rats using a social dominance tube test. Prefrontal cortex was collected under deep anesthesia to examine social behavior-related gene and inflammatory biomarkers using real-time RT-PCR method, histological and immunohistochemical analyses.

Results and Discussion: DE-SOA-exposed rats showed socially dominant behavior compared with the control and DE-exposed rats. The mRNA expression levels of serotonin receptor (5-HT_{2B}) and brain-derived neurotrophic factor (BDNF) were down-regulated whereas interleukin (IL) 1 β , and heme oxygenase (HO)-1 were up-regulated in the prefrontal cortex of DE-SOA-exposed rats compared to the control rats. In addition, the expression of mast cells and microglia marker ionized calcium-binding adapter molecule (Iba)1 were increased in the prefrontal cortex of DE-SOA-exposed rats.

Conclusion: We suggest that the potential toxic substances contained in DE-SOA may reach the brain via placenta during fetal period and via the olfactory nerve route or systemic circulation during neonatal period and induce neuroinflammation. Our results indicate that perinatal exposure to DE-SOA may cause autism-like behavior such as social dominance behavior of male rats by modulating social behavioral-related genes and proinflammatory markers in the prefrontal cortex. Among the constituents of DE-SOA, organic carbon is the potential candidate to induce neurotoxicity.

P10-03

Effects of retinoic acids on the differentiation of human embryonic stem cell-derived neural stem cells *in vitro*

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Retinoic acids and their signalling are crucial during early development of vertebrates^[1]. Furthermore, retinoid signalling is recognized as an endocrine mode of action, though validated tests to address retinoid endocrine disruption are not available yet^[2].

Retinoid signalling is mainly mediated via retinoid receptors: retinoic acid receptor (RAR) and retinoid X receptor (RXR). The model and most potent endogenous ligand for RAR is all-*trans* retinoic acid (atRA), for RXR 9-*cis* retinoic acid (9cRA) is a specific high-affinity ligand.

Besides endocrine disruption, developmental (neuro)toxicology is still lacking validated alternative *in vitro* tests for human health hazard and risk assessment, despite the need for such methods being identified and formulated more than a decade ago^[3–6].

In this study, we use a differentiating human neural stem cell (hNSC) *in vitro* model^[7] to study the effects of two model retinoic acids, atRA and 9cRA on the expression of neural differentiation-related biomarkers and neural patterning. Upon initial attachment and conditioning, cells were exposed after 4 days *in vitro* (DIV4) to 8–1000 nM of either retinoic acid for up to 18 days (DIV22). We monitored gene and protein expression at two timepoints during exposure (DIV13, DIV22); network patterning was observed by immunocytochemical staining at DIV22.

Both, atRA and 9cRA dose-dependently increased the expression of retinoic acid-specific cytochrome P450 monooxygenase CYP26A1, with atRA leading to a higher induction (1000 nM atRA: 45.9-fold

induction, DIV22) than 9cRA (1000 nM 9cRA: 32.9-fold induction, DIV22) compared to time-matched solvent-control.

At DIV22, biomarkers of neural stem cells (paired box 6, neuroectodermal stem cell marker NESTIN, SRY-box transcription factor 1 and 2) were decreased. Despite the activation of retinoic acid metabolism (as confirmed by CYP26A1 induction) and atRA being a model inducer of, and promoter for neural differentiation, extended exposure (DIV22) to both retinoic acids did not increase the expression of neural biomarkers (doublecortin, β III tubulin, microtubule-associated protein 2, synaptophysin); if observed, significant dose-dependent changes were rather a decrease in neural biomarkers. Interestingly, the early glial biomarker S100 β was significantly increased on the gene expression level but decreased on protein level; the changes were not evident for glial fibrillary acidic protein (astrocytes).

Furthermore, increasing concentrations of retinoic acid decreased the degree of network patterning, that was observed in non-exposed cells, the solvent control, and differentiation-positive control.

Overall, exposure to 8–1000 nM retinoic acid for up to 18 days (DIV22) resulted in a phenotype resembling non-differentiated neural stem cells at the highest concentration on all levels (gene expression, protein expression, cell culture morphology/network pattern).

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P10-04

Rodent thyroid toxicity and potential child neurodevelopmental impairment

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Human clinical evidence suggests a link between altered serum levels of thyroxine (T4) and/or thyroid stimulating hormone (TSH) in pregnant mothers and child neurodevelopmental outcomes. However, thresholds for altered maternal serum T4/TSH levels resulting in neurodevelopmental impairment are currently unknown, and it remains unclear which neurodevelopmental parameters in safety studies are best suited to predict effects in children. Further, it is currently unknown, to what extent species differences in thyroid hormone metabolism and transport impact the transferability of observations seen in *in vivo* rodent studies to human safety. Following an extensive literature review of published relevant human and animal studies the ECETOC Thyroid Task Force (TF) held a meeting with external regulatory and academic experts in November 2019 and is currently in the phase of publishing the results of the review through a small series of publications (Sauer *et al.*, 2020).

Rodent toxicity studies showed that substances identified to disrupt the thyroid system often do so through induction of liver enzymes (mainly uridine diphosphate glucuronosyltransferases (UGTs), but possibly also sulfotransferases), that lead, via increased thyroid hormone (TH) conjugation, to increased TH clearance, decreased circulating TH, and consequent alterations in the thyroid gland itself. By contrast, the available human evidence is insufficient to establish such a causal link, as UGT or liver enzyme measurements in humans are not collected in the clinic, thus unavailable for comparison with the extensive laboratory rodent data.

One of the major scientific and regulatory uncertainties is to establish whether such substance-mediated maternal UGT-induced TH clearance may ultimately lead to neurodevelopmental impairment. Therefore, the TF work aims to identify which experimental data are key to determine whether a substance-induced increase in liver enzymes and TH clearance does (or does not) result in impaired neurodevelopment.

In accordance with the European Commission's Endocrine Disruptor Criteria, the TF examined whether thyroid-related adverse outcome pathways (AOPs) could provide useful information on a chemical's potential to cause adverse neurodevelopmental outcomes. Molecular initiating events (MIEs) and/or key events (KEs) of these AOPs were reviewed with the goal to identify non-routine parameters that, coupled with standard rodent toxicity studies, could identify potential developmental neurotoxicants. Overall, the TF concluded that MIE/KE evaluation can support the identification of potential thyroid modes-of-action and could add relevant information to the weight of evidence assessment. However, to interpret the relationship between a thyroid MIE and DNT, increased knowledge of KE relationships (KERs) is required, including a quantitative understanding of KERs and the impact of species differences in thyroid signaling pathways.

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P10-05

Investigation of the impact of genotoxins on the proliferation and differentiation capacity of human neural stem/progenitor cells *in vitro*

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Due to highly complex and time-dependent mechanisms, the developing nervous system is considered to be a sensitive target for chemical insults, including genotoxins. It is well known that these DNA-damaging substances can reduce cell proliferation and induce apoptosis, e.g. in the developing rat brain, processes that are crucial for normal brain development. Moreover, differentiation capacity of the affected cells can be altered if the DNA damage is not successfully repaired. Thereby, modes-of-action (MoAs) and cellular responses differ depending on the genotoxin. In this study we analyze the effects of model genotoxins on developing primary human neural progenitor cells (hNPCs), a multicellular test system with high relevance for the developing human brain.

Human NPCs allow assessment of distinct neurodevelopmental key events (KE), including NPC proliferation, radial glia migration, neuron and oligodendrocyte differentiation, their migration and neuronal morphology. Using the neurosphere assay, we analyzed the adverse effects of the selected genotoxins, i.e. N-Ethyl-N-Nitrosourea (ENU), Hydroxyurea (HU), 5-Azacytidine (5Aza) as well as Etoposide (ETOP), which act by different MoAs. As a positive control we used ionizing radiation (IR).

The studied genotoxins altered distinct neurodevelopmental KE in the range of 0.08 – 20 µM. Thereby, HU, 5Aza and ETOP reduced the proliferation of NPCs, while all four genotoxins also impacted NPC differentiation into neurons and/or oligodendrocytes. Strikingly, ETOP influences radial glia migration leading to a phenotypically altered migration pattern.

We generated genotoxin-specific phenotypic data by applying the high-content human neurosphere assay for hazard assessment of this compound class. In the future, more genotoxic-related endpoints like cell-cycle and DNA repair analyses will be performed for understanding the cell type-specificity of the genotoxins during human brain development.

P10-06

Is ochratoxin A a potential neurodegenerative toxin?: mechanistic *in vitro* and *in vivo* studies

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Ochratoxin A (OTA) is a mycotoxin contaminating a huge variety of crops. Its main target organ is the kidney and it is a known rodent

carcinogen. Humans are continuously exposed to OTA and maximum limits have been laid down in several food matrices based on its nephrotoxic effects. Parkinson's disease (PD) is a neurodegenerative disorder pathologically characterized by the loss of dopaminergic (DA) neurons in the *substantia nigra* and the presence of Lewy bodies aggregates, which contain phosphorylated alpha-synuclein. The etiology of PD is unclear, however some exposure scenarios related with farming, pesticide exposure or living in rural areas have been associated with the disease. OTA has been previously been reported to be neurotoxic and to cause striatal dopamine depletion in mice, although at doses higher than the doses leading to minimal renal changes in the pivotal studies used for human health risk assessment.

To further identify if OTA exposure could have a role in the development of PD, wild type mice were orally treated with low doses of OTA (0.21 or 0.5 mg/kg b.w.) four weeks. The long-term effects of OTA on PD related molecular features (alpha-synuclein pathology, dopaminergic dysfunction and motor deficits) were evaluated six months after the end of the treatment. Mechanistic analyses were carried out in brain and gut. The brain is the target organ for PD. The gut is the first barrier against food contaminants and has been related to some early symptoms of PD. Indeed, it has been linked to the initiation of the pathology via the spread of alpha-synuclein from the enteric nervous system to the lower brainstem, a process that might precede DA neurons degeneration.

In vivo immunohistochemical and biochemical analyses demonstrated a loss of DA cells associated with a decrease of dopaminergic innervation in the striatum responsible for motor impairments. Phosphorylated alpha-synuclein levels were increased in brain and gut. Similar to PD, LAMP-2A protein levels were decreased in brain, as well as in the gut.

The mechanism was further validated *in vitro* in a human SH-SY5Y neuroblastoma cell line. Cell cultures exposed to OTA exhibited decreased LAMP-2A protein levels, impairment of the chaperone-mediated autophagy pathway and decreased alpha-synuclein turnover which was linked to the deregulation of miRNAs, all reminiscent of PD.

Our results provide preliminary data pointing to a more sensitive endpoint than the nephrotoxicity used in the current risk assessment, however more studies are needed to unravel a potential neurodegenerative effect of OTA.

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P10-07

Development of an automated cognitive behavioral testing method for juvenile mice in a group-housed environment and its application to developmental neurotoxicological study

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Behavioral assessment using animal models is essential to evaluate the effects of drugs and industrial chemicals on cognitive functions because it is still challenging to fully substitute *in vitro* or *in silico* tests. However, conventional behavioral tests have been pointed out that improvements are needed in terms of the 3Rs principle. One of the main arguments calls that behavioral tests should avoid direct and repeated human-animal contacts, which may seriously stress the animal and thereby compromise the reproducibility of the test. Furthermore, there are currently few standardized, high-

throughput behavioral tests on young animals, as required by OECD testing guidelines. This study aims to develop an automated cognitive behavioral toxicity testing method for group-housed juvenile mice without human intervention. We modified the commercially available IntelliCage system to apply to 3 to 5-week-old mice (approx. 8 to 20 g in body weight) as follows: (1) we made hardware attachments to fill the space at each cage corner and the tubular radio frequency identification (RFID) antennas, both of which ensure telecommunication between the sensors and RFID. (2) we revised a cognitive-behavioral task protocol developed for adult mice in IntelliCage to fit into juvenile mice to assess their higher cognitive functions. (3) we applied this modification of the IntelliCage system to developmental neurotoxicology testing. The present study consists of two experiments. Experiment 1: 3-week-old mice (a total of 24 mice) were subjected to a behavioral sequencing task and its repetitive reversals to obtain a reward in the modified IntelliCage. They accomplished the reversals more than 30 times by seven weeks of age. Experiment 2: Pregnant mice were administered methylmercury chloride at a dose of 5 mg/kg or saline on gestation days 7 and 14. Pups born to these groups of dams were subjected to the same behavioral task as Experiment 1. The methylmercury-exposed group of pups showed significantly lower explorative behavior and increased perseverative behavior to the reward. In conclusion, our modified IntelliCage system and behavioral task were proven to successfully analyze the cognitive behavior of mice from weaning till adulthood and to have the potential to be incorporated into a test battery for developmental neurotoxicity. These animal experiments were approved by Animal Use and Care Committee at Phenovance Research and Technology LLC (Experiment 1) and the National Institute for Environmental Studies (Experiment 2).

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P10-08

Refining EDC hazard assessment by integrating an *in vitro* testing battery for endocrine disruptor (ED)-induced developmental neurotoxicity (DNT)

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Endocrine disrupting chemicals (EDCs) have been intensively studied regarding their harmful effects on human health. Although there is increasing evidence that early developmental exposure to EDCs causes developmental neurotoxicity (DNT), systematic testing for DNT is not included in the hazard and risk assessment process of EDCs. Therefore, a refinement of the EDC test guidelines, including testing strategies for DNT, is urgently required. Currently, the identification of chemicals causing adverse neurodevelopmental effects is mainly based on animal studies. However, insufficient test throughput, species differences, and ethical concerns demand alternative *in vitro* models with high predictivity for humans. Therefore, a DNT *in vitro* testing battery has been established including a multiplexed

high-content assay based on human primary neural progenitor cells (hNPCs), the Neurosphere Assay.

As a member of ENDpoiNTs, a H2020 research consortium comprising 16 international partners, we focus on the development of alternative *in vitro* assays to identify endocrine disruption (ED)-induced DNT. The established ED-DNT *in vitro* assays will meet the requirements for screening putative EDCs regarding their impact on hormone-dependent neurodevelopmental processes. To study which key events of human brain development are regulated by hormone receptor signaling, we investigated the effects of specific hormone receptor agonists and antagonists on the neurodevelopmental processes represented within the Neurosphere Assay. Key events of human brain development like NPC proliferation, migration, and terminal differentiation into neurons and oligodendrocytes were significantly affected by hormonal modulation. Strikingly, oligodendrogenesis proved to be the most sensitive endpoint, being influenced by activation of aryl hydrocarbon receptor (AhR), liver X receptor (LXR), retinoid X receptor (RXR), retinoic acid receptor (RAR), peroxisome proliferator-activated receptor alpha, beta, and delta (PPAR α , β , δ), thyroid hormone receptor (THR), and progesterone receptor (PR) signaling. By incorporating both male and female hNPCs in our testing strategy, we could identify sex-specific dependencies of hormonal activity during brain development, especially concerning progesterone, glucocorticoid, and prostaglandin E₂ receptor signaling. Since hormone-regulated neurodevelopmental processes provide putative targets for EDCs, the established ED-DNT *in vitro* assays will be used to screen libraries of known and putative EDCs and identify ED-induced DNT. Due to the dramatic consequences of DNT for human development, testing putative EDCs for their developmentally neurotoxic potential is of highest importance for several stakeholders including regulatory bodies, industry, and the civilian population. Therefore, with the developed ED-DNT *in vitro* assays we aim for acceptance at the regulatory level.

P10-09

***In vitro* evaluation of the effects of the synthetic cannabinoids ADB-FUBINACA and AMB-FUBINACA on mitochondrial function during neurodifferentiation**

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Synthetic cannabinoids (SCs) comprise a broad group of new psychoactive substances with stronger psychoactive effects compared to tetrahydrocannabinol (Δ^9 -THC). Potential SC use by pregnant women or women of child-bearing age is especially alarming due to the possible onset of neurodevelopmental disorders in their offspring. This work aimed to assess 1) the effects of two commonly seized SCs, ADB-FUBINACA (ADB) and AMB-FUBINACA (AMB), on the neurodifferentiation of NG108-15 neuroblastoma x glioma cells; and, given the key role played by mitochondria in regulating neurogenesis, 2) the involvement of mitochondria in SC-induced disruption of neurodifferentiation.

Neurodifferentiation of NG108-15 cells was induced in serum-starved (1% FBS) cell culture medium supplemented with 30 μ M forskolin and 10 μ M retinoic acid.^[1] Neurite outgrowth was analysed by measuring the differentiation ratios (number of neurites/total cell number) and total length of primary neurites after addition of SCs (kindly supplied by TicTac Communications Ltd, UK), once (at day 0) or every 24h for 3 days (repeated exposure) at human-relevant concentrations (1pM–1 μ M). ATP levels and mitochondrial membrane potential (MMP) were analysed following a single SC addition after 24 and 72h exposure by luciferase-based luminescence assay and TMRE labelling, respectively. Cytosolic Ca²⁺ levels were monitored for

1h (Fluo-4 AM labelling) immediately, and at 24 and 72h after a single SC exposure. SR141716A, a selective CB1 antagonist, was used to assess the involvement of CB1 in the observed SC effects.

Both SCs increased differentiation ratios around 1.6–1.8-fold after exposure to all concentrations tested, with no significant differences observed between single and repeated exposure. Notably, this effect was prevented when incubated in the presence of SR141716A. Most conditions tested (except 1 μ M and 1pM AMB, single and repeated additions, respectively) increased total neurite length between 1.6–2.5-fold. A reduction in MMP was noted after a 72h exposure to both SCs, especially at the lowest concentration tested (1pM), but not at an earlier timepoint (24h), indicating a time-dependent effect. Pre-incubation with SR141716A did not revert these effects, suggesting that CB1 does not mediate SCs' actions on MMP. No significant changes in intracellular Ca²⁺ or ATP levels were detected at any of the concentrations and time points tested.

Overall, ADB and AMB enhanced *in vitro* neurodifferentiation of NG108-15 cells in a CB1-dependent manner, which was paralleled by decreased MMP. However, further research is required to clarify the potential relationship between these phenomena, and other mechanisms involved in SC-induced neurodifferentiation.

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P10-10

Are serum thyroid hormone changes in rats a reliable indicator for human neurodevelopmental effects? – Evaluation of different cases

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The thyroid modality is one of the four endocrine modalities evaluated under European pesticides and biocides regulations. Following the criteria on endocrine disruption (ED) and the ECHA/EFSA Guidance (2018) on ED, compounds altering the circulating levels of triiodothyronine (T3) and/or thyroxine (T4) with or without thyroid histopathological changes present a potential concern for adverse neurodevelopment.

While in humans a causal link between altered plasma levels of T4 and/or TSH (thyroid stimulating hormone) in pregnant women and changes in the neurodevelopment of their children is suggested by clinical evidence, it is doubtful that T4 and/or TSH alternations seen in adult rodents represent a concern for human neurodevelopment due to significant and well-established species differences.

In this context, the ECETOC Thyroid Task Force evaluated toxicological data sets of compounds with six different molecular initiating

events (MIEs) associated with thyroid toxicity: i. TPO (thyroid peroxidase) inhibition, ii. NIS (sodium-iodide symporter) inhibition, iii. Dio (deiodinase) inhibition, iv. nuclear receptor activation, v. interaction with transthyretin (TTR), and vi. interaction with thyroid hormone receptor (TR). More specifically, the key event relationships between serum thyroid hormone changes, tissue thyroid hormone changes, brain gene and/or histopathological changes and adverse neurodevelopmental as adverse outcomes were assessed. Further, the predictivity of rodent brain and/or behavioral parameters and the relevance of histopathological thyroid changes for humans regarding adverse neurodevelopment was investigated.

It could be shown that the adverse outcome pathway (AOP) is best investigated for the TPO- and Dio 1 inhibiting compound propylthiouracil (PTU), which induced serum and brain thyroid hormone changes, brain gene and histopathological changes as well as effects on electrophysiology, learning and behavior. Other compounds with the same or different MIEs did not induce this sequence of events (although in some cases less thoroughly investigated).

This raises the questions, whether the alteration of serum thyroid hormone levels in rodents beyond a certain threshold is a sound predictor of adverse neurodevelopment, and which additional parameters obtained in which population would be sensitive and more decisive for predicting adverse neurodevelopment for compounds being active in a thyroid-related AOP.

As an outlook, the ECETOC Thyroid Task Force will propose a testing scheme on how to comprehensively assess compounds being active in a thyroid-related AOP, taking into account mechanistic information, key events (KE) and key event relationship (KER), significant species differences, and the appropriate determination of markers for the adverse outcome of neurodevelopmental effects.

P10-11

Gene-Environmental Interactions in Autism with iPSC-derived brain organoids: microRNA and Metabolic Biomarkers of the Synergy

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The strong increase of autism in recent years worldwide cannot be explained solely by genetics or exposure to environmental chemicals, but rather as interplay between both. A combination of three cutting edge technologies (the induced pluripotent stem cell (iPSC) technology, CRISPR-CAS9 gene editing and 3D human relevant microphysiological systems) allows for the first time to produce a human brain model with autistic genetic background, which recapitulates the critical phase of early brain development. Autism is a multifactorial disease that makes it challenging to find disease causation. Mutation in the chromodomain helicase DNA binding protein 8 (CHD8) is one of the major genetic risk factors linked to ASD. CHD8 is an important regulator of brain development and regulates a number of ASD-related genes. Here, we identify a potential synergy of two putative autism risk factors, i.e., CHD8 mutation and the pesticide chlorpyrifos (CPF). A heterozygous CHD8^{+/-} knockout iPSC cell line was generated using CRISPR-CAS9 technology. CHD8^{+/-} mutant iPSC alongside with the control iPSC were differentiated in 3D to form BrainSpheres. Brain organoids were exposed for 24h to chlorpyrifos at 4 weeks of differentiation. BrainSpheres derived from CHD8^{+/-} iPSC were more sensitive to CPF than control, as identified by viability assay and de-

creased neurite outgrowth. Increased oxidative stress was observed in CHD8^{+/-} brain organoids. Both, CHD8 mutation and CPF increased levels of tyrosine hydroxylase, which suggest perturbation of dopamine synthesis. L-DOPA but not dopamine levels were elevated due to mutation and due to exposure. The ratio of SAM/SAH was perturbed in mutant brain organoids and due to treatment, suggesting perturbations in methylation and the trans-sulfuration pathway. The ratio of glutamate and GABA was changed. We also observed down-regulation of neuroprotective microRNA *mir-7* upregulation of hypoxia-related *mir-210*, due to mutation and due to treatment. Further experiments are being conducted using multi-omics approaches to identify molecular mechanisms of potential synergy between CHD8^{+/-} genetic background and chlorpyrifos exposure.

P11 – Developmental toxicology

P11-01

This abstract has been withdrawn.

P11-02

ReproTracker: A Human Stem Cell-Based Biomarker Assay for *In vitro* Assessment of Developmental Toxicity

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Testing for developmental toxicity according to the current OECD guidelines requires large numbers of animals, making these tests very resource intensive and time-consuming, as well as raising ethical concerns. Over the past years, several alternative *in vitro* assays have been developed, but these often suffer from low predictability and lack of mechanistic information.

Here, we present ReproTracker, a human induced pluripotent stem cell (hiPSC)-based biomarker assay that follows the differentiation during early embryonic development. The hiPSCs were directed to differentiate into three germ layer-specific cell types, hepatocytes, cardiomyocytes, and neural rosettes. The differentiation processes were followed by morphological profiling and expression patterns of the cell-specific biomarkers. In this system, a decrease in the expression of the biomarker genes and morphology disruption of the differentiated cells following compound treatment indicated teratogenicity.

The assay was validated with over 40 well-known *in vivo* teratogens and 20 non-teratogenic compounds at non-cytotoxic concentrations. In ReproTracker, *in vivo* teratogenic compounds markedly disrupted morphology and decreased the expression pattern of the biomarker genes in at least one of the three cell types. Non-teratogenic chemicals generally had no effect on the morphology of the differentiated cells, nor on the expression of the biomarker genes. Compared to the *in vivo* classification, the assay achieved high accuracy (80%), sensitivity (77%), and specificity (84%).

In conclusion, ReproTracker is a state-of-the-art *in vitro* assay that is able to identify the teratogenic potential of new chemicals and drugs with high accuracy and provide a signal as to the likely outcome of *in vivo* test systems. The assay can best serve as an early phase teratogen screening platform, or as a late phase verification for animal testing outcomes.

P11-03 Zebrafish larvae as an alternative model for nephrotoxicity testing

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A wide variety of compounds including drugs, fertilizers, and natural pollutants are known to have a major impact on nephrogenesis and renal functionality. The unmet need for nephrotoxicity assessment to elucidate potential human health hazards likely to arise from chemical exposure has been the motive of this research. In this context, the zebrafish embryo model proposed herein aims to fill the gap between *in vitro* methods, with a lack of physiological context, and traditional *in vivo* models, entailing ethical concerns.

Zebrafish has evolved as a promising alternative *in vivo* model for toxicity evaluation due to their fast development, transparency, amenability for drug administration, and high homology to humans. Taking advantage of the natural transparency that zebrafish embryos present during the first days of life, we have developed an assay to evaluate the nephrotoxicity of a variety of chemicals based on pronephros morphology and function. Before the evaluation of nephrotoxicity, an acute toxicity assay was run in zebrafish embryos exposed to the test compounds from 6 hpf to 120 hpf to define LC10 and LC50 values, to avoid any interference by non-specific toxicity. For the assessment of kidney morphology, a 6αF antibody immunostaining was performed to evaluate the proximal convoluted tubules morphology (PCT). Embryos were exposed from 6 hpf to three concentrations of the test compounds ($\frac{1}{4}$ LC10, $\frac{1}{2}$ LC10, and LC10) and at 120 hpf the structure of the PCT was classified based on the convolution stage. A transgenic line *Tg(wt1b:GFP)* was used to also evaluate pronephros morphology. Embryos were exposed as above mentioned and at 48 hpf were imaged in a widefield microscope. Measures of the distance between tubules and the angle between the neck segments and the PCT were performed. Finally, kidney function was assessed using a rhodamine-dextran clearance assay, where the elimination of the fluorescent dextran out of the bloodstream is used as a read-out of kidney filtration activity. For this, embryos treated from 6 hpf with the test compounds were injected in the cardiac cavity with the fluorescent probe at 48 hpf, and changes in the fluorescent intensity were evaluated at 3 and 24 h post injection.

A total number of 17 test compounds were included in this study. The sensitivity of the assay reached 100% indicating good reliability of our model to detect nephrotoxic compounds. The specificity of the method was 60% for kidney development, mainly limited by the challenging search of non-nephrotoxic compounds. This issue could be tackled including a larger number of non-nephrotoxic compounds.

To conclude, the presented strategy corroborates the usefulness of zebrafish embryos in the evaluation of nephrotoxicity. However, further work should be done in order to optimize the method and automate it.

P11-04 Zebrafish, an innovative alternative model in preclinical studies

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Over the past decades, drug discovery has faced various fluctuations. In the 19th century, it relied on the serendipity of medicinal ingredients. Thereafter, in the 20th century, New Drug Entities emerged, contributing to the era of antibiotics that covered unmet needs. This involved fundamental pillars such as target identification & validation, hit identification, lead generation & optimization, and finally

the development of elected candidates. Drug discovery also included chemical synthesis, formulation optimization, toxicological studies in mammals, clinical trials, and regulatory approval. Currently, the cost of mammalian studies is high and results are not always translatable for humans. In addition, significant social and ethical dilemmas have also risen. Fortunately, the 3Rs principle (Reduction, Replacement, and Refinement) has resulted in the development of alternative methods to the use of mammals. Scientific improvements in the fields of toxicology and drug development have diminished the need of mammalian models. Given the need for a reliable, cost-effective methodology to detect toxicity during the development of New Chemical Entities, laboratories are joining forces to harmonize the use of zebrafish. Whilst mammals remain crucial to providing evidence that translates into clinical stages, the zebrafish community works tirelessly carrying out inter-laboratory initiatives to boost zebrafish validations and get the approval of regulatory agencies. In this perspective, the aim of the present study is to shed light on the implementation of an innovative automated high-throughput screening method using zebrafish to predict teratogenic effects in humans. To do so, zebrafish embryos were exposed to a library of 31 known compounds classified as teratogens and non-teratogens in mammals and 16 phenotypical parameters related to embryo early development were assessed: heart and yolk-sac edema, pigmentation, body length, eye and yolk size, development delay, tail bending, scoliosis, fin absence, hatching ratio, jaw malformations, necrotic tissue, defects in otic vesicle, otoliths and body axis. Results showed high correlation with results obtained in murine models, detecting 20 teratogenic compounds and 11 non-teratogenic compounds, with a 94.44% sensitivity, 90.91% specificity and 87.10% accuracy. Moreover, the model shows to have a high correlation with humans 87.50% sensitivity, 81.82% specificity and 74.19% accuracy, increasing the prediction level reported in rodents for the same compounds. Deeper analysis revealed potential modes of action involved in physiological and developmental disorders for some compounds such as retinoic acid, DEAB, ochratoxin A, warfarin, valproic acid, and bisphenol A among others. The high predictivity degree of zebrafish shown in this report highlights the great potential of this alternative model in toxicological preclinical studies.

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P11-05

Juvenile minipigs: a suitable model for the pediatric safety assessment of antisense oligonucleotides

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We investigated the suitability of the juvenile Gottingen minipig as a non-rodent model for the nonclinical safety assessment of oligonucleotide therapeutics intended for pediatric use. Thirty-three juvenile minipigs received weekly subcutaneous injections of saline or 20 mg/kg of RTR5001, a locked nucleic acid (LNA) single-stranded antisense oligonucleotide (ASO), injected subcutaneously caudal to the pinnae starting on Post Natal Day (PND)1. Designated piglets (1 to 4 per occasion) were sacrificed on PND2, 9, 16, 23, 29–30, 37, 43 and 51. The study included toxicokinetic investigations, in-life monitoring (including physical development assessment) and clinical pathology (including complement factor activity). Each piglet was submitted to full necropsy and histopathological evaluation. To investigate the biodistribution of RTR5001 to the kidney, liver, and lymph nodes, immunohistochemistry with an antibody for ASO and *in-situ* hybridization with a probe targeting RTR5001 were performed.

Plasma AUC of RTR5001 and liver concentration (in the Kupffer cells) increased throughout the course of the study. Kidney concentrations (in the renal tubular cells) were similar at all ages. RTR5001 was also detected in the lymph node macrophages. All animals survived up to the scheduled euthanasia and RTR5001 was clinically well tolerated. No injection site reactions were observed macroscopically. Body weight was slightly reduced in the RTR5001 group. There were no effects on food consumption nor on the physical development of the piglets (as assessed by pinna unfolding, eye opening and incisor eruption). Changes in hematology, coagulation and clinical chemistry parameters consisted of increases in circulating numbers of neutrophils, lymphocytes and white blood cells, along with an increase in fibrinogen concentration and in complement activity (CH50), indicative of inflammation. A mild increase in aspartate aminotransferase was observed with no microscopic correlate. A mild decrease in cholesterol (total and low-density lipoprotein) was also noted. At microscopic examination, tubular degeneration/regeneration in the kidney and minimal to slight dermal/subcutaneous inflammation at injection sites were observed. In the lymph nodes, foamy/granular macrophages were considered to reflect the accumulation of the ASO in the lysosomes.

Overall, these findings were comparable with those previously reported with RTR5001 in adult Gottingen minipigs. There was, however, some variability in plasma exposure, biodistribution and toxic-

ity profile in the piglets compared with adult minipigs. Based on these results, the juvenile minipig is considered a suitable non-rodent species for the non-clinical safety assessment of ASOs intended for pediatric use.

P11-06

Characterization of the Human Induced Pluripotent Stem Cell (HIPS)-Test to Predict Embryotoxicity

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Mammalian development can be disrupted by exogenous noxae, making it essential to test substances for their embryotoxic potential. Up to date embryotoxicity is studied within the Organisation for Economic Co-operation and Development (OECD) or the United States Environmental Protection Agency (U.S. EPA) test guidelines (TG) *in vivo*. Due to the high number of substances that have to be tested, testing using the mentioned TGs is logistically and financially not feasible, bears the problem of species differences and additionally raises ethical concerns due to a high number of animals needed. Therefore, there is an urgent need for human-based alternative methods assessing embryotoxicity. This approach is supported by the European Center for Validation of Alternative Methods (ECVAM). We established and characterized a human test method for embryotoxicity evaluation based on human induced pluripotent stem cells (hiP-SC), which is called the HIPS-Test. The test is based on a 2D monolayer culture that is challenged with substances during the differentiation period of 10 days. The protocol is based on modulating the WNT pathway resulting in beating cardiomyocytes at day seven. During the time course of differentiation, different markers (mRNA and protein) of early and later cardiomyocyte development are expressed in the cultures and were evaluated. Endpoint analyses for this test method are: (i) a cell viability assay, (ii) analysis of video recordings and (iii) PCR analysis. Video recordings were analyzed with an in-house developed software to evaluate the beating frequency and area of the cardiomyocyte layer. To set up the HIPS-Test as a test method it was challenged with 5-Fluorouracil (positive control) and Penicillin G (negative control) analogous to the mouse embryonic stem cell test which was validated by ECVAM.

In conclusion, the HIPS-Test is a promising human *in vitro* assay to evaluate substances for their potential to induce embryotoxicity.

P11-07

A proposal of an innovative microfluidic platform for testing developmental toxicity

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The official Guideline for testing developmental toxicity is the OECD TG 414. One single assay performed observing this OECD TG 414 requires several hundreds of rats or rabbits with a cost of around 100,000 €. There are no alternative methods for testing developmental toxicity among the OECD Test Guidelines for testing chemicals and therefore the development and validation of novel alternative methods for such purpose is needed.

Organs-on-chip are advanced *in vitro* systems designed to recapitulate the functional units of a given organ allowing the modelling of organs' functions and physiological responses. The authors represent a consortium involving so far up to sixteen different partners including research/academic entities and small-medium companies from nine different European countries. We propose to develop a foetal-placental organ-on-chip with functional immortalised foetal and placental human cells.

In our proposal, the effect of chemicals will be tested passing the substances through placenta and foetal cells in microfluidic conditions mimicking the physiological environment. The proposed organ-on-chip will allow assessing the role of placenta as barrier for the foetal cells and in the biotransformation of chemicals. The effect of chemicals on foetal cells could be assessed under a number of readouts as microscopy, omics, microscopy, electrophysiology, etc.

Some of the potential application of the proposed organ-on-chip in Regulatory Toxicology will be to avoid the OECD TG 414 test in a second species, to support results of old test performing without OECD TG 414 observance or to support decisions based on read-across. Basic research in Toxicology will also benefit from this organ-on-chip since it would help to elucidate AOPs, to address gender perspective using XX and XY cells and to avoid inter-species extrapolation since human, and not animal cells, will be used.

Finally, it is also remarkable that this organ-on-chip tool is expected to have a strong bioethical impact since it will allow a notable reduction on animal experimentation for testing developmental toxicants with both regulatory and basic research purposes.

P11-08

Transcriptomic and morphological study of the influence of all-trans retinoic acid's duration exposure on the zebrafish developmental mechanisms.

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As we move forward with non-animal testing for chemical safety in the future, having strong mechanistic knowledge and robust *in vitro* tools becomes crucial. The zebrafish embryo model offers the possibility of studying embryogenesis in a complete vertebrate organism, in an animal-free regulatory context. This model is relevant to humans when studying highly conserved developmental mechanisms. For example, the retinoic acid (ATRA) pathway is known to play a major role in axial patterning, craniofacial morphogenesis, and neural tube closure, across species and may therefore offer biomarkers for the study of embryotoxicity. The ATRA pathway influences other important morphogenic regulators (e.g. *fgf8*, *wnt*, *shh*, and *bmp*), by its regional-specific expression and local concentration gradients. ATRA itself is regulated by a strictly maintained balance between its synthesizing enzymes such as *aldh1a2* and metabolizing enzymes of the *cyp26* family. We studied the influence of ATRA on zebrafish developmental mechanisms after different exposure durations, in order to find biomarkers of dysmorphogenesis in the zebrafish embryo. We investigated both the transcriptomic and morphological changes after ATRA exposure (7,5nM) for different durations (2hrs, 4hrs, 6hrs, 24hrs, 48hrs, 72hrs, 120hrs). The results show an impact of the duration of exposure on both the morpho-

logical and transcriptomic readouts. The zebrafish showed significant changes in morphology after 24h of exposure. Transcriptomic changes occurred as early as 2hrs after exposure, firstly with genes implicated in the metabolism on the ATRA pathway (*cyp26a*, *aldh1a2*), the *hox* genes (*hox1*, *hox2b*) and the genes responsible for the neural tube formation/ closure (*gli1*, *dlx5*). These results show the morphologic fate of the embryo is determined by gene expression at a much earlier age than 24 hpf, when dysmorphogenesis is usually assessed. It also demonstrates that the toxicity of a compound is not only dependent on concentration but also on exposure duration. These findings allow us to select ATRA pathway-related genes as biomarkers for developmental toxicity, which we anticipate will be responsive to a wide array of dysmorphogens.

P11-09

Zebrafish Model to Predict the Developmental Toxicity Potential of Fragrance Materials

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At the Research Institute of Fragrance Materials (RIFM), our role is to ensure the safe use of fragrance materials for the general population, while being proactive in looking for alternatives to animal testing. There is a need to develop new methods to either replace or reprioritize testing on animals, and specifically for systemic toxicity and developmental and reproductive toxicology as they are heavily dependent on animal testing. Zebrafish has been a well know model for developmental toxicity in the past few decades. Biobide's Teratotox Assay analyzes zebrafish embryo at 2- and 4-days post fertilization (dpf) for developmental toxicity by looking at specific malformations. A blinded study was conducted on 10 fragrance materials which already had *in vivo* developmental toxicity data. Materials analyzed in this study ranged from different functional groups, some had CMR status and some materials which did not show any developmental or reproductive toxicity. All the materials analyzed in Teratotox Assay were evaluated at 8 different concentrations with 15 zebrafish embryos per treatment group. Based on LC50 (mortality) and EC50 (number of abnormal embryos), a teratogenic index (TI) was calculated for all the 10 materials. According to the Biobide's Teratotox Assay, a TI value of above 2 is considered to be likely teratogenic. Methyl acetoacetate showed several malformations in zebrafish embryos like microcephaly, pericardial edema, yolk sac edema, and trunk alteration. Hence it was classified as toxic based on the results. However, there are no animal data to indicate that this material causes developmental toxicity. On the other hand, Phenylacetic acid did not show any malformations in zebrafish embryos and was not classified as teratogenic or toxic which aligned with the *in vivo* results. Overall, mixed results from the Teratotox Assay for the 10 materials were observed, where some materials tested in the Teratotox Assay aligned with already existing *in vivo* data and some did not. Although currently there is no regulatory approved *in vitro* assays for developmental toxicity, the current research will help to predict potential toxicity and prioritize testing if required. In conclusion, the results show that Teratotox Assay showed promising results to get an initial idea about developmental toxicity of a chemical and in future more data might be required to validate and help in replacing or to reprioritize testing on animals.

P11-10**Effects of chrysene and benzo-a-pyrene on placental estrogen receptors**

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Purpose: Placenta is a unique and complex organ, structured by two different individual genetic organisms, including the mother's uterine wall and the fetus's own tissue; which has a crucial function in the continuation of the pregnancy and the development of fetus. During pregnancy, production of 17-beta-estradiol (E2) increases to approximately eighty times and placenta specifically binds estrogens. Despite the fact that the human placenta binds to estradiol (E2) and estrogens affect various aspects of placental function and fetal development in humans and primates, there are no studies which placenta is shown as a target for estrogens. Polycyclic aromatic hydrocarbons (PAHs) are hundreds of toxic organic chemicals found everywhere in the environment and in foodstuffs. BaP is metabolised to dihydrodiols, phenols and quinone derivatives by cytochrome P450 enzymes. Among these metabolites, some hydroxylated species have structural similarity with 17b-estradiol, and some of the hydroxylated metabolites interact with ERs. The aim of this study was to evaluate the effects of BAP and chrysene on the expression of ER in placental cell line HTR-8/SVneo cells.

Methods: To determine IC₅₀ values on HTR8/SVneo cell line, benzo-a-pyrene and chrysene were applied individually between concentrations of 2,5–0,001 µg/ml and MTT assay was performed. The cells were later treated with the IC₅₀, IC_{50/2}, IC_{50/4} and IC_{50/8} concentrations of the pollutants to evaluate the ESR1 and ESR2 expression using Western Blot and qPCR analysis.

Results: IC₅₀ concentrations of BaP and chrysene were found as 110.201 ± 14.734 ng/ml and 109.766 ± 5.829 ng/ml respectively. BaP induced ESR1 expression in HTR8/SVneo cells. 110.201–27.550 ng/ml treatment were found to upregulate ESR1 expression. Fold changes in ESR1 mRNA expression were 2.86 ± 0.06, 2.87 ± 0.011 and 3.36 ± 0.09 respectively (p < 0.001).

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P11-11**Toxicological effects of different antioxidant compounds abundant in plants: a comparative study in zebrafish towards glaucoma treatment**

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Genetic and environmental factors contribute to glaucoma, many of which cause an imbalance between oxidants and antioxidants in the affected ganglion nerve cells in the eye. This oxidative stress leads to mitochondrial dysfunctions that contribute to symptoms linked to cell ageing and degeneration. The importance of botanical compounds to develop new therapeutic approaches for treating glaucoma has been recognized. However, there is a major gap in understanding how these compounds work and their toxicological effects, which limit their potential. Acute toxicological effects of Ferulic acid (FA), L-ascorbic acid (AA), Syringic acid (SA), Chlorogenic acid (CA), Querce-

tin (Q), Gallic acid (GA), Kaempferol (K), Resveratrol (R), and Catechin (C), abundant in plants, were assessed in wild-type zebrafish larvae. Adult zebrafish were set up (1♀:1♂) for mating, and fertilized embryos with healthy morphology were randomly selected and transferred to 48-well plates containing 500 µL/well of the antioxidant solution. The concentrations tested were: 5, 10, 20, 30, 40, and 50 mg/L FA; 20, 40, 60, 80, 100, 120 mg/L AA, SA, and CA; 30, 60, 120, 240, and 480 mg/L Q, GA, K, R, and C; and E3 embryo medium (0mg/L) as control. Experiments were performed in triplicates (n = 60 fish/concentration). The embryo-larval development was assessed individually at 24, 48, 72, and 96-hours exposure. Death (coagulation, dead embryo/larvae); hatching; pigmentation patterns; somite and eye formation; heart beating; tail blood circulation; absorption of the yolk sac; hearth and yolk sac edema; balance; and tail, spine, head, and eye deformities were quantified. LC₅₀_{96h} values were calculated, and ammonia levels and pH were monitored during the experiments. The 96-hours mortality had a concentration-response relationship for AA (LC₅₀_{96h} = 80.76 mg/L) and FA (LC₅₀_{96h} = 12.48 mg/L). Embryos treated with SA, R, GA, K, Q, CA and C did not show concentration-dependent mortality, and their LC₅₀_{96h} values are > 480 mg/L. The control groups showed normal morphology and low mortality (< 10%). The mortality observed was exhibited at different experimental times: at 24-hours exposure for all concentrations of R, GA, Q, K, C, and CA, and ≥ 40 mg/L FA; at 48-hours for 30 mg/L FA; at 72-hours for 20 mg/L FA, ≥ 100 mg/L AA, and ≥ 20 mg/L SA; at 96-hours for 10 mg/L FA, and 80 mg/L AA. Embryos treated with AA, SA, R, GA, K, and Q exhibited hatching delay, bradycardia, yolk sac and cardiac edema; and spine malformation just for embryos exposed to Q. FA, CA and C showed balance disorder, cardiac edema and hatching delay, mainly at the highest concentrations. Thus, undesired effects of these compounds were identified at different concentrations and experimental times, which are relevant for proposing safe and effective antioxidant treatments for oxidative stress related diseases, such as glaucoma.

P12 – Epidemiological toxicological studies**P12-01****Dioxin-like activity in serum of Danish and Greenlandic Inuit pregnant women and fetal growth indices***M. Long¹, M. Wielsøe¹, E. C. Bonefeld-Jørgensen^{1,2}

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Background: Human exposure to lipophilic persistent organic pollutants (POPs) is ubiquitous due to their resistance to both biotic and abiotic degradation. Because of different diet, lifestyle and genetic factors, the POPs profile differ between Danes and Greenland Inuit, where Inuit have higher body burdens of POPs. The human exposure to complex POP mixtures is life-long beginning during critical fetal developmental. Exposure to lipophilic POPs such as dioxins, i.e. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and non-ortho and mono-ortho polychlorinated biphenyls (PCBs) can cause a series of negative effects both in animals and in humans. The aryl hydrocarbon receptor (AhR) mediates the species- and tissue-specific toxic responses of dioxins and dioxin-like PCBs.

Aim: i) to measure the combined serum dioxin-like activity of 800 Danish pregnant women and 473 Greenlandic Inuit pregnant women sampled during 2011–2013 and 2010–2015, respectively; ii) to assess the association between maternal serum dioxin-like activity and infant growth indices.

Methods: The serum lipophilic POPs fraction was extracted by Solid Phase Extraction (SPE) and clean up on Supelco multi-layer silica column and florisil column. The combined dioxin-like activity in the serum lipophilic POP fraction was determined as AhR transactivity using an AhR reporter gene bioassay and expressed as pg TCDD equivalent (TEQ) per gram serum lipid [AhR-TEQ (pg/g lipid)]. Fetal growth indices, including birth weight, birth length, head circumference and gestational age, were obtained from the clinical record. One-way ANOVA was used to assess the relation between dioxin-like activity and different characteristics and lifestyles. Multivariable linear regression model was used to assess the possible association of dioxin-like activity with the maternal serum levels of POPs and fetal growth indices.

Results and Discussion: In Danish pregnant women 93.9% of the serum extracts had detectable AhR-TEQ with the median level of AhR-TEQ 185 pg/g lipid. For Greenlandic Inuit pregnant women 87.7% had detectable AhR-TEQ with the median AhR-TEQ level of 86.2 pg/g lipid.

The AhR-TEQ positively correlated with serum POP levels in both Danish and Greenlandic Inuit pregnant women.

In Danish maternal serum, the AhR-TEQ levels were positively associated with infant birth weight, birth length and gestational age. Whereas, for the Greenlandic Inuit, the maternal AhR-TEQ levels tended to be inversely associated with the fetal growth indices.

Differences in POPs profile, genetic factor and lifestyle of Danish and Greenlandic Inuit might play roles in the observed results.

P12-02

Assessment of Impacts on the Lung of Long-term Exposure to Copper-Containing Dust at a Copper Smelter – Historical Spirometry Data, Examination via Body Plethysmography and Inflammatory Biomarkers in Blood and Sputum

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Occupational exposure limits for Cu dusts are under discussion in Europe and North America. Actual human exposure to Cu in the occupational context is complex, and may occasionally be considerably higher than, for example, the reduced limit value of 10 µg/m³ (respirable fraction) as proposed by SCOEL in 2014^[1]. However, Cu-related adverse health effects have rarely been reported in occupational medicine. Addressing this discrepancy and the relative lack of human data, two studies were conducted on workers of a Cu smelter:

(1) Reviewing the medical files, especially spirometry data, within a historical *health surveillance study* and (2) complementing the information with state of the art examinations on current employees addressing lung function and inflammatory parameters within a *cross-sectional study*.

Health surveillance study

Health surveillance data of 476 Cu-exposed and 172 control subjects were collected in 2018 covering on average > 16 years of follow-up. Declines in FEV₁ were compared between both groups and among different exposure levels within the exposed group. Historical exposures were estimated based on airborne monitoring data and cumulative Cu exposures were derived for each subject.

Declines in FEV₁ among never smokers in both exposed and control groups were similar to a typical age-dependent decline of 29 mL/yr.^[2] A similar outcome was observed for the highest exposure tertile

sub-group (cumulative Cu exposure of > 5.5 mg/m³-years). These findings indicate that cumulative Cu exposures averaging 4.61 mg/m³-years as inhalable fraction, which is approximate to 1.06 (0.32–1.80) mg/m³ years as respirable fraction, do not adversely impact lung function.

Cross-sectional study

174 currently employed workers (104 exposed and 70 control subjects) volunteered to participate in the cross-sectional study in 2019, which included the following examinations:

- General medical parameters such as height, weight, blood pressure, pulse, etc.;
- Whole body plethysmography including diffusion capacity of the lung tissue;
- Measurement of exhaled nitric oxide (FeNO);
- Blood sample for analysis: Differential blood count and inflammatory parameters;

Furthermore on a separate date, sputum was collected from a subsample of 40 subjects (28 of the exposed group and 12 of the control group), identified as non-smokers. In addition, information on, i.e. tobacco consumption and usage of respiratory protective equipment was obtained from a survey handed to the participants prior to the examination.

Lung function and inflammatory values of the exposed and control group were compared and put into context with published ranges of healthy controls. The findings did not indicate any effects that could be attributed to Cu-containing dusts.

In conclusion the findings of the current studies have not raised concerns regarding functional or inflammatory effects on the lung of workers exposed long-term to Cu-containing dust in a Cu smelter.

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P12-03

Kidney damage associated with tobacco consumption. Role of oxidative stress

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Tobacco consumption is related basically to various types of cancer, in addition to other diseases of the cardiovascular and respiratory systems. Although new research relate tobacco consumption with the development of kidney damage, the classic markers currently used nowadays, such as plasma creatinine, are not useful to show this kidney injury. In this way, innovative diagnostic methods, among which there are biomarkers of subclinical kidney damage, have been developed. Moreover, in clinical studies carried out in our laboratory, it was found that smoking patients had subclinical kidney damage shown by the excretion of these urinary biomarkers. The aim of this work was to elucidate whether oxidative stress is involved in subclinical kidney damage caused by tobacco consumption.

A transversal study, in which there were included 300 smokers and non-smokers without kidney damage and without risk factors for kidney disease, was carried out. They came from Primary Care of a Health Center. Urine samples were taken. In these samples were

determined by ELISA or colorimetric techniques, the biomarkers of subclinical kidney damage: N-acetyl- β -D-glucosaminidase (NAG), lipocalin associated with neutrophil gelatinase (NGAL) and kidney damage molecule 1 (KIM-1), as well as oxidative stress biomarkers: malondialdehyde (MDA), Vanin-1 and 8-isoprostane. Furthermore, the total antioxidant capacity of the patients was analyzed in the urine samples.

The results obtained showed a higher excretion of both the biomarkers of subclinical kidney damage and the oxidative stress biomarkers in the urine of smoker patients compared to the non-smoker ones. In addition, a significant correlation between kidney damage and oxidative stress was demonstrated. However, the total antioxidant capacity of the body was also higher in the smoker patients, which could be a defense mechanism against kidney damage. Our results indicate that oxidative stress could play a relevant role in subclinical kidney damage caused by tobacco consumption.

P12-04

This abstract has been withdrawn.

P12-05

Biomonitoring of metals on maternal-fetal unit and its possible association with oxidative stress biomarkers

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Human population is constantly exposed to environmental pollutants, for example, heavy metals and metalloids which are directly related to industries and mining areas, within which mercury (Hg), lead (Pb), arsenic (As) and cadmium (Cd) stand out, considered as the main pollutants by the European Commission (2006). Biomonitoring allows us to identify and eliminate possible sources of exposure, studying possible relationships between these pollutants and health problems and identifying groups of populations vulnerable to certain pollutants. Among all population groups, pregnant women are one of the most vulnerable groups since these substances can cross the placental barrier, having consequences on the health of the newborn. The aim of this work was biomonitoring the levels of metals (As, Cd, Cr, Cu, Fe, Hg, Ni, Pb, Se, and Zn) in a cohort of mother-child pairs in Seville (Spain) and its possible relationship with biochemical parameters such as oxidative stress biomarkers (lipid peroxidation, protein oxidation and the activity of antioxidant enzymes such as superoxide dismutase, catalase and total antioxidant capacity), and levels of vitamins D and B. Samples of maternal and cord blood have been taken at the time of delivery, previous signing of the informed consent, and filling the epidemiological questionnaire. It should be noted that in the preliminary results presented, participants were grouped by areas of residence, age and health habits (mainly smoking and dietary habits). Results showed no correlations between groups and metal levels with the exception of Hg and fish consumption, where a positive correlation was observed. The highest levels of Hg detected in maternal blood samples were above 5.8 $\mu\text{g/L}$, which is the threshold limit established by WHO. Another remarkable result was that about 40% of our participants presented hypovitaminosis D with levels below the normal value of 50 nmol/L.

P12-06

(Bio)monitoring Wildland Firefighters' Occupational Exposure

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Climate changes is expected to increase the frequency of unstable weather conditions which may result in additional number of forest fires, with longer fire seasons and more potent fire. Portugal has been severely affected by extraordinary and uncontrolled wildfires, particularly the northern and center districts. Wildland firefighters are heavily exposed to a wide range of health hazardous gaseous chemicals and particulates, most of which are known carcinogens. IARC have inclusively classified occupational firefighting activity as possibly carcinogenic to humans. However, firefighter is among the least studied occupations in terms of exposures and the relationship to occupational diseases. The difficulty involved in the collection of data on personal exposure during firefighting activities has largely contributed to this scientific gap. The integration of exposure data with biomarkers information is of great importance for accessing the short- and long-term health risks in occupational health. Thus, Bio-4FOX main goals are to a) characterize firefighter's occupational activities and behaviours through questionnaires; b) assess personal exposure levels to health-relevant hazardous pollutants through metabolites in exhaled air; c) evaluate early effects at molecular and cellular levels in systemic and local-target tissues and d) integrate data for a comprehensive assessment of firefighter's occupational exposure and related health impacts. The study will comprise three different phases (pre-exposure, during exposure, and post-exposure to fire season). Around 200 wildland northern Portuguese firefighters will be engaged in the study. A characterization of study population will be conducted through collected data via questionnaires. Volatile organic compounds will be assessed by the means of metabolites in exhaled air by the e-Nose equipment (dose biomarkers). Genomic instability will be assessed through effect biomarkers, namely, micronucleus test in buccal and urothelial cells and DNA primary and oxidative damage in blood by comet assay. Statistical analysis will be performed to evaluate the differences within the three study phases, linking information on dose and effect biomarkers and individual data. The results obtained will contribute to a better characterization of firefighters' occupational exposure, providing valuable data from (bio)markers of exposure and early-effect biomarkers. We expect that improvements herein proposed may contribute to better definition of policies and prevention strategies for occupational exposure, as well as for implementation of safety and hygiene measures greatly needed in this sector.

P12-07

Maternal tobacco smoke exposure and determination of the optimal urinary cotinine cut-off values: a cross-sectional birth study

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Tobacco consumption and environmental tobacco smoke (ETS) exposure during pregnancy remain an important public health concern worldwide. Population-based epidemiologic studies often rely on self-reported data to assess smoking and ETS exposure status, although tobacco smoke exposure prevalences have been hindered by high rates of misreporting, particularly among pregnant women. Cotinine, which is the main metabolite of nicotine, is commonly used as a biomarker of tobacco smoke exposure. Within the frame of the NEOGENE project, the aim of this study was to identify the optimal UC cut-off values to discriminate smoking and ETS exposure status and to assess the validity of self-reported smoking and ETS exposure status using urinary cotinine (UC) as a biomarker.

This cross-sectional birth study enrolled 595 pregnant women who received prenatal care in a public hospital in Porto (Portugal), from April 2017 to July 2018. A structured questionnaire, including sociodemographic characteristics, tobacco consumption and exposure before and during pregnancy was completed for all the participants, during the hospital stay in a face-to-face interview. Cotinine concentrations were measured by solid-phase competitive enzyme-linked immunosorbent assay (ELISA), in maternal urine samples, collected on the day of delivery.

The optimal UC cut-off value identified to distinguish smokers from non-smokers in the third trimester of pregnancy was 74.1 ng/mL (sensitivity = 96.7% and specificity = 98.0%). On the other hand, 1.6 ng/mL was the optimal UC cut-off value identified to discriminate within non-smokers those who were exposed to ETS from those non-exposed (sensitivity = 66.2% and specificity = 75.7%). In this study, the concordance of maternal self-reported and urinary cotinine-based smoking status was very good (kappa coefficient = 0.919, $p < 0.001$), while there was a substantially lower agreement between self-reported and urinary-cotinine based ETS exposure (kappa coefficient = 0.386, $p < 0.001$).

The UC cut-off values here established are the first to be identified for pregnant women in Portugal and represent current levels of exposure. Besides, they contribute to a more accurate assessment of in utero tobacco smoke exposure, particularly in the case of passive smoking in which information is still scarce; and are fundamental in a population-based epidemiologic study as this one, to avoid further biased risk assessments.

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P12-08

Development and Validation of a Method for Selenium Determination by Flame Atomic Absorption Spectrometry in Dietary Supplements and Food Samples

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Introduction: Selenium is an essential micronutrient necessary for the proper functioning of the human body. Selenium is an integral part of selenoproteins including important antioxidant enzymes. Health-related properties of selenoproteins include among others proper functioning of antioxidant system, immune and reproductive

system as well as the thyroid gland. Insufficiency of selenium can lead to dysfunction of all the above-mentioned important systems in the human body. Studies have shown that Estonian soils are naturally poor in selenium and the average selenium blood level among the Estonian population is below the recommended level.

Therefore, the current research aimed to develop and validate a suitable method for the determination of selenium by flame atomic absorption spectrometry (FAAS) in dietary supplements and food samples.

Methods: The most commonly used methods for selenium determination are inductively coupled plasma spectroscopy (ICPMS) and graphite furnace atomic absorption spectrometry (GFAAS). Considering the cost-efficiency, a large number of samples and the availability of apparatus at THCC, the FAAS method was chosen for development.

Results: The following validation parameters were determined: repeatability, reproducibility and measurement uncertainty. The developed and validated method was successfully applied to the determination of selenium in dietary supplements and food matrices. The selenium content of the analysed selenium supplements corresponded to the nominal value of the package considering the extended measurement uncertainty of the methodology ($U_{(k=2)} = 20\%$). The average selenium content of the analysed Brazil nuts sold in Estonia was 42 µg/g and 158 µg per one nut, which is sufficient to cover the daily recommended amount of selenium.

Discussion: Due to Estonia being located in the selenium-poor region, it is very important to know the selenium contents of the food sold in Estonia. It is therefore important to develop a fast and inexpensive method for more extensive analysis in food products. As the FAAS method seems to be suitable for determinations of concentrations over 1 µg/1g, it would be sufficient to determine selenium in foodstuffs.

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P13 – Geno-toxicology & carcinogenesis

P13-01

Application of newly validated *in vitro* 3D skin genotoxicity and hen's egg micronucleus test assays to support the safety assessment of cosmetic ingredients

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The current *in vitro* test battery recommended by the EU Scientific Committee on Consumer Safety for genotoxicity testing of cosmetic ingredients consists of the Ames and *in vitro* micronucleus (MNvit) tests. While this 2-test battery has a high sensitivity for prediction of *in vivo* genotoxic/carcinogenic agents it tends to over-predict the genotoxicity hazard, resulting in misleading positive results. Therefore, new *in vitro* models have been established as follow up assays to improve the prediction in the absence of *in vivo* data. The reconstructed skin (RS) Comet and RS micronucleus (RSMN) assays were designed for topically exposed chemicals (e.g., shampoo ingredients); whereas, the hen's egg micronucleus test (HET-MN) was developed for use scenarios that more readily lead to systemic exposure (e.g., oral – lipstick and toothpaste ingredients). The strategy is based on an endpoint-triggered follow up of positive results from the 2-test battery. For chemicals in topically applied products, the RSMN assay is recommended as a follow-up for MNvit positive results; whereas, Ames positives should be followed-up with a 3D Skin Comet assay. For MNvit positive chemicals in products that lead to systemic exposure, the HET-MN is recommended. We report on the validation of all three assays, which are now complete. The skin assays exhibited a very good sensitivity and specificity: 77% and 88% for the 3D Skin Comet (32 compounds) and 80% and 87% for the RSMN assay (47 compounds). A combination of the two skin-based assays enables all three types of DNA damage (mutation, clastogenicity and aneugenicity) to be addressed. Importantly, since many of the true positive chemicals were positive in Ames and MNvit, the sensitivity increases to 89% if the above endpoint-triggered strategy is applied (both RS assays would be performed). Both skin assays were accepted into the OECD guideline development program in April 2019. The HET-MN assay, applied for substances with expected systemic exposures, also exhibited a very good sensitivity and specificity: 83% and 98%, respectively (32 compounds). In conclusion, the observed high predictivity of the expected genotoxicity *in vivo* across the three assays supports their use as follow up tests to the standard 2-test battery. This tiered strategy shows great promise as an *in vitro*-only approach for genotoxicity testing of cosmetic ingredients.

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P13-02

Effects of cold atmospheric pressure plasma on various plant seeds

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Climate change, environmental pollution, and pathogen resistance to available chemical agents are part of the problems that the food industry has to face in order to ensure healthy food for people and

livestock. One of the promising solutions to these problems is the use of cold atmospheric pressure plasma. Cold atmospheric pressure plasma is partially ionized gas where the energy is stored mostly in the free electrons and the overall temperature remains low^[1]. It has become a subject of significant research effort in recent years. In addition to common areas of plasma applications, such as surface finishing or removal of gaseous pollutants, there are many scientific teams focused on the study of plasma interactions with cells and microorganisms. Current results demonstrate the possibility of plasma application in medicine, agriculture, pharmaceutical, and food industry^[2,3]. In agriculture, plasma is suitable for efficient surface decontamination of seeds and food products, germination enhancement, and obtaining higher yields in agricultural production^[4]. However, the plasma effects vary due to plasma source, treatment conditions, and seed type.

In our experiments we used Diffuse Coplanar Surface Barrier Discharge for plasma treatment of various plant seeds (pea, barley, and soybean). As plasma represents low doses of oxidative stress, activities of antioxidant enzymes and genotoxic effects after plasma treatment were determined. Plasma has also a positive effect on seed germination, therefore various germination parameters and lytic enzyme activities were analysed after plasma treatment.

Our results suggest that plasma in low doses is suitable for enhancement of seed germination, and it does not have genotoxic effects on plant organisms. However, appropriate conditions (exposure time or working gas) of plasma treatment are different for each type of seed. In general, the most harmful effects of plasma treatment were observed using nitrogen as working gas. On the other hand, plasma generated in ambient air and oxygen in low exposure times had positive effects on all seeds used in experiments. Therefore, cold atmospheric pressure plasma application can represent a new green technology for the germination improvement of various seeds and grains, but its potentially harmful effects need to be further investigated.

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P13-03

Genotoxicity, DNA damage response and cell fate in human lung cells after the treatment of diverse genotoxic compounds

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Objectives: DNA damage is associated with various pathophysiological processes including cardiovascular diseases or cancer. DNA damage response (DDR) is activated upon genotoxic stress to promote DNA repair, inhibit cell cycle progression, and alternatively trigger apoptosis or senescence. In the present study, we aimed to compare a genotoxic potential of model polycyclic aromatic hydrocarbons (benzo[a]pyrene, BaP, and 1-nitropyrene, 1-NP) and anticancer drugs (doxorubicine, DOX, and bromodeoxyuridine, BrdU) and to study their

effects on persistence and dynamics of DDR and cell fate decision in human lung cells BEAS-2B.

Methods and results: An alkaline version of Comet assay was used to determine the level of DNA strand breaks and oxidative DNA damage. The greatest extent of DNA damage, particularly oxidized lesions, was observed in cells treated with BaP and 1-NP following 4h exposure while DOX and BrdU induced a comparatively lower level of damaged sites. DNA strand breaks generally tended to decrease with a longer period of exposure (24h) while oxidative damage remained unchanged. Flow cytometry was used to evaluate the effect on cell cycle and apoptosis following different incubation periods (24, 48, and 48+72h). DOX treatment resulted in marked accumulation of cells in the G2 phase indicating G2 arrest at each time point while BrdU caused a mild increase of cells in the G1 phase and BaP and 1-NP had no effect. The proportion of apoptotic and necrotic cells was massively increased in response to DOX after 48+72h incubation. Detection of key proteins involved in DDR by Western blotting and immunocytochemistry surprisingly did not show activation of γ H2AX after the treatment of any tested compound suggesting the absence of double strand breaks. Expression of p21 and p53 was elevated in response to 48h and 48+72h treatments of DOX and BrdU. β -galactosidase activity assay was used to study the pro-senescence effect of the tested compounds. We observed an increase of senescent cells after the 48+72h exposure to DOX and BrdU. No effect was found after BaP and 1-NP treatment.

Conclusions: Although DOX and BrdU exhibited lower genotoxic potential, both compounds activated DDR proteins in BEAS-2B cells, affected cell cycle, increased number of apoptotic cells, and induced senescence. In contrary, cells incubated with BaP and 1-NP, more potent genotoxins, displayed a better survival rate despite a high level of DNA damage.

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P13-04

MWCNTs induced epithelio-mesenchymal transition of BEAS-2B cells

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In the field of nanotechnology, the use of multi-walled carbon nanotubes (MWCNTs) is growing. Pulmonary exposure during production, use, and handling of these nanomaterials rises concern about their potential adverse health effects. Because of their physico-chemical properties as well as their biopersistence, they share similarities with asbestos fibres, what rises questions regarding their carcinogenic potential. Because of their small size, they can easily go deep into the respiratory tract and deposit on the lung epithelium surface.

The purpose of this study is to assess how MWCNTs physical characteristics such as diameter and/or length can play a role in cellular (geno)toxicity and be linked to their potential carcinogenicity. Our experimental design is based on the treatment of human bronchial epithelial cells (BEAS-2B) for six weeks with low concentrations (0.125 – 1 $\mu\text{g}/\text{cm}^2$) of MWCNTs with opposite characteristics: the short and thin NM-403 and the long and thick Mitsui-7. After 6 weeks of treatment, a recovery period of 4 weeks allows the reversibility of the effects.

Following treatment with both MWCNTs, about 15% of cells in mitosis showed mitotic abnormalities from the first week. In addition, a genotoxicity test showed a time-independent increase of

micronucleus-positive cells. In parallel, we observed a cytotoxic effect, which is delayed in cells treated with NM-403. After 4 to 6 weeks of treatment, clear cellular morphological changes from epithelial to fibroblast-like phenotype were also noted. Concomitant with this observation, a modification of cell population composition appears gradually with time and concentration of MWCNTs. BEAS-2B cells undergo a conversion of epithelial to mesenchymal state as we observed a decrease of the epithelial marker E-cadherin at the mRNA and protein level and an increased expression of mesenchymal markers i. e. N-cadherin, Vimentin, Fibronectin. After 4 weeks of recovery, we have shown that the induced epithelio-mesenchymal transition can be reversed and that the degree of reversibility depends on the length and the diameter of the MWCNT.

P13-05

Standardisation of mutagenicity testing approaches, tailored to the evaluation of engineered nanomaterials

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Introduction: The hypoxanthine-guanine phosphoribosyltransferase (HPRT) forward mutation assay is a standardised method for assessing mutagenicity induced following exposure of mammalian cells to chemicals. However, this assay has not been standardised for the assessment of engineered nanomaterials (ENMs). The present study therefore aims at achieving this harmonisation for testing ENM mutagenicity using the HPRT assay in two separate laboratories. An interlaboratory approach was employed incorporating two OECD-approved cell lines; Human lymphoblastoid (TK6) and Chinese hamster lung (V79-4) cells to investigate the mutagenic effects of titanium dioxide (TiO₂) zinc oxide (ZnO) nanoparticles. The highly mutagenic agent methyl-methanesulfonate (MMS) was used as a positive control.

Results: When the HPRT assay was conducted with TK6 cells by Laboratory 1, ZnO and TiO₂ ENMs did not induce significant mutagenicity; however significant ($p < 0.05$) cytotoxicity was recorded at 10 $\mu\text{g}/\text{ml}$ and 50 $\mu\text{g}/\text{ml}$ for ZnO and TiO₂ respectively. TK6 cells tested at Laboratory 2 showed no significant cytotoxicity or mutagenicity following exposure to both ENMs. When the V79-4 cells were used by Laboratory 1, no cytotoxicity was noted with either ENM, but significant mutagenicity was observed following exposures to ZnO and TiO₂ from 5–20 $\mu\text{g}/\text{ml}$ and 10–100 $\mu\text{g}/\text{ml}$ respectively, with up to 2-fold increases in point mutation frequency over background levels. V79-4 experiments at Laboratory 2 similarly indicated no significant cytotoxicity but also no observable mutagenicity. Independent statistical analysis demonstrated that there was good concordance across the data sets originating from the two laboratories with this assay, with no significant differences in accuracy or precision between both groups. However, the difference in detection of mutagenicity when using the V79 cells indicated that further standardisation of scoring approaches will be necessary.

Summary: The data generated in this project has shown good concordance between the two laboratories pertaining to HPRT data generated using two OECD-approved cell lines. Whilst the SOP has been optimised and harmonised for ENMs, it is evident that additional training and guidance for the colony scoring practices are necessary, as this is the primary source of inconsistency between the two laboratories.

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P13-06

Impurity mediated genotoxicity of glyphosate technical grade active ingredients

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Glyphosate-based formulations are used as broad-spectrum herbicides and crop desiccants. The data on the genotoxicity of glyphosate are contradictory.

The genotoxicity of 3 glyphosate technical grade active ingredients (TGAs) (the purity of TGAs 1, 2, and 3 – 96.6%, 95.8 and 95.7%, respectively) and the glyphosate-based formulation (360 g/l) was assessed using the Ames test, the micronucleus (MN) test *in vivo* and DNA-comet assay.

Glyphosate TGAs did not induce reverse gene mutations in bacteria *S. typhimurium* (TA97, TA98, TA100, TA102, TA1535 strains).

In MN-test on CD-1 mice, the various effects were obtained. TGAs 2 and 3 did not cause a significant increase in the frequency of micronucleated polychromatic erythrocytes (mPCE) in the bone marrow. TGA1 induced a low but statistically significant genotoxic effect at all doses. A statistically significant linear dependence of the frequency of mPCE on the doses was revealed ($p=0.002$).

A comparison of the impurity contents in TGAs was carried out. Two impurities – nitrosoglyphosate and formaldehyde were of particular interest from the point of view of genotoxicity. According to the certificates of analysis, nitrosoglyphosate was not detected in any of TGAs. The formaldehyde content in TGAs 1, 2 and 3 was 0.13%, 0.024% and 0.06%, respectively. The formaldehyde level in glyphosate TGAs of 0.13% is the maximum acceptable level according to the FAO specification. However, according to the amended annex I of Directive 91/414/EEC, the content of formaldehyde impurity in glyphosate should not exceed 1 mg/kg.

It was shown that the frequency of mPCEs in mice bone marrow depends on the content of formaldehyde in glyphosate TGAs. A statistically significant genotoxic effect and a significant linear dependence of the frequency of mPCEs on the dose were revealed only in the presence of 0.13% formaldehyde in the TGA1. At lower concentrations of this impurity, no significant induction of MNs formation in bone marrow cells was revealed.

DNA-comet assay shown that administration of TGA1 to mice at dose 2000 mg /kg b.w. caused a statistically significant increase in the percentage content of DNA in the bone marrow cells ($p<0.01$).

Thus, the genotoxic activity of glyphosate may depend on the content of impurities in the TGAs, in particular, formaldehyde. The obtained data are an additional justification for lowering the upper limit of the formaldehyde content in glyphosate TGAs.

The glyphosate-containing formulation did not induce reverse gene mutations in bacteria and the formation of micronuclei in bone marrow PCEs of laboratory animals. However, the formulation had higher toxicity than the technical product.

P13-07

Carcinogenicity assessment of cyclohexanone through inhalation exposure

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Cyclohexanone is an industrially used substance and is produced over 4 million tons per year worldwide. It is widely used in the semiconductor industry, which is the core field of the 4th industrial revolution, electronic industries and medical supplies manufacturing. In Korea, cyclohexanone is a substance subject to work environment measurement, but carcinogenicity through inhalation exposure has not been evaluated. Therefore, this study was conducted to evaluate the carcinogenicity of cyclohexanone through inhalation exposure.

In a 90-day repeated dose toxicity study, significant effects of cyclohexanone were observed at concentrations of 200 and 625 ppm. In male rats, an increase in ALT and AST values with an increase in the liver weight and proliferation of the hepatic bile duct were observed. A dose-responsive increase in BUN value and a lesion of tubular basophilization in the kidney were observed. And the exposure concentration in the working environment is specified as 20 ppm for TLV and 50 ppm for STEL in ACGIH. Therefore, the exposure concentrations in this study were set to 20, 60 and 200 ppm including the control group in consideration of these effects and the exposure regulation of cyclohexanone.

This study was performed to evaluate the carcinogenicity of cyclohexanone by exposing the set concentration in F344 rats for 6 hours a day, 5 days a week, and 104 weeks. As a results of this study, exposure-related effects of cyclohexanone in clinical signs, body weight changes, food consumptions, hematology and blood biochemistry examinations were not observed, and no increase in neoplasm and tumors incidence related to exposure was observed.

In the results of ACGIH documentation, thyroid adenoma-carcinoma was observed in the male high-dose group(6500 ppm) in the 2-year oral carcinogenicity study of cyclohexanone. But thyroid tumorigenesis is not related to humans at comparably low levels of the chemicals.

Based on these results, the effect of cyclohexanone was not observed up to the high concentration of 200 ppm in F344 rats, so NOAEL(No Observed Adverse Effect Level) was evaluated over then 200 ppm. In addition, no increase in neoplasm or tumor incidence was observed, so the carcinogenicity of cyclohexanone was not confirmed under this study condition.

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P13-08**Comparison of genotoxic activity of benzimidazole pesticides**

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Benzimidazole pesticides are systemic fungicides. This group includes carbendazim, benomyl, thiophonate methyl, thiabendazole, and fuberidazole. The comprehensive research of carbendazim and benomyl revealed serious adverse effects, including genotoxicity^[1-2]. But some countries continue to use these pesticides. The results of most genotoxicity studies of thiabendazole were interpreted as being negative but there are limited data of its positive activity on *Salmonella typhimurium* and mice (micronucleus test)^[3].

The purpose of this study was to evaluate and compare the genotoxic effects of TGAs of carbendazim and thiabendazole.

Two carbendazim TGAs and two thiabendazole TGAs were studied using the Ames test, mammalian erythrocyte micronucleus test, and comet assay.

Thiabendazole TGAs did not cause genotoxic effects in any of the tests. TGA I and TGA II of carbendazim showed negative outcomes in *Salmonella* strains TA100, TA102, and TA1535 ($\pm$ S9). In the case of TGA I, a dose-related and reproducible statistically significant increase in the mean of revertant colonies per plate on TA98 was observed. The fold increase of revertants at the peak of the dose-response was greater than 5 times the concurrent vehicle control. TGA II was negative in TA98 ($\pm$ S9). We found weak but reproducible and dose-related effects of both carbendazim TGAs in TA97 in the presence of S9 mix. The increase in the number of revertants at the highest dose was 1.7–1.8-fold.

In vivo, both TGAs of carbendazim caused similar genotoxic effects. A dose-related and statistically significant increase in the incidence of micronucleated polychromatic erythrocytes (mPCEs) in bone marrow was found (20.0-fold relative to negative control at the high dose of 2000 mg/kg b.w.). Comet-assay did not reveal DNA damage confirming the aneugenic effect of carbendazim. Furthermore, carbendazim TGAs caused a dose-dependent increase in the proportion of macrocytes and dumbbell-shaped cells with constrictions among PCEs and normochromic erythrocytes probably due to distortion of cytokinesis. The accumulation of polychromatophiles with pyknotic nuclei in bone marrow was observed. This suggests that carbendazim delays or disrupts the process of nuclear extrusion.

Comparison of genotoxic activity of the tested benzimidazole fungicides shows that carbendazim but not thiabendazole damages the genetic structures in prokaryotic and eukaryotic cells. Carbendazim not only disrupts the karyokinesis (aneugenic effect) but also inhibits cytokinesis and the nuclear extrusion during the erythroid cell maturation. The observed abnormalities could possibly be mediated by similar mechanisms based on the interaction of carbendazim with tubulins.

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P13-09**Quantitative dose response modelling of historical ToxTracker data to evaluate *in vitro* genotoxicity and mode of action**

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ToxTracker is a *in vitro* mammalian stem cell-based reporter assay that detects activation of specific cellular signalling pathways upon chemical exposure using flow cytometry. ToxTracker contains six different GFP-tagged reporter cell lines that together allow the discrimination between induction of DNA damage, oxidative stress and/or protein damage in a single test. Genotoxicity is detected by the Bsc12-GFP reporter for pro-mutagenic DNA lesions and DNA replication stress, and the Rtkn-GFP reporter for DNA double strand breaks. The Srxn1-GFP and Blvr-GFP reporters are activated upon oxidative stress, activation of the Btg2-GFP reporter correlates with p53 activation and the Ddit3-GFP reporter is activated by protein stress.

Here we used quantitative dose response modelling to analyse the multivariate concentration-response data. First we defined a fold-change threshold for identification of a significant positive response and defined endpoint-specific Benchmark Response (BMR) values. Next, we used the BMD (Benchmark Dose) combined-covariate approach for potency ranking of a set of more than 120 compounds, and used PCA (Principal Component Analysis) to investigate functional and statistical relationships between the ToxTracker reporters.

The bootstrapping approach yielded fold-change cut-offs of 1.5 for the 95th percentile and 1.7 for the 99th percentile. The 1.5-fold matches with the value used for weak responses and this analysis also demonstrates that the 2-fold cut-off for a positive response in ToxTracker is very conservative. For 120 compounds, the BMD was applied for potency ranking by comparative identification of the most potent inducers of each reporter. The PCA results, based on the BMD results for all substances, indicated the DNA damage (Rtkn, Bsc12) and p53 (Btg2) reporters are functionally redundant and indicative of genotoxic stress. The oxidative stress and protein stress reporters are orthogonal indicators of cellular stress and essential for toxicological profiling using the ToxTracker assay. PCA axis scores reflected the toxicological MOA (Mode of Action) of the tested compounds; hitherto unknown MOAs can therefore be inferred using PCA axis-plot proximity to well-studied compounds.

Overall, dose response modelling of multivariate ToxTracker data can be used to calculate potency and predict the MOA of substances.

P13-10**Denervation of the urinary bladder in spinal cord injury patients as an independent bladder cancer risk factor**

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Bladder cancer in spinal cord injury (SCI) patients, associated with a dramatically worse prognosis, is thought to be due to adverse effects of chronic indwelling catheters like chronic local irritation and urinary tract infections. We challenged this commonly accepted expla-

nation by investigating two large study groups of traumatic spinal cord injury patients managed without chronic indwelling catheters. We investigated all 40 bladder cancer cases of 7396 long-term traumatic SCI patients, 4 bladder cancer cases diagnosed at the initial urological workup on the occasion of the acute spinal cord injury, all in- and out-patients of our Hamburg centre observed from 01.01.1998 to 12.31.2019, and 135 SCI bladder cancer cases from a multicentre study in 28 centres in the German speaking area, managed by different types of bladder management, except chronic indwelling catheters. The urinary bladder cancer data of the German population were obtained from the German Centre for Cancer Registry Data at Robert Koch Institute (RKI) in Berlin. T category and grading of the 40 Hamburg cases were significantly poorer, compared to bladder cancer cases from the general population (p lower to 0.0001, each) and the 4 incidental cases at initial urological workup, resulting in a median survival of 12.0 months. However, these findings are comparable to those in SCI patients managed with chronic indwelling catheters. In the multicentre study, the mean latency time between the onset of SCI and bladder cancer diagnosis was significantly longer in patients with catheter-free emptying methods, compared to patients with intermittent catheterization. The described findings in SCI bladder cancer patients managed with and without chronic indwelling catheter point to the disturbance of the innervation of the urinary bladder as an independent bladder cancer risk factor.

P13-11

Acute exposure to non-specific organophosphate metabolites induce genotoxicity in bone marrow cells of Balb/c mice and human cells *in vitro*

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The organophosphate (OP) compounds are synthetic substances used mostly as pesticides, flame retardants and plasticizers. OP pesticides still represent one of the most used pesticides in agricultural and domestic environments, increasing the OP environmental distribution. During OP pesticides degradation they produce dialkylphosphates (DAPs), that are their non-specific metabolites, and are often employed as exposure biomarkers only^[1]. Previous *in vitro* studies showed that DAPs can also produce genotoxic and immunotoxic effects at low concentrations, and concentrations similar to those reported in occupationally exposed populations^[2–4], which may participate in the general toxic effects related to OP pesticides exposure.

Here, we evaluate the genotoxic effects of dimethyldithiophosphate (DMDTP), dimethylthiophosphate (DMTP), diethyldithiophosphate (DEDTP), and diethylthiophosphate (DETP), compared to their parent compounds malathion (methylated DAPs), and terbufos (ethylated DAPs), respectively. We used bone marrow cells obtained from Balb/c mice exposed to OP compounds *i.p.* for 24 h. *In vivo* micronucleus assays showed that both DMDTP and DMTP (0.05 mg/kg) induced a higher genotoxic response than malathion in female mice, while exposure to DEDTP and DETP (0.05 mg/kg) induce a genotoxic effect similar to that of terbufos exposure (0.005 mg/kg). Interestingly, DAPs genotoxicity was higher in female exposed mice than in males, in which only DMTP and DEDTP induce micronucleus. These results confirm previous reports of *in vitro* genotoxic effects of DMTP, DEDTP and DETP observed in human cell lines using the comet assay. We also evaluated the genotoxic effect of DMDTP using the comet assay in HepG2, and WRL-68 cells lines, and in human peripheral blood mononuclear cells exposed to 1–100 μ M DMDTP. The results in human cells *in vitro* were similar to those observed in the animal model. However, DAPs induced genotoxicity was evident at lower concentrations in human cells than in the murine model.

Altogether, these results demonstrate that the genotoxic effect induced by DAPs exposure occurred in an animal model *in vivo* and in human cell models *in vitro*. These results highlight the importance of including the evaluation of DAPs during OP risk assessment, not only as an exposure biomarker, but as a biomarker of effect because DAPs-induced genotoxicity may play an important role in cancer development associated with chronic exposure to OP compounds or directly to DAPs from other sources.

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P13-12

Citotoxicity, mutagenicity, and genotoxicity of two metabolites of propyl-propane-thiosulfonate (PTSO) and thiosulfinate (PTS): Glutathione conjugate (GSSP) and cysteine conjugate (CSSP)

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Propyl-propane-thiosulfonate (PTSO) and propyl-propane-thiosulfinate (PTS) are two of the main organosulfur compounds present in *Allium* essentials oil with a widely documented antiyeast, antibacterial and antifungal activities. For this reason, they could be used as a food and feed additive in agri-food industry. Following the recommendations of different international organizations, a genotoxic evaluation of substances present in food is necessary to guarantee the health of the consumer. In this sense, PTSO and PTS have shown muta/genotoxicity *in vitro* when S9 fraction is used. Thus, we aimed to show if glutathione conjugate (GSSP) and cysteine conjugate (CSSP) could be responsible of these effects. Thus, taking into account cytotoxicity tests of these compounds, an *in vitro* micronucleus (MN) test (OECD 487) and the standard and enzyme-modified comet assays were performed. Mutagenicity was also tested using the AMES test (OECD 471). The cytotoxicity tests indicated that there was no decrease in cell viability up to solubility concentrations of both metabolites in Caco-2 and L5178Y cells. Regarding the AMES test, there was no significant increase in the number of colonies after exposure of both compounds for the strains 97, 98, 100, 102, and 1535. For the MN test, there was also no increase in the number of binucleated cells for both compounds assayed, and for standard and enzyme-modified comet assays there was also no increase in % DNA tailed and no oxidative DNA-genotoxic damage was found neither. Therefore, we can conclude that the GSSP and CSSP are non-genotoxic nor mutagenic compounds, demonstrating their safety.

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P13-13**Adaptation of the *in vitro* Micronucleus Assay (OECD 487) for the Assessment of the Mutagenic Potential of Nanoparticles**

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Numerous genotoxicity testing methods have been applied to nanoparticles in the last decade, but there is still, no harmonized, validated and approved OECD test guideline for nanoparticles mutagenicity testing. Thus, the current OECD guidelines require adaptation of the respective protocol for nanoparticle testing.

In a joint effort organized by the EU Joint Research Centre (JRC) protocols were developed for testing of nanoparticles in the *in vitro* micronucleus assay. Two protocols were adapted one using the (adherent) cell line V79 and another one using primary human lymphocytes (suspension cultures using human buffy coat as well as whole blood cells). The protocol adaptations concerned the test substance application (dispersion of the nanoparticles according to the "Nanogen-tox" protocol, as well as limiting the top concentration to 100 µg/mL), testing only in the absence of S9 mix and the treatment period (corresponding to 1 cell cycle followed by a 24 h Cytochalasin B treatment).

The mutagenic potential of five nanoparticles (BaSO₄, CeO₂, Au_{5nm}, Au_{30nm} and SiO₂) was analysed using these protocols. Concurrently, Tungstencarbide-Cobalt (WC-Co) nanoparticles were used as a potential particulate positive control and Ethylmethane sulphonate (for V79 cells), Mitomycin C (for human lymphocytes) and Colcemide (for both cell types) as small molecules positive controls. The cellular dose of nanoparticles was quantified by the Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) method after the treatment period.

None of the five tested nanoparticles induced a treatment-related increase in the micronucleus frequency. The putative particulate positive control WC-Co increased the micronucleus frequencies only in the human lymphocyte cultures, but not in V79 cells. Buffy coat cells showed a better reproducibility of a positive response to WC-Co than whole blood cultures and thus are considered more reliable. LA-ICP-MS data showed that the particle mass associated with the target cells increased with the external exposure, the concentrations of the nanoparticles in the cell culture media.

In conclusion nanomaterials can be tested for their mutagenic potential *in vitro* using modified OECD standard protocols – human buffy coat cells are preferred over V79 cells. Concomitantly the nanoparticle burden associated with the target cells can be quantified by LA-ICP-MS. WC-Co can be used as a particulate positive control for these nanomaterial mutagenicity tests.

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P13-14**Assessment of antioxidative and cyto/genoprotective properties of chestnut honey against UVB radiation *in vitro***

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Ultraviolet B (UVB) radiation causes a number of harmful effects in cells including direct DNA damage, oxidative stress, signalling pathway disorders, inflammatory response and photocarcinogenesis. Along with research into the biological basis of malignant cell transformation, great efforts are being made on research into various natural and synthetic compounds that can provide protection against the harmful effects of UV radiation. Natural compounds have a number of advantages because they mostly show low toxicity and good efficacy. In addition to being able to enter the body through food, they are also components of various cosmetic products that are applied directly to the skin. Honey is a completely natural and nutritionally rich product of honey bees. Its content of minerals, vitamins, antioxidants, but also sensory properties, depend on the geographical location of the apiary in the period of active food collection. Previous findings indicate that chestnut honey is characterized with a high content of phenolic components, and consequently exerts a good antioxidant effect. Present study was undertaken to investigate potential geno- and cytoprotective effects of the chestnut honey *in vitro*. The experiment was conducted on the human peripheral blood lymphocytes and plasma. We proposed the hypothesis that treating cells with chestnut honey can increase the survival rate and decrease primary DNA damage as well as the oxidative stress levels after exposure to UVB irradiation. Experimental design included two exposure scenarios: (I) 2 hours of pretreatment of cells and whole blood with honey followed by exposure to UVB irradiation and an additional 1 hour incubation of cells/whole blood at 37°C, (II) treatment including exposure to UVB irradiation and incubation of cells/whole blood with honey for 2 hours at 37°C. The effects of the honey were assessed at concentrations that correspond to the daily acceptable honey intake dose and a 10-fold higher concentration than the daily acceptable dose. Primary damage of lymphocyte DNA, as well as the protective effect of honey, was evaluated using the alkaline comet assay, while the plasma samples were used to determine markers of oxidative stress (lipid peroxidation, levels of reactive oxygen species and glutathione). Obtained results confirmed our assumptions. Chestnut honey showed good cytoprotective and genoprotective, as well as antioxidative effect after exposure of lymphocytes and whole blood to UVB irradiation. The beneficial effect of honey was more pronounced after the treatment of irradiated samples. The optimal concentration of honey was shown to be 1 mg/mL, while the lowest (0,2157 mg/mL) and the highest (10 mg/mL) concentrations showed a prooxidative effect in measuring oxidative stress markers. Our results suggest that chestnut honey ameliorated detrimental effects of UVB radiation, which has to be further studied on other *in vitro* and *in vivo* model systems.

P13-15***In silico* unravelling of descriptors for cytotoxicity and genotoxicity for hazard identification of nanomaterials**

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Introduction: Hazard characterization and safety assessment of engineered nanomaterials (ENMs) demand robust and validated test methods, as well as read-across approaches for grouping. Successful determination of hazards associated with (ENMs) depends on an intimate knowledge of their interactions with target biological materials.

Methods: We have explored the relationships between physico-chemical characteristics of a range of ENMs and their biological effects – cytotoxicity, oxidative stress and genotoxicity (strand breaks and oxidized DNA bases) – using a grouping analysis approach. A large set of data on characterization and biological effects has been collected and analysed with the aim of elucidating modes of action. Most of these data were obtained during the NANoREG and Nanoreg2 projects.

Results: Our results showed that ZnO NMs (NM-110 and NM-111) and Ag-NMs (NM-300K and NM-302) were cytotoxic in both the Alamar Blue (AB) and colony forming efficiency (CFE) assays. The TiO₂ and SiO₂ NMs were not found to be cytotoxic. For genotoxicity testing, TiO₂ (NM-100), ZnO (NM-110), SiO₂ (NM-203) and Ag (NM-300K) were categorized as positive inducing strand breaks (Sbs). The nanoparticle tracking analysis (NTA) was used to measure size of NMs in stock and in media different time points. The modelling and chemoinformatic analyses identified several descriptors to be of importance for cytotoxic potential, mainly related to NMs electron properties and their overall chemical reactivity (HOMO-LUMO energy, redox potential, ionization potential and electron affinity). Similarly, for the genotoxic potential the NMs HOMO-LUMO energy parameter, ionization potential together with pristine size (TEM) are more influential. For the induction of oxidized base lesions, the presence of surface coating was the best determinant (detected by the enzyme-linked comet assay).

Conclusions: An integrated approach combining experimental and *in silico* studies was shown to be of value in identifying hazards associated with ENMs. In particular, grouping analysis can give support where crucial empirical information is lacking.

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P13-16

Overcoming the Restricted Therapy Options and Monitoring Challenges in Metastatic Breast Cancer

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The lower incidence but higher mortality rate of breast cancer in low- and middle-income countries compared to the developed ones indicates the importance of accessibility to treatment and monitoring breast cancer. Current study aimed to evaluate an alternative adjuvant therapy against metastatic breast cancer and to monitor the disease progression as well as the therapy efficiency with a novel approach. Drug re-purposing approach have been adopted to assess a cost-effective therapy option. Agomelatine, an approved melatonin receptor agonist against major depressive disorder have been evaluated for its cytotoxicity (MTT and LDH methods), anti-proliferative (colony formation method), and inhibitory effects on migration and invasion ('scratch' and 'transwell' experiments) in non-tumorigenic

MCF-10A, ER (+) tumoral MCF-7, triple negative tumoral MDA-MB-231 human breast cells and ER (+) 67NR and ER (-) 4T1 murine breast cancer cell lines. Furthermore, anti-metastatic action of agomelatine was assessed in metastatic syngeneic tumor model via tumor growth measurements as well as immunohistochemistry analyses. To obtain low-cost monitoring strategy for breast cancer progression and therapy efficiency, cellular bioenergetic changes of peripheral blood mononuclear cells were investigated in both ER (+) 67NR and ER (-) 4T1 syngeneic tumor models. In addition to agomelatine treatment in both ER (+) and ER (-) tumor models, radiation therapy was used as a positive control in the ER (-) tumor model. The response of mononuclear cells in peripheral blood to treatment was monitored via extracellular flux analyses. According to the results, agomelatine which was found to have *in vitro* cytotoxic, anti-proliferative, migration and invasion inhibitory effects, did not inhibit primary tumor growth on syngeneic tumor models, however, reduced the metastatic sites in animals observed in immunohistochemical analysis. The data also indicated that bioenergetic changes of peripheral blood mononuclear cells is a promising method for monitoring metastatic breast cancer.

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P13-17

Models of Renal Carcinogenesis: Characterisation and Novel Therapeutics

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Increased exposure to chemicals in day-to-day life in the form of pesticides, cigarette smoke and food additives, is associated with side effects such as chemical carcinogenesis^[1]. Due to their important role of filtering blood in order to eliminate endogenous and exogenous compounds, including toxicants, kidneys are exposed to different environmental carcinogens. Potassium bromate (KBrO₃), a salt of the bromate ion, is a nephrotoxic and neurotoxic agent in humans. It is a proven carcinogen in animals^[2] and a possible human carcinogen (IIb) according to the International Agency for Research on Cancer (IARC)^[3]. Although banned in some countries, it is often used as a food additive, specifically in the bread-making process, in the cosmetic industry and water disinfection process^[2]. The ability of KBrO₃ to induce kidney cancers was tested and confirmed in many *in-vitro* and *in-vivo* experimental models^[4,5,6], in which KBrO₃ acted as a tumor initiator and promoter^[7]. The effects of the carcinogen KBrO₃ were studied in a Renal Proximal Tubule Epithelial Cell line (RPTEC) alone and in the presence of chemopreventative compounds. One of these compounds seemed to be particularly effective in reducing cell viability of an *in-vitro* model of renal cell carcinoma, the most common type of renal cancer.

Methods: RPTEC/TERT1 cells were exposed to the carcinogen KBrO₃ individually and in combination with chemopreventative compounds. Toxicity of these treatments was assessed through morphological changes, viability and cytotoxicity assays. Oxidative stress was also assessed by measuring the intracellular concentration of H₂O₂. Oxidative stress-induced DNA damage was estimated quantitatively by measuring 8-OHdG concentrations. Western blot analysis was used to detect the changes in particular oxidative stress-induced proteins.

Results: The carcinogenic effects induced by KBrO₃ were reduced by the co-administration with all of the chemopreventative agents used.

Oxidative stress markers, H₂O₂ and DNA adduct formation 8-OHdG, were reduced.

Conclusion: Targeting and downregulation of TRAF3, NF- κ B and IL-1 gene expressions by KBrO₃ might be one mechanism to counteract apoptosis and cellular dedifferentiation by the nephrotoxic carcinogen, KBrO₃.

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P13-18

Effect of thimerosal over hypoxia-related factors in glioblastoma

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Introduction: Gliomas are the most common and fatal tumors of the CNS but have a limited number of approved drugs, due to low efficacy^[1]. Glioblastoma multiforme (GBM) resistance to therapy can be related to tissue hypoxia, an important element in tumor progression and at overexpression of antioxidant systems such as the thioredoxin system (Trx)^[2]. This system regulates several aspects of tumor biology including DNA synthesis, cell survival and can influence in tissue hypoxia increasing the hypoxia-induced factor 1 α (HIF-1 α) protein and the expression of HIF-regulated genes, such as the vascular endothelial growth factor (VEGF)^[3]. HIF-1 α can also be influenced by the activation of signal transducer and activator of transcription 3 (STAT3) in pSTAT3, an oncogenic pathway^[4]. Mercury compounds are the strongest known inhibitors of the thioredoxin system and, thimerosal (TmHg) with an history of clinical use, arises as potential candidate to GBM therapy^[5].

Objectives: The aim of this study was to assess the effect of thimerosal, a Trx system inhibitor, on hypoxia related factors and angiogenesis in glioblastoma cells.

Materials and Methods: For the study, U87 cell line was used and the effect of TmHg, its metabolite ethylmercury (EtHg) and temozolomide (TMZ; standard therapeutic for GBM) over cellular viability was evaluated by MTT assay.

Cells were treated with sub-lethal levels of TmHg, EtHg, TMZ, including co-exposure groups (TmHg and TMZ). The expression of HIF-1 α , VEGF, STAT-3 and p-STAT3 were analyzed by Western Blot in whole cell lysates. The scratch assay was performed to check cell migration ability after exposure.

Results: The GI50 for a decrease in MTT reduction was 7.53 μ M for TmHg, 10.48 μ M for EtHg and >800 μ M for TMZ after 24 hours of exposure. Expression of HIF-1 α and VEGF were decreased when cells were exposed to TmHg and EtHg (<5 μ M), and the decrease was higher when compared to the co-exposure to TMZ. The phosphorylation of STAT-3 was also altered. Additionally, cell migration was inhibited when cells were exposed to TmHg, EtHg and co-exposure to TmHg and TMZ.

Conclusions: The Trx system inhibitors, TmHg and EtHg, have a notorious dose-dependent inhibitory effect over hypoxia related factors and cellular migration. Co-exposure with TMZ demonstrated a stronger impact on those factors. These are significant results since current therapeutic options for glioblastoma have quite low success and therapy resistance is very common. Therefore, hard repurposing of thimerosal could be a good option to control and fight GBM.

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P13-19

This abstract has been withdrawn.

P13-20

Anti-androgens in the treatment of prostate cancer and risk of secondary cancers: Overview of non clinical models and regulatory perspectives

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Over the ten past years, non-metastatic and castration-resistant prostate cancers have been gradually brought under control, in particular thanks to second-generation anti-androgens which are increasingly present on the market. The ICH S9 guideline for advanced, refractory or recurrent cancers is applied on a case-by-case basis for the non-clinical evaluation of treatments for castration-resistant prostate cancer. This directive allows faster access to treatments for patients without therapeutic alternatives and eliminates the need for several preclinical studies, in particular genotoxicity and carcinogenicity. Considering the lengthening of the life expectancy of patients with nmCRPC, the application of the ICH S9 directive to this pathology is under discussion at the French, American and European regulatory authorities. Repeated dose carcinogenicity or toxicology studies have been carried out for some anti-androgenic treatments which have induced numerous secondary tumors in animal models. In order to better understand the relevance of the animal models used in carcinogenicity studies, and the risk incurred or not from the application of the ICH S9 guideline, bibliographic searches in the lit-

erature, in clinical trials and in pharmacovigilance databases were carried out in order to find out the incidence of these secondary cancers in humans.

Several events show that there is a potential risk of secondary cancers with antiandrogens in humans. An epidemiological study will be carried out in France by Epiphare (epidemiological unit of the National Agency for the Safety of Medicines and Health Products) on anti-androgens and their implication in secondary cancers. Thus, it will be easier to steer the choice of regulation for the CRPC and to judge the relevance of the non-clinical results observed.

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P14 – Immune toxicology

P14-01

This abstract has been withdrawn.

P14-02

Examination of immune status of rats fed with diet, containing Black soldier fly larvae biomass, during 93 days of ontogenesis

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The purpose of the study was focused on the immune status of rats fed with diet, containing Black soldier fly larvae biomass, during 91–93 days of ontogenesis (from the moment of the oocyte fertilization until day 70 of postnatal life). The work was carried out as part of a comprehensive study of the novel insect-derived food safety.

The experiment was performed on 2 groups of male Wistar rats, 20 animals each, of F2 generation bred from animals fed a basic semi-synthetic casein diet for 110 days (control group) or a diet including dry biomass of *Hermetia illucens* larvae (test group). From the moment of separation from their mothers (at 25 day of postnatal life) until day 70, F2 animals from both groups received experimental diets.

Immune status was assessed by the content of cytokines in blood serum (pro-inflammatory, anti-inflammatory, interleukins produced by Th1 and Th2 cells). The studies were performed using Bio-Rad, USA Bio-Plex Pro Rat Cytokine Th1/Th2 Panel, 12-Plex, 1x96, Cat.No:171-K1002M. The results were read on a Luminex 200 multiplex flow analyzer.

The content of pro-inflammatory cytokines (IL-1 α , IL-1 β , IL-6, IL-12(p70), TNF- α , IFN- γ) in serum of the control group rats was 0.234 ± 0.030 , 0.089 ± 0.009 , 1.252 ± 0.175 , 2.740 ± 0.565 , 1.156 ± 0.190

0.892 ± 0.131 pg/ml, the test group rats – 0.222 ± 0.016 , 0.082 ± 0.014 , 1.097 ± 0.106 , 1.849 ± 0.250 , 0.844 ± 0.117 , 0.750 ± 0.077 pg/ml, respectively. The content of anti-inflammatory cytokines (IL-4, IL-10, IL-13) in serum of control group rats was 0.562 ± 0.102 , 0.450 ± 0.081 , 2.519 ± 0.399 pg/ml, the test group rats – 0.467 ± 0.061 , 0.352 ± 0.037 , 1.963 ± 0.205 pg/ml, respectively. There were no differences between the groups in all the indices studied ($P > 0.05$).

Thus, the examination of immune status of rats fed with diet, containing Black soldier fly larvae biomass, during 93 days of ontogenesis did not reveal any effect of the studied product on the cellular and humoral components of immune system.

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P14-03

Allergological research of novel insect-derived food on the model of systemic anaphylaxis in the *in vivo* experiment

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The Russian approach to the safety assessment of novel food involves the study of specific types of toxicity in *in vivo* experiments. In accordance with established research practice, a comprehensive approach has been used to provide the most complete and reliable information on the potential reprotoxic, immunotoxic, and allergenic effects of novel food. This publication presents the results of allergological studies of insect-derived food on a model of systemic anaphylaxis in rats. A dry biomass of Black soldier fly (*Hermetia illucens*) larvae was used as a material for research.

The 30-day experiment was performed on male Wistar rats with initial body weight ~194 g. During the experiment the rats received a balanced semi-synthetic casein diet; larval biomass was included in the feed at the rate of 18 g/100 g of the diet. The investigated product was introduced into the diet, excluding the amount of casein and lard equivalent in calories and nutrient content. The potential allergenicity evaluation included the detection of (1) systemic anaphylaxis reaction severity and (2) circulating sensitizing antibodies level (IgG1+ IgG4 subclasses) during intraperitoneal sensitization of adult rats with a food antigen, chicken egg ovalbumin, followed by intraperitoneal injection to the sensitized animals with a resolving dose of the same protein. The humoral immune response was assessed by IgG1 and IgG4 concentrations using an indirect solid-phase enzyme immunoassay.

During the period of sensitization and feeding no animal death was observed. The severity of the active anaphylactic shock reaction in the control group was 16% (fatal outcome detected in 3 rats out of 19), in the test group – 5% (fatal outcome detected in 1 out of 19 rats). The intensity of the humoral immune response in the test group rats didn't have statistically reliable differences from the same parameters of the control group rats: the IgG1 content was 215 ± 24 and 264 ± 22 ng/ml; the IgG4 content was 251 ± 38 and 307 ± 56 ng/ml, respectively. Analysis of the indices' distributions in the groups with the use of ANOVA criterion indicates their homogeneity ($p > 0.1$).

The results of the assessment of active anaphylactic shock severity and the humoral immune response intensity in rats fed with the biomass of Black soldier fly larvae indicate the absence of allergenic action of the studied product. Thus, the allergological research of novel insect-derived food on the model of systemic anaphylaxis in the *in vivo* experiment hasn't reveal any immunomodulatory, sensitizing, and allergenic properties of the *Hermetia illucens* larvae biomass.

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P14-04

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P14-05

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P14-06

Cytotoxicity and anti-inflammatory properties of a novel lingonberry-based dietary supplement

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Purpose: Studies of the immunopharmacological activities of different plant species have provided proof on different extracts with high therapeutic potential, which represents an encouraging alternative to reduce the inflammatory processes and thus diseases related to inflammation. Several scientific studies strongly recommend that diet plays an important role in inflammation, and that certain dietary factors can act as preventive or treatment methods to reduce inflammation. In our study, a novel lingonberry-based dietary supplement has been investigated for the ability to suppress the inflammatory response in activated monocytes/macrophages.

Materials and methods: The monocyte/macrophage cell line (ATCC 9855) was used to evaluate cell viability (MTS test), cytotoxicity (LDH test) and *in vitro* anti-inflammatory capacity (xMAP array) of our novel lingonberry-based dietary supplement. Using Luminex xMAP array multiplexing technology, the immune response to dietary supplement treatment was investigated by analyzing a 10-plex inflammatory cytokine panel. Considering the xMAP analysis results, our study continued with the selection and validation (ELISA) of pro-inflammatory cytokines which recorded the most remarkable activity.

Results: Based on cell viability/ proliferation and cytotoxicity tests, concentrations between 40–130 µg/mL of extracts showed high viability/ proliferation effect and no cytotoxic activity in monocyte/macrophages cells. To further investigate the anti-inflammatory potential of our novel lingonberry-based dietary supplement, we studied the effect of the extract, with concentrations ranging between 40–130 µg/mL, on inflammatory response in LPS (lipopolysaccharide) – stimulated macrophages. We found that the extract exhibited a strong anti-inflammatory potential by inhibition the expression of major inflammatory cytokines (IL-6, IL-8 and TNFα) in activated monocytes/macrophages cells. Subsequently, IL-6 and IL-8 expression was validated by enzyme-linked immunosorbent assay (ELISA).

Conclusion: In conclusion, we have demonstrated that our novel lingonberry-based dietary supplement has no cytotoxicity and could suppress inflammation, and thus can be considered a natural important tool for inflammation control.

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P14-07

In vitro assessment of cytotoxicity, immunotoxicity and regeneration activities of collagen-based biomaterial

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Purpose: The use of biomaterials in biomedical areas can change the direction of research in tissue engineering; the goal is the self-repair of the organism based on collagen scaffolds that may contain different bioactive molecules. Our aim was to evaluate *in vitro* cytotoxicity, immunotoxicology and regenerative effects of novel collagen-based biomaterial, obtained through innovative biotechnological processes.

Methods: Novel collagen-based biomaterial from natural sources, compared to commercial collagen was investigated using *in vitro* methods regarding cytotoxicity, immunotoxicology and regenerative activities. Several cell types: human chondrocytes (HC), human fetal osteoblastic (hFOB), monocytes (CRL9855), and human fibroblast cell line (HS27) were used. Cytotoxicity and proliferation testing were performed using LDH and MTS assays, in standard conditions, at different cell concentrations (10,000–20,000 cells/well), and different times of exposure (24 h, 48 h, 72h). Real-time monitoring of cellular behavior and regenerative effects were achieved with xCELLigence platform (electrical impedance measurements of cell adhesion and proliferation) and BioStation IM (timelapse videomicroscopy). xMAP array (Luminex²⁰⁰ platform) was used to evaluate the 10-plex molecules: cytokines, chemokines, and growth factors release after cell treatment, these molecules confirm modulation in proximal events of inflammation, immune response, and repair.

Results: Two collagen-based biomaterial types were investigated, on monocytes cell line, and did not express significant cytotoxic effects (**LDH and MTS assays**). Our results showed comparative effects of novel tested biomaterial and commercial type. In order to ensure a normal healing process and no adverse reactions, classical biocompatibility tests have been extended with complex experiments performed on real-time monitoring. **xCELLigence measurements** showed a more pronounced proliferative effect/ cellular index (CI) in the case of treatment with the novel collagen-based biomaterial compared to commercial type, in hFOB cells, at 72h. **Nikon BioStation IM** used to closure monitor a wound via the scratch assay, revealed that our collagen-based biomaterial have comparative effects with commercial type, at 24h, in HS27, HC and hFOB cells. **xMAP array** (Luminex²⁰⁰) revealed an increased level of IL-8, TNFα and MIP-1α molecules on HC cells supernatants, at 24, 48 and 72h of treatments, emphasizing the role of these molecules in bone regeneration.

Conclusion: Our results confirmed that this novel collagen-based biomaterial could be used in a wide variety of medical fields including regeneration and wound healing, due to collagen excellent biocompatibility and weak antigenicity. Thus, collagen-based biomaterials are expected to become beneficial scaffolds for various biomedical applications in the future.

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P14-08**Human dendritic cell maturation induced by synthetic amorphous silica nanoparticles is modulated by pulmonary surfactant**

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Introduction: Synthetic amorphous silica (SAS) nanoparticles, are widely employed in pharmaceuticals, cosmetics, food or concretes. A better assessment of their toxicity is needed. Workers are mostly exposed via respiratory route and the challenge is to simulate the *in-vivo* situation through optimized *in-vitro* models. In the deep lung airborne, nanoparticles (NPs) first encounter alveolar lining fluids such as the pulmonary surfactant (PS) before being in contact with innate immune cell. Consequently, the interaction of PS with inhaled particles may influence subsequent biological outcomes through corona formation.

Dendritic cells (DC) capture and present sampled antigens in an immunogenic or tolerogenic way. In the presence of “danger signals”, DC undergo a maturation process resulting in their migration to regional lymph nodes where they activate naive T-lymphocytes. Our hypothesis is that nanomaterials could be considered as danger signals. The aim of this work was to determine whether PS could affect DC response to SAS.

Results: Human DC were exposed for 16 hours to 12.5 and 25 µg/mL fumed silica NPs, with or without a PS substitute (Curosurf®). Our preliminary results showed that pyrolytic SAS alters DC phenotype with a significant increase of CD80 and CD86 co-stimulation markers and of the CD83 maturation markers. When DCs were incubated with the PS substitute, neither an increase in cell death nor modifications in the CD80, CD83 and CD86 markers expressions were observed. However, when NP were pre-coated with the PS substitute, the resulting DC markers expressions were 20% lower compared to pristine NPs' phenotypic modifications, in a PS concentration-dependent manner and at both NP concentrations tested.

Conclusion: As Curosurf® concentrations increase, the mechanism leading to DC activation following NP treatment seems to be prevented.

Summary: The pulmonary surfactant, which could affect NP corona composition and biological identity, modulates DC activation in response to SAS.

P14-09**Role of CinA in the regulation of inflammatory cytokines in keratinocytes: is Nrf2 the only regulator?**

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Keratinocytes (KCs) are the main component of the epidermis protecting us from UV light, pathogens or chemical products. KCs are a key player in the barrier function of the epidermis, notably through

their metabolic activity and the detection of danger signals. Following contact with a chemical sensitizer (CS), the transcription factor Nrf2 is activated and participates to cellular detoxification. CS also induces the production and secretion of proinflammatory cytokines such as IL-1β, a key cytokine in allergic contact dermatitis. Recently, an anti-inflammatory role of Nrf2 has been described. Indeed, Nrf2 can negatively regulates gene transcription of certain pro-inflammatory cytokines. In this study, the role of Nrf2 in KCs was investigated in the control of the inflammatory response induced by Cinnamaldehyde (CinA), a frequently used contact allergen.

For this purpose, a KC human cell line, KERT, was stimulated by CinA using two concentrations, a low concentration (100 µM) and a high concentration (250 µM) inducing no more than 30% or 50% cell death, respectively. Our results showed that a low concentration of CinA induced a rapid accumulation of Nrf2 while a high concentration did not. Nrf2 expression correlated with HO-1 and NQO1 gene expression for the lowest CinA concentration. Regarding the expression of inflammatory genes, CinA (100 µM) inhibited IL-1β transcription without modifying the gene expression of the other inflammatory cytokines studied (IL-6, IL-8, IL-23, IL-24 & TNF-α). In contrast, CinA (250 µM) induced an increase of the expression of all the inflammatory cytokines tested. Furthermore, the low concentration of CinA-inducing Nrf2 is able to attenuate the zymosan-induced inflammatory response in KCs.

Nrf2 invalidation using shRNA showed that Nrf2 controlled the levels of all inflammatory cytokines tested. At the basal level, the absence of Nrf2 led to an increase in all these inflammatory cytokines tested and to the induction of IL-1 β in response to the lowest concentration of CinA. These results showed that CinA-induced regulation of IL-1β is dependent on Nrf2 activation.

In conclusion, this work underlies a dichotomous regulation of inflammatory cytokines in KCs depending on CinA concentrations. The mechanisms involved in the inactivation of Nrf2 following a high concentration of CinA need further investigation.

P14-10**Statins repress multi-wall carbon nanotube-stimulated IL-1β release through inhibiting the uptake by macrophages**

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Purpose: Multi-wall carbon nanotubes (MWCNT), which are asbestos-like in shape and size, induce pathological responses similar to those produced by asbestos, including inflammation, following pulmonary or peritoneal exposure. Studies have shown that MWCNT induces the production of IL-1β, which plays a critical role in the development of inflammatory pathologies. Statins are 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors that lower atherogenic LDL-cholesterol. Statins exert clinically relevant anti-inflammatory effects, although the underlying molecular mechanism remains unclear. Our aim was to investigate the effect of statins on MWCNT-stimulated IL-1β production and clarify the mechanism.

Methods and Results: We evaluated the effect of statins on IL-1β production in THP-1 macrophages and the role of inflammasomes. We found that various statins, including pitavastatin, atorvastatin, fluvastatin, and lovastatin, but not squalene synthase inhibitor, inhibited MWCNT-stimulated IL-1β production, whereas IL-1β release induced by ATP or nigericin, was unaffected. Statin decreased AIM2 mRNA expression, while the mRNA levels of MEFV and NLRP3 were unaffected. However, specific siRNA knockdown revealed that MWCNT-stimulated IL-1β release was dependent on the expression of NLRP3, but not AIM2, NLRC4, or MEFV, indicating the critical role of

NLRP3 inflammasome. Statin-induced repression was accompanied by reduced levels of mature caspase-1 and IL-1 β . Flow cytometry analysis showed that statin decreased MWCNT uptake into cells. Supplementation of mevalonate, geranylgeranyl pyrophosphate, or farnesyl pyrophosphate prevented the reduction in IL-1 β release, suggesting that reduction in isoprenoid intermediates, but not in cholesterol itself, is responsible for statin-induced repression. The statin-induced repression of MWCNT-elicited IL-1 β release was observed in THP-1 derived and mouse peritoneal macrophages, but not in bone marrow-derived macrophages.

Conclusion: Statins can prevent activation of the NLRP3 inflammasome and IL-1 β release induced by MWCNT through inhibition of the uptake into cells. Our findings reveal a novel mechanism underlying the anti-inflammatory effect of statins, implying the possibility of using statins to treat sterile inflammation induced by MWCNTs.

P14-11

Immunomodulatory potential of combined *Alternaria alternata* mycotoxins in non-cancerous epithelial colon cells

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The human gastrointestinal tract (GIT) constitutes the first barrier toward ingested food contaminants and, as such, it is regularly confronted by a multitude of xenobiotics. This includes natural and man-made contaminants, as well as their metabolites. To cope with these challenges, the GIT benefits of tailored immune responses to maintain tissue homeostasis and support structural integrity.

Secondary metabolites produced by *Alternaria alternata* fungi, such as the mycotoxins alternariol (AOH) and alt毒素 II (ATX-II) can be found in a wide variety of food commodities and are known, as individual molecules, to impact cellular immune response *in vitro*. Besides, *Alternaria alternata* toxins were previously shown to activate the aryl hydrocarbon receptor (AhR) pathway [1], which is essential for the maintenance of the epithelial barrier function. Building on this, we decided to explore the toxicological potential of AOH and ATX-II, alone or in combination, on the several endpoints relevant for intestinal immunocompetence and tissue integrity.

Hence, we chose the non-transformed colonic epithelial cell line HCEC 1CT to study the toxins' immunomodulatory potential succeeding inflammatory stimulation. Both, AOH and ATX-II revealed immunosuppressive capacities as single compounds and in a binary mixture. In particular, the toxins modulated the mRNA transcription of the pro-inflammatory cytokines TNF- α and IL-8 as well as the matrix metalloproteinase MMP-2. The picture was completed by the evaluation of down-streaming effects on proteins relevant for structural integrity. Immunofluorescence experiments revealed regulation of membrane bound proteins such as integrin β 1, claudin 4 and zona occludens 1. Besides, microscopy experiments confirmed both toxins to trigger the AhR translocation, alter the cellular distribution of the Nrf-2 – ARE pathway transcription factor Nrf2 and the pro-inflammatory NF- κ B protein p65.

To conclude, this study reveals that *Alternaria* toxins are capable of impacting signalling pathways that connect phase-I-metabolism, cellular antioxidant capacity, the colonic immune response and subsequently epithelial barrier function.

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P14-12

Accumulation of Nrf2 controlled by CK2 in dendritic cells plays a role in the chemical sensitizer-induced inflammation response

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Allergic contact dermatitis (ACD) is one of the most frequent inflammatory disorder with increasing worldwide prevalence. ACD is predominantly caused by chemical sensitizers (CS) that are present in the daily and/or professional environment. These small organic molecules exhibit significant chemical reactivity properties. Pathogenesis of ACD involve the activation of innate and adaptive immunities leading to the priming of specific effector T cells, which in turn mediate skin inflammation. Both phases of ACD are regulated by the nuclear-related factor 2 transcription factor (Nrf2) through the regulation of oxidative stress and inflammatory pathways.

Among the cells present in the skin, dendritic cells (DCs) are playing a key role in the cellular immune response elicited by CS during the sensitization phase. Our previous work identified casein kinase 2 (CK2) as a novel kinase strongly affecting DCs activation and maturation in response to CS. Moreover, phosphorylation of Nrf2 by CK2 has previously been shown to be a critical event for its nuclear translocation and activation.

To address the link between CK2 activity and Nrf2 accumulation for the control of the inflammatory response, an adoptive transfer of DCs followed by an animal model of contact hypersensitivity (CHS) has been performed. Briefly, bone-marrow derived DCs (BM-DCs) whose CK2 activity has been pharmacologically inhibited with CX-4945 have been loaded or not with a strong CS, DNBS and transferred to naive mice. Five days later, mice were elicited with an application of DNCB to both ears and ear thickness were measured daily during 3 days after elicitation. These results of the passive CHS showed that CK2 inhibition was responsible for an exacerbated response to CS, somehow mimicking the level of inflammation demonstrated in a model of CHS involving mice lacking Nrf2 (Nrf2 KO).

To understand the increase in inflammation *via* passive injection of BM-DCs inhibited for CK2, we investigated the level of Nrf2 and the inflammatory status in BM-DCs. Our preliminary data showed that Nrf2 accumulation was clearly lower in BM-DCs inhibited for CK2 compare to normal BM-DCs while inflammation cytokines levels such as IL-1b and TNF- α were augmented. CK2 seems to be responsible for the accumulation of Nrf2 in BM-DCs allowing the regulation of inflammatory cytokines upon CS exposure.

Constitutive CK2 activity in DCs could therefore play an important role in controlling their activation threshold in response to CS, probably by orienting them towards a less inflammatory phenotype *via* Nrf2 activation. To go further, the membrane phenotype of these BM-DCs will be explored to link Nrf2 and CK2 in the orientation of an immunogenic phenotype.

P14-13

A potential immunotoxic effect of imidacloprid on raw 264.7 cells

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Imidacloprid is a neonicotinoid insecticide raising in the market due to the resistance related to other classes of insecticides and the low-risk potential for mammals because of greater specificity for insects. This study aimed to investigate the cytotoxicity of imidacloprid in murine RAW 264.7 macrophage cell line. Cytotoxicity was evaluated after 24 h and 96 h incubations through the MTT reduction and neutral red uptake assays. Reactive oxygen and nitrogen species production, mitochondrial membrane potential, and DNA damage were also evaluated. It was observed a concentration-dependent increase in cell death both after 24h and 96h of incubation for MTT reduction (EC₅₀ 519.6 and 324.6 mg/L, respectively) and neutral red uptake (NR) (EC₅₀ 1139.0 and 324.2 mg/L, respectively) assays. There was no change in the production of reactive species. However, mitochondrial membrane potential decreased significantly for all tested concentrations compared to the control. DNA damage in low molecular weight DNA assay was observed, which is a consequence of insecticide-induced cytotoxicity. Overall, the results showed evidence of the immunotoxic potential of imidacloprid, indicating mitochondria as one of the intracellular targets of the insecticide.

P15 – Liver toxicology

P15-01

This abstract has been withdrawn.

P15-02

Towards Elucidating the Mechanisms of Macrolide-Induced Liver Injury: Investigating the Role of the Mitochondria

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Mitochondrial dysfunction is a key mechanism of drug-induced liver injury (DILI). Antibiotics are the drug class most commonly associated with DILI, and one sub-class frequently implicated are the macrolides. Macrolide antibiotics inhibit bacterial protein biosynthesis via selective and reversible binding to the bacterial 50S ribosomal subunit. It is hypothesized that they may induce DILI via mitochondrial dysfunction, due to the structural similarities between bacterial and mitochondrial ribosomes. In order to investigate mitochondrial involvement, we have explored the ability of five macrolide antibiotics to induce mitochondrial dysfunction *in vitro* using the human-derived hepatic cell lines HepG2 and HepaRG.

Acute metabolic switch assays were performed in HepG2 and HepaRG cells in order to unmask the mitochondrial liabilities of drugs [1,2]. Briefly, by replacing the glucose in media with galactose, the dependence of the cells on oxidative phosphorylation is increased and susceptibility to drug-induced mitochondrial toxicity can be revealed. Cells were dosed with a macrolide (azithromycin, clarithromycin, erythromycin, roxithromycin, or telithromycin), linezolid or chloramphenicol (known to induce mitochondrial toxicity via the inhibition of mitochondrial protein synthesis), or rotenone (a known mitochondrial toxicant). Cell viability was determined via ATP and LDH assays and normalised to protein per well. Assays were conducted in duplicate, and three separate assays were performed in each case. Cell viability was calculated as a percentage of that of the respective vehicle control. ANOVA followed by Dunnett's post hoc tests were used to determine statistical significance of raw data when

comparing drug to vehicle, and ANOVA with Tukey post hoc tests were used to assess the significance of variation between cells maintained in glucose-containing or glucose-free media.

No mitochondrial toxicity was observed within 24 hours in HepG2 cells after administration of clinically relevant macrolide concentrations. There was no significant reduction in viability of cells switched to glucose-free media compared to cells maintained in glucose-containing media ($P < 0.05$ for all antibiotics tested). Switching to galactose media was found to increase mitochondrial sensitivity to some compounds after chronic dosing in HepaRG cells. After 28 days exposure to $> 33.3 \mu\text{M}$ erythromycin, cells cultured in galactose media produced 50% less ATP than the vehicle control ($P < 0.033$). These observations after chronic dosing in HepaRG indicate that a short (24–48 hr) dosing period (a limitation of HepG2 cells *in vitro*) may not allow sufficient time to detect macrolide-induced mitochondrial dysfunction. Clinically, macrolides are often given for 3–14 days to treat infections, but are also used in the treatment of chronic conditions such as COPD and acne vulgaris, meaning these extended dosing studies remain clinically relevant.

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P15-03

Okadaic acid-induced changes in xenobiotic-metabolizing enzyme and cytokine expression

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Okadaic acid is a marine biotoxin produced by dinoflagellates of the genus *Dinophysis* spp. and *Prorocentrum* spp. and accumulates predominantly in filter feeding shellfish and other marine animals, and is therefore also introduced in the human food chain. It causes the diarrhetic shellfish poisoning (DSP), which leads to severe gastrointestinal disorders. Due to climate change, the occurrence of okadaic acid-producing dinoflagellates has significantly increased, which leads to an increased necessity to understand molecular mechanisms and metabolic changes induced by okadaic acid in the human body.

Okadaic acid is taken up through the gastrointestinal barrier and is metabolized by intestinal and liver cells. It has a major influence on the expression of xenobiotic-metabolizing enzymes of phase I (CYP), phase II (SULT, UGT, GST) or phase III (ABC transporters) in liver. The underlying mechanisms are only partially understood up to now; in parts the regulation occurs via nuclear receptors (CAR, PXR, AHR), which are responsible for transcriptional activation of their target genes. Furthermore, okadaic acid activates NF- κ B, which is then able to regulate cytokine expression.

To determine changes in expression of xenobiotic-metabolizing enzymes and in cytokines (interleukins), HepaRG cells were incubated with okadaic acid and the nuclear receptor activators Benzo[k] fluoranthrene (for AHR), SR12813 (for PXR) and CITCO (for CAR). Gene expression was analyzed using a RT-qPCR. Relative expression of interleukins on protein level will be investigated by ProcartaPlex™ assay.

The cells showed an overall downregulation of CYP enzymes after addition of okadaic acid. The effects of the nuclear receptor activators on CYP expression was compensated by okadaic acid. Cytokine expression, however, was induced. Furthermore, the expression of TNF α , which is generally activated by NF- κ B and activates cytokines, was upregulated.

In summary, okadaic acid seems to decrease expression of CYP enzymes and other xenobiotic-metabolizing enzymes, but activates interleukins, which leads to the assumption of liver inflammation after exposure to okadaic acid.

P15-04

Modulation of hepatic xenobiotic metabolizing enzymes following chronic low-dose exposure to PFOA in mice

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Perfluoroalkylated substances (PFAS) are used in a wide range of industrial applications including coatings for cookware, food contact papers, waterproofed clothing and fire-fighting fluids. PFOA (perfluorooctanoic acid) has raised major public health concerns over the last few years. Although its industrial use is decreasing, consumers are still exposed to this extremely persistent and bioaccumulative substance. Dietary intake is reported as the main source of human exposure to PFOA, with seafood and freshwater fishes being the highest contributors. Epidemiological surveys and results from *in vivo* studies in rodents suggest that PFAS can induce hepatotoxicity and exert metabolic effects, including effects on lipid metabolism. However, the mechanisms underlying these effects remain largely unexplored.

In this study, we assessed the effects of low doses of PFOA on mice hepatic xenobiotic metabolizing enzyme (XME) activities. XMEs are involved in the biotransformation of xenobiotics, playing an essential role in the protection of organisms, and are also involved in a number of endogenous anabolic and catabolic processes.

Adult C57BL/6 male mice were exposed *via* drinking water to 0, 1.5, 150 or 15,000 ng/kg body weight/day ($n = 10$ per group) for 90 days. Livers were collected, perfused and subcellular fractions (microsomal and cytosolic fractions) were prepared. Following protein content assessment, *in vitro* incubations with specific probe substrates and cofactors were carried out to measure key phase I and II activities. Specific cytochrome P450 (CYP450) isoform activities were assessed in microsomal fractions, including CYP2C37 (probe substrate: diclofenac; monitoring of 4'-hydroxydiclofenac formation by HPLC-UV); CYP4A10 ($[^{14}\text{C}]$ -lauric acid; formation of $[^{14}\text{C}]$ -12-hydroxylauric acid; Radio-HPLC); CYP3A11 ($[^{14}\text{C}]$ -testosterone; formation of $[^{14}\text{C}]$ -6 β -OH-testosterone; Radio-HPLC). Phase II enzyme activities such as glutathione S-transferases (GST) were also investigated using microsomal and cytosolic fractions, by measuring 1-chloro-2,4-dinitrobenzene conjugation by spectrophotometry.

A significant impact of PFOA exposure on hepatic CYP2C37 activity, an isoform involved in liver fatty acid and eicosanoids metabolism (endogenous substrates), was demonstrated. CYP2C37 activity was inhibited by PFOA at the highest dose. Conversely, PFOA exposure had no significant effect on CYP4A10 activities, involved in ω -oxidation of medium-chain fatty acids and eicosanoid metabolism. Phase II cytosolic and microsomal GST activities were not significantly modulated by PFOA exposure, whatever the exposure level. In conclusion, our study has demonstrated that *in vivo* exposure to PFOA impacted liver phase I XME activities. This research is ongoing and further experiments will be performed to gain a broader overview of the modulation of these enzymes, consecutive to low-dose *in vivo* exposure to this PFAS, with the aim to improve the understanding of its mode(s) of action.

P15-05

Molecular mechanisms of hepatotoxic cholestasis by clavulanic acid in human hepatocytes

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Purpose: To elucidate the molecular mechanism of the cholestatic drug-induced liver injury (DILI) triggered by Augmentin® (an amoxicillin (AMO) and clavulanic acid (CLAV) mix), which is the most frequent cause of idiosyncratic DILI^[1].

Methods: Sandwich-cultured human Upcyte hepatocytes^[2] were treated with AMO and CLAV for 1-72h. Cytotoxicity, gene expression, reduced/oxidized glutathione ratios (GSH/GSSG) and total cholesterol were quantified by MTT assays, microarray and RTqPCR, and biochemical assays, respectively, at different times of treatment. The direct effect of CLAV on FXR activity was evaluated by luciferase reporter assays in cells transfected with an FXR-responsive element reporter plasmid (pGL3-FXREx4) (Upcyte hepatocytes, U2OS cells) and a human FXR-coding plasmid (U2OS only).

Results: CLAV alone, but not AMO, at sub-cytotoxic concentrations, caused a dose-dependent decrease (> 50%) in the expression of key genes involved in bile acid (BA) transport (BSEP, NTCP and OST α) and synthesis (CYP7A1 and CYP8B1). Microarray expression analysis of 24h CLAV-treated Upcyte hepatocytes evidenced 618 genes with fold-change ≥ 2 and FDR p -value < 0.05. Downregulation of pathways involved in BA homeostasis, cholesterol and fatty acid biosynthesis; and induction of antioxidant and detoxification pathways were observed. CLAV caused oxidative stress and reduced the GSH/GSSG ratio at 1, 3, 5 and 24h by 20%, 47%, 41% and 50%, respectively. Consequently, an early (5h) induction of the NRF2 antioxidant pathway, including several NRF2 direct target genes, were observed: detoxification (AKR1B10 20.3-fold), antioxidant GSH-based system (SLC7A11, GCLM, and GCLC; 3.3-, 2.5- and 2.2-fold) and antioxidant TXN-based system (TXNRD1 3.6-fold). Activation of the NRF2 antioxidant response by sulforaphane resulted in NTCP downregulation. CLAV inhibited FXR reporter activity by 60% in GW4064-treated hepatocytes and by 35% in CDCA-treated U2OS, which is in agreement with the observed BSEP and OST α downregulation. Finally, CLAV treatment for 48h significantly reduced total cholesterol by 15%.

Conclusions: We demonstrate for the first time that CLAV (but not AMO) may directly inhibit FXR activation mediated by BAs, thus blocking the consequent up-regulation of BSEP^[3] and OST α ^[4]. Moreover, CLAV may inhibit NTCP expression by triggering oxidative stress and NRF2 signalling^[5]. Both could be effective mechanisms to hamper BA uptake and canalicular efflux by hepatocytes exposed to AMO+CLAV. Moreover, reduced cholesterol content could also hinder membrane dynamics and BA transporter functioning^[6]. On top of that, increased ROS production and GSH depletion, despite the early NRF2 response, may limit the BA-independent bile flow^[7] and aggravate the cholestatic DILI mediated by this antibiotic mix.

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P15-06

Dose-dependent toxicological effects in rats following a 90-day dietary exposure to PCB-156 include retinoid disruption

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The toxicity of PCB-156 (2,3,3',4,4',5-hexachlorobiphenyl) was investigated in rats following subchronic dietary exposure. Groups of 10 male and 10 female Sprague-Dawley rats were administered PCB in the diet at 0, 0.01, 0.1, 1 or 10 ppm for 13 weeks. Results were analysed by group-wise comparison and benchmark dose-modelling. The latter revealed dose-related decreases of final body weight, growth rate and thymus weight. Additionally, rats receiving PCB-156 showed dose-dependent weight increases of liver, lungs and kidneys. Biochemical changes included increases in liver EROD, PROD and UDPGT enzymatic activities, as well as increases in uro-porphyrin. Retinoid (Vitamin A) quantification showed a clear and treatment-related reduction of the levels in the liver and lungs, as well as increased levels in the kidneys. A lowest-observable-adverse-effect level (LOAEL) of 0.01 ppm was established, based on effects in the liver apolar retinoids concentration, corresponding to dietary exposure of 0.7 and 0.8 µg PCB-156/kg body weight per day in male and female rats, respectively. Benchmark dose-modelling corroborated effects in the retinoid system, in both sexes, at even lower intake levels. The lower confidence limit (BMDL) for a 5% decrease in the concentration of liver apolar retinoids was 0.00086 (males) and 0.00068 ppm (females), corresponding to a daily exposure of 0.06 µg PCB-156/kg body weight for both sexes. This BMDL₅ is approximately 10-fold lower than the LOAEL for PCB-156. Based on the retinoid system's susceptibility to PCB-156 exposure, we recommend effects on this system to be considered as critical for risk assessment of PCB-156 and other PCB congeners.

P15-07

Leveraging the Power of High Content Screening Using Iterative Data Mining for Ethnically-diverse, Predictive, Animal-free Hepatotoxicity Platform.

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Multiparametric high-content screening (HCS) approaches, such as the Cell Painting assay, are growing in popularity for many applications including, but not limited to, *in vitro* pharmacology and toxicology. HCS combines rapid automation and imaging of fluorescent markers to capture unique cellular phenotypes. This is particularly useful for gaining insights into mechanism-of-action by comparing the phenotypic profiles of various compounds with reference drugs or controls. Cytochroma uses HCS approaches to provide *in vitro* toxicology services without the use of animal models. However, HCS approaches precipitated a growing problem where the capacity for data analysis was overwhelmed by the speed at which the data was being generated. Here, we present a workflow in which a web-based platform called StratoMineR™ was used to accelerate the analysis of Cytochroma's RAPIDTox™ platform, a human induced pluripotent stem cell derived hepatocyte toxicity assay. We tested a library of 160+ compounds in quadruplicate at multiple concentrations ranging from 0.75 µM to 200 µM. 48 hours after drug induction, an established Cell Painting protocol was used for direct visualisation and segmentation using fluorescent markers (i.e., Hoechst 33342, Syto14 (CellMask), Phalloidin / AlexaFluor and Mitotracker Deep Red). Images were acquired using the Perkin Elmer High Content Opera Phenix Imaging System, and analysed by Harmony software v4.9, extracting > 350 features based on intensity, texture, size, and morphology. The output of the image quantification was uploaded into StratoMineR™ for rapid data mining and analysis. Moreover, StratoMineR™ also allowed data normalization and transformation, data reduction and unsupervised hit selection. These functionalities allowed Cytochroma to identify a hit rate of 59.84% with 1149 hits that gave significant phenotypes relative to the controls. Using phenotypic distance score ranking from the hit selection, Cytochroma can identify key compounds of interest as well as plotting dose response curves and IC values (e.g., Acetaminophen at 25µM). StratoMineR™ also offers clustering analysis to find similar/dissimilar mechanisms of action. These results demonstrate that the proposed workflow allows combining iPSC technologies, HCS and StratoMineR™ to yield robust and reproducible results for predictive *in vitro* hepatotoxicity.

P15-08

Steatosis as risk factor for drug-induced liver injury (DILI)

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Background: The sensitizing effect of steatosis and NASH as general risk factors for human DILI is receiving a lot of attention. Reasons are the high incidence of patients with metabolic diseases in our population and the need for a more differentiated patient specific risk assessment of new drug candidates. Patients with steatosis might be more sensitive and more fragile as healthy subjects. Until now the relationship between DILI and underlying steatotic diseases has not been systematically investigated. The steatosis DILI risk factor hypothesis appears very plausible but still needs clinical endorsement, as well supportive data from *in vitro* experiments.

Methods: Human 3D liver microtissue, containing scaffold free co-culture of primary hepatocytes, Kupffer cells and liver endothelial cells when exposed to free fatty acids in media, high levels of sugars

and insulin displayed substantial accumulation of lipids within 7 days of treatment as detected by Nile red staining and triglyceride levels. 3D human liver microtissues have been validated by 108 clinically tested FDA-annotated DILI drugs by the determination of cellular ATP levels showing high specificity and sensitivity (Archives of Toxicology, vol. 91, 2849–2863, 2017).

Results: It was the purpose of the present study to evaluate the cholestatic drug Chlorpromazine and the non-cholestatic drug Acetaminophen under steatosis and non-steatosis conditions in human 3D liver microtissues. Under steatosis conditions the cytotoxicity of the cholestatic drug Chlorpromazine was significantly enhanced compared to non-steatosis conditions. The cytotoxicity of the non-cholestatic Acetaminophen under same conditions was not enhanced.

Conclusion: These preliminary results suggest that drug-induced cholestasis together with underlying steatosis can enhance DILI and might be a risk factor for the expression of DILI in patients. The increased cytotoxicity of cholestatic drugs might come from the intracellular accumulation of cytotoxic bile acids. To better understand this relationship, further investigations of cholestatic and non-cholestatic drugs under steatotic and non-steatotic conditions are necessary.

P15-09

Associations between the results of hepatotoxicity-related *in vitro* assays and rat repeated-dose liver toxicity of chemical compounds

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Introduction: Although current chemical safety evaluation largely depends on the results of toxicity studies using experimental animals, the establishment of alternatives to animal experiments are needed to reduce the number of animal experiments and developing cost and time. However, it is very difficult to develop such a method for complex toxicity, such as repeated-dose toxicity (RDT), which is crucial for chemical safety evaluation. In 2017, a 5-year project, termed “AI-SHIPS”, supported by the Ministry of Economy, Trade and Industry of Japan, was launched. The aim of this project is to develop a system to predict rat RDT, especially liver, kidney and blood toxicity, based on chemical structures using machine learning and cheminformatics, and to provide toxicity mechanism information. To this end, we performed multiple *in vitro* tests with chemical compounds whose rat RDT test results are available and investigated the association between the results of *in vitro* tests and *in vivo* tests. [Methods] Test compounds (326 compounds) were selected from HESS (NITE, Japan), from which the results of rat 28-day RDT and 42-day combined RDT and reproductive/developmental toxicity tests were available. Inhibition of rat cytochrome P450s (P450s), including CYP1A1, CYP1A2, CYP2B1, CYP2C6, CYP2D1, CYP2E1 and CYP3A2, and total UDP-glucuronosyltransferase activity were assessed with recombinant enzymes or rat liver microsomes and luciferin-derived substrates. Inhibition of rat BSEP and MRP2 were investigated with fluorescent substrates and transporter-expressed vesicles. Changes in CYP2B1, CYP3A1 and CYP4A1 mRNA levels in rat primary cultured hepatocytes were determined with MAGPIX system. LDH leakage, cell viability (ATP content) and cellular GSH levels in HepG2 cells were investigated using commercial kits. Effects on cell organelles (nucleus, mitochondria and actin) and the accumulation of triglycerides and phospholipids in HepG2 cells were evaluated by high-content analysis (HCA) with fluorescent probes. Statistical analyses were performed with JMP Pro 14. [Results and Discussion] The association between 67 *in vitro* test parameters and 6 liver-related *in vivo* endpoints (cell death/inflammation, reduced liver function, enhanced

liver function, bile duct-associated lesion, hypertrophy, and dyslipidemia) were investigated using Fisher’s exact test after binarization. The results demonstrated significant associations between some *in vitro* parameters and *in vivo* toxicity. For example, strong associations between reduced liver function and various cell toxicity-related parameters, between hypertrophy and CYP2B1 mRNA levels and P450 inhibition, and between dyslipidemia and lipid accumulation determined by HCA were observed as expected. Meanwhile, unexpected associations were also observed. These results suggest that the *in vitro* tests performed in this study are useful for developing a RDT prediction model with mechanistic information.

P15-10

Cysteine conjugate of paracetamol serves as substrate for kidney cysteine S-conjugates β -lyase: implications for drug-induced acute kidney injury

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Paracetamol (acetaminophen; PAR) is considered safe at therapeutic doses; however, severe hepatotoxicity occurs when it is ingested at higher doses. In addition to hepatotoxicity, PAR overdose induces kidney injury too. Recent data revealed that previously supposed 1% incidence of PAR-induced nephrotoxicity may be between 10% and 79% in overdose cases, and about 50% of patients have significantly higher circulating creatinine in their plasma, which is a reliable biomarker of kidney injury. We recently showed that PAR-induced nephrotoxicity does not follow the same molecular events as in PAR-induced hepatotoxicity (Akakpo *et al.*, 2020). One of the possible mechanisms is Cysteine S-conjugate β -lyase-mediated activation of PAR-cysteine (PAR-Cys) in kidney proximal tubular cells, which may further cause cell death. In the present study, we investigated whether PAR-Cys acts as a substrate for renal, as well as hepatic β -lyase in mice, rats, and humans. Results showed that PAR-Cys degradation is catalysed with higher affinity by mitochondrial form of the enzyme compared to cytosolic form. This finding may be significant regarding unknown mechanism of paracetamol-induced nephrotoxicity because mitochondria has been identified as the major target.

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P15-11

Hepatic nonparenchymal cells isolated from rodent livers and characterized for the development of primary hepatic cocultures

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Liver cells are established as *in vitro* models for toxicology studies in 2D and 3D cultures. The goal is to approach the *in vivo* situation as good as possible. To expand the specificity of the liver models, the total cell population of the liver has to be taken into account. Cultivation and verification of these nonparenchymal cells, like Liver Sinusoidal Endothelial Cells (LSEC), Kupffer cells (KC) and Stellate cells (SC) remain an issue. Percoll gradients for cell separation of nonparenchymal cells have been established, but the yield of NPC per gram liver tissue can be low. At PRIMACYT, internal experiments comparing Percoll- and Iodixanol gradients in rat liver showed a slightly higher yield (20%) of the whole NPC population when cells

were isolated by an Iodixanol gradient compared to Percoll. In mice, this effect was more obvious by providing about 54% higher NPC yield with Iodixanol. We examined assays to prove purity and viability of the KC, LSEC and SC fractions of the rats and mice after purification by Iodixanol. In a first step, Kupffer cells were tested over culture time for their stimulation by LPS, followed by a phagocytosis test. LSEC fractions were tested equally to proof the absence of phagocytic cells. Stellate cells were tested for the presence of lipid droplets by Oil Red O staining. Further analysis was done by immunofluorescence by CD68 (KC), CD31 (LSEC) and Vimentin (SC). Finally, the fluorescence was used to count the cell nuclei against positively stained cells by a cell counter system to indicate the proportions of cells present in the purified cell fractions. Together, the tests could give rise to a clear characterization of the liver cells. Finally, the approach to utilize tissue-like cocultures should reduce the number of laboratory animals used for toxicity studies and drug development.

P15-12 Improved QSAR model for cholestasis built with FDA drug label data

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The liver is one of the essential organs of the human body in transforming and deactivating xenobiotic chemicals. Major liver toxicity events include drug-induced liver injury, cholestasis, liver damage, bile duct disorders, liver enzyme abnormalities, etc. Among these, cholestasis is an important condition in which bile cannot flow from the liver to the duodenum. However, availability of good QSAR ready data is a bottleneck. Most of the current research work are based on noisy data from FAERS reports, including our existing QSAR model for Cholestasis. Therefore, we collected data from three publicly available sources PubChem bioassay #1079936 on Choleostatic liver toxicity, Kotsampasakou E, Ecker GF research paper (data from these sources are clinical and invivo) and Drugs@FDA Data files containing FDA drug labels.

While extracting data from first two sources was relatively straightforward, retrieving QSAR ready information from the FDA Drug labels was challenging. We extracted data from digital FDA Drug labels utilizing concepts of natural language processing and automatically assigned outcomes based on keywords, resulting in a significant increase in the number of chemicals with a positive signal. The data set size increased from 596 compounds to 1498 with an additional external test set of 136 compounds.

The updated QSAR model was built using structural fragments and machine learning. Bootstrap cross-validation improved sensitivity from 57.6% to 79.4%, specificity 61% to 76.1% and AUC from 0.592 to 0.852. The count of identified alerts also increased from 53 in the existing model to 336 in the new model. The external set ROC improved from 0.509 to 0.655.

The dataset covers compounds with various functional groups relevant to liver injury, e.g., fused tricyclic structural moiety, sulfonamides, hydrazines, etc., reflecting the suitability of the data for this purpose. Statistical alerts, automatically discovered for this QSAR model closely match these specific classes of chemicals with known mechanisms of hepatotoxicity.

In summary, we could make significant improvements in our Cholestasis model with data augmentation from drug labels and existing open-source data. In addition, the drug label data extraction process can be applied to any other endpoints by simply modifying the relevant keywords.

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P15-13 Rescuing a troubled tolcapone: nanomedicine on hepatotoxicity fight club

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Purpose: Tolcapone is an orally active catechol-O-methyltransferase (COMT) inhibitor, with a short serum half-life ($t_{1/2}$ = 2.3 h), that is used as adjuvant therapy in Parkinson's disease. Despite being effective and potent, tolcapone has a highly hepatotoxic profile, which led to the introduction of a “black box” warning by the FDA and to intensive monitoring requirements during its administration^[1,2]. Nanomedicine emerged as a solution to overcome several drug drawbacks, namely at drug-induced toxicity level, by using functional nanomaterials with controlled morphology and well-defined properties^[3]. Therefore, the aim of the present work was to develop a polymeric carrier with controlled drug release properties that could reduce tolcapone-induced hepatotoxicity and prolong the drug serum half-life.

Methodology: Tolcapone was encapsulated in PEGylated (PLGA) nanoparticles (NPs) synthesized using the nanoprecipitation method after a brief optimization process^[4]. Several physicochemical parameters, such as nanoparticle size, Z-potential and drug-loading capacity, were evaluated. Drug release studies were carried in PBS medium for 1 week, at 37°C, using the dialysis bag method. Cytotoxicity of free tolcapone, empty and tolcapone-loaded NPs (concentrations up to 50 µM) was assessed by measuring the metabolic activity (resazurin assay) and lysosomal activity (neutral red assay) [MR1] in HepG2 cells, in cell culture media with and without glucose. Timepoints of 2, 6, 12 and 24 hours were considered.

Results: Tolcapone-loaded NPs presented a size of 173.8 ± 11.8 nm and a superficial charge of -26.8 ± 3.1 mV in PBS medium. The drug loading capacity was $5.4 \pm 0.2\%$. Drug release studies revealed a typical biphasic pattern, with 98% of tolcapone being released within the first 24 hours, presenting a good correlation with the First-order ($R^2 = 0.986$) and Korsmeyer-Peppas ($R^2 = 0.991$) models of drug release. The *in vitro* cellular assays showed an overall decrease in drug-induced cytotoxicity [MOU2] [MR3] upon drug encapsulation, particularly in the higher concentration tested ($p < 0.0001$ for both resazurin and neutral red assays in the 4 timepoints and 2 cell culture media tested). No cytotoxicity was observed for the placebo NPs

Conclusions: This work demonstrated the advantages of tolcapone-loaded NPs as a controlled release system over free tolcapone in terms of *in vitro* cytotoxicity. However, work has to be done on the

improvement of the NPs drug-loading capacity and in the assessment of their therapeutic efficacy, both *in vitro* and *in vivo*.

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P16 – Metabolic toxicology

P16-01

This abstract has been withdrawn.

P16-02

How the skin microbiome influences ingredient action of cosmetics. Through their effects on metabolism, bacteria in the skin affect whether active ingredients will be safe and effective for human health

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A Pathway-Based Toxicology from the first Molecular Initiating Event(MIE) through key events (KE) leading to clinical benefits, demonstrates skin's microbiome is the key actor and creates the first MIE with our Active Ingredient (AI). Our (AI) 'Gardenia extract' is concentrated in crocin well known for its antioxidant, anti-inflammatory benefits, and that protects the natural skin melatonin cycle. Our researchers demonstrate the Conversion of crocin into crocetin by the skin microbiome (*in vitro*) with sampling of skin's microbiome performed on human volunteers.

The following key event (KE) consists in triggering the same receptors as melatonin (MT1R). Docking studies were performed to evaluate the possibility of crocetin versus crocin to interact with a melatonin specific receptor (MT1R). Crocetin has a high affinity with MT1R while crocin shows a disfavoured interaction. The conversion of crocin by the skin microbiome into crocetin with a score close to the one of melatonin itself, means it may bind to this receptor and trigger the same biological cascade of mechanisms as melatonin: activation of the antioxidant defences and positive activity on the circadian rhythm.

Next key events about Cellular response have been investigated. A KeratinoSensTM assay with a reporter cell line to detect potential skin sensitizers by their ability to induce the Nrf2-response has been performed. The conclusion as a non-sensitizer is clearly supported by the analysis of the dose-response curve with overall no induction of the luciferase reporter gene.

In case of UV exposure a 3T3 NRU photo toxicity assay demonstrates no phototoxic potential.

Next key events about Tissue response have been investigated. An *In vitro* irritation study on human reconstructed epidermis (EpiDerm SIT) has been performed to predict skin acceptability by measuring the relative conversion of MTT correlated with cell viability. Mean Viability of 95% with the neat AI demonstrates a very good tolerance.

Next key events about organ responses have been studied. In case of Absorption, results indicate that orally administered crocins are hydrolyzed to crocetin before or during intestinal absorption. Results have revealed that in animal studies, crocin and crocetin at the pharmacological doses didn't show relevant damage on main body organs

Next and last key events about organism response have been studied with clinical responses. Consumers home usage test demonstrate a Perceived improvement of skin quality, looking healthier skin, and a global Well-being sensation with Improvement of sleep quality.

All these benefits are made possible with the great help of skin microbiome: 1st key actor that activates the cascade of mechanism with a vegetal melatonin-like molecule activating skin repair and defences, connected to the circadian rhythm, and providing well-being.

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P16-03

This abstract has been withdrawn.

P16-04

The effect of alkyl substitution on Polycyclic Aromatic Hydrocarbons (PAHs) that may be present in the mineral oils on their oxidative metabolism

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Hydrocarbons may be found in foodstuffs. Although some of these hydrocarbons are of natural origin, mineral oil residues originating from food packaging, additives, processing aids, and lubricants may also be present. An emerging concern in modern food safety is the possible carcinogenicity of aromatic hydrocarbons that may be present in mineral oil and end up in foodstuffs. The bioactivation of non-substituted PAHs has been extensively studied in relation to carcinogenicity. However very few data on possible metabolic activation of alkyl substituted PAHs, which are predominantly present in mineral oils, have been reported. The present study investigates the oxidative metabolism of PAHs and their alkylated congeners by rat and human rat liver microsomes to get a better insight in the relative balance between bioactivation and detoxification. Model compounds included in the study were naphthalene, phenanthrene, benzo[a]pyrene and some of their methyl-, ethyl-, n-hexyl-, and n-dodecyl-substituted analogues. Metabolite formation of each test compound in rat and human liver microsomal incubation was measured and quantified using UPLC and GC-MS/MS. The results showed alkyl substitution shifted the oxidative metabolism from the aromatic ring to the alkyl side chain, in favor of detoxification and excretion. Furthermore, elongation of the alkyl side chain reduced the overall metabolism of PAHs. In summary, alkylation of PAHs may increase the chances of alkyl chain oxidation and detoxification and as a consequence reduce the genotoxic risk.

P16-05**Oxidative Carbonylation of Proteins as a Mechanism of the Toxic Action of Pesticides**

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For several decades, glyphosate-based herbicides (GBH) have been among the most widely used in the world but the safety of glyphosate and its commercial formulations is still controversial. The development of free radical oxidation is generally recognized as one of the manifestations of the toxic effect [1]. The assessment of the level of carbonyl derivatives of proteins makes it possible to simultaneously assess the damage to amino acid residues by free oxygen and/or nitrogen radicals, as well as by the products of lipid peroxidation. Proteins are recognized as prime targets for reactive oxygen and nitrogen species due to their high sensitivity to free radicals and their abundance in biological materials.

Purpose of the work: evaluation of the oxidative carbonylation proteins of liver tissue in a subchronic 90-day study of GBH *in vivo*.

GBH was administered intragastrically with doses 100 (study group 1) and 280 mg/kg b.w. (group 2) in a subchronic 90-day study on male Wistar rats. The animals were taken out of the experiment in stages by euthanasia (CO₂). Subcellular fractions of liver tissue homogenates were obtained by sequential centrifugation. To assess the degree of oxidatively modified proteins, the level of carbonyl derivatives of the obtained homogenate fractions was measured by the method of R. Levine modified by E. Dubinina [2,3] in spontaneous (SP) and metal-induced (MI) variants with registration of products in the absorption ranges of dinitrophenylhydrazones (DNPH) with spectrophotometer SF-2000. The results expressed in units of optical density per 1g of protein. Based on the values of the areas under the curve of total carbonyl derivatives, their total content (S_t), the content of primary markers – aldehyde forms of DNPH (S_A) and secondary markers – ketone forms of DNPH (S_K) and reserve adaptation potential (RAP,%) were determined. RAP was calculated as the difference between the S_t in the MI version taken as 100% and the percentage of the S_t of SP oxidation in it. Statistical processing was performed with Statistica 10.0, p=0.05.

In the 2 group, a statistically significant increase in S_t was formed in the cytoplasmic fraction of the liver tissue due to an increase in the content of aldehyde- and keto-DNPH in 2 weeks, 1 and 3 months. At the same time, the RAP statistically significantly decreased in the 2 group at all stages of the experiment. A similar tendency was observed in the 1 group at all stages of observation against the background of a statistically significant decrease in RAP, with the exception of values after 3 months from the start of priming. There were statistically significant differences between S_A and S_K in the 2 group compared to the 1 group in 1 and 3 months, which was reflected in a significant difference in S_t.

These changes may indicate the triggering of adaptive mechanisms, including the induction at this stage of the processes of proteolytic utilization of oxidized proteins or additional protein synthesis.

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P16-06**Zebrafish as a Model for Chemical Induced Adipogenesis and Related Metabolic Diseases**M. Den Broeder¹, N. Habraeken¹, L. Kamminga², J. Kamstra¹, *J. Legler¹¹ Utrecht University, Utrecht, Netherlands;² Wageningen University, Wageningen, Netherlands

Humans are widely exposed to endocrine disrupting chemicals (EDCs). Current research suggests that developmental exposure to specific EDCs, the so-called metabolism disrupting chemicals (MDCs), may play a role in the latent onset of obesity and other metabolic disorders like type 2 diabetes mellitus and non-alcoholic fatty liver disease. Within the European H2020 project GOLIATH (Generation Of Novel, Integrated and Internationally Harmonised Approaches for Testing Metabolism Disrupting Compounds), we aim to for better understanding of molecular mechanisms of MDCs, and develop methods to test chemicals for metabolic disruption. Our ultimate goal is an integrated approach to testing and assessment of this class of chemicals.

Our laboratory uses zebrafish (*Danio rerio*) and an emerging model organism to study human metabolic diseases. The goal of this study is to analyze the effects of early developmental exposure to chemicals on adipogenesis, as well as pancreas and liver development in the zebrafish. We have developed a zebrafish model of adipogenesis and obesity, and use advanced imaging technology and fluorescent lipid stains to visualize the development of adipocytes in living larvae at 15 days of age. With this approach we have shown that developmental exposure to the biocide tributyltin stimulates adipogenesis, similar to previous reports in rodent models. Here, we take this approach further and use a transgenic fish line to examine effects of MDCs on the endocrine and exocrine pancreas, as well as lipid metabolism in the liver, in combination with a lipid stain to assess alterations in adipogenesis.

This project (GOLIATH) has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 825489.

P16-07**Exposure to acrolein disrupts the molecular regulation of mitochondrial metabolism in epithelial cells of the human airways***C. Tulen¹, C. H. Schiffrers², C. van de Wetering², H. W. Cremers³, E. Duistermaat³, P. H. Fokkens³, W. N. Klerx³, N. Weibolt³, N. L. Reynaert^{2,4}, M. A. Dentener^{2,4}, Y. C. Staal³, F. J. van Schooten¹, A. Opperhuizen^{1,5}, A. H. Remels¹¹ Maastricht University Medical Center+, NUTRIM School of Nutrition and Translational Research in Metabolism, Department of Pharmacology and Toxicology, Maastricht, Netherlands;² Maastricht University Medical Center+, NUTRIM School of Nutrition and Translational Research in Metabolism, Department of Respiratory Medicine, Maastricht, Netherlands;³ National Institute for Public Health and the Environment (RIVM), Center for Health Protection, Bilthoven, Netherlands;⁴ Maastricht University Medical Center+, PLUC facility, Maastricht, Netherlands;⁵ Netherlands Food and Consumer Product Safety Authority, Utrecht, Netherlands

Exposure of the human airways to cigarette smoke (CS) is the major etiological risk factor for developing devastating lung diseases such as Chronic Obstructive Pulmonary Disease (COPD). The complex mixture of circa 6000 chemicals present in CS includes the chemical class of aldehydes, reactive components generated during the pyrolysis

and combustion of tobacco. Acrolein, an $\alpha\beta$ -unsaturated aldehyde, has been associated with a substantial proportion of the smoking related non-cancer hazard. Although previous studies demonstrated aberrant mitochondrial morphology and impaired mitochondrial function in response to exposure of human airway epithelial cells to CS, whether or not acrolein is involved and to what extent the pathways regulating mitochondrial content and quality control are impacted is unknown. Therefore, we assessed the activity and abundance of key constituents of mitochondrial metabolic pathways, as well as transcript and protein levels of critical molecules regulating mitochondrial biogenesis and mitophagy in undifferentiated human primary bronchial epithelial cells (PBECS; provided by PLUC facility MUMC+, Maastricht⁴) of COPD (n=5) and non-COPD (n=7) subjects, in undifferentiated PBECS of non-COPD subjects exposed to CS extract (CSE) (0.1–2%) (n=4) or acrolein (0–25–50 μ M) (n=2), and in air-liquid interface differentiated PBECS of non-COPD subjects (n=4) exposed to CS (1 Marlboro Red cigarette) or a mixture of aldehydes (acetaldehyde, acrolein, formaldehyde; at concentrations as present in 1 Marlboro Red cigarette) according to the Health Canadian Intense Regime. Evaluation of the abundance of prominent regulators controlling mitochondrial metabolic pathways revealed no outstanding differences at gene and protein level in PBECS of COPD versus non-COPD subjects. Although mitochondrial DNA copy number was unaltered, CSE exposure, dose- and time-dependently, downregulated mRNA levels of mitochondrial biogenesis markers and upregulated protein and transcript abundance of autophagy regulators in undifferentiated PBECS. Moreover, we observed increased metabolic enzyme activity in response to CSE exposure, respectively of hydroxyacyl-coenzyme A dehydrogenase and phosphofructokinase. These alterations were, at least in part, recapitulated in acrolein exposed undifferentiated PBECS, as well as in differentiated PBECS in response to CS or a mixture of aldehydes. Collectively, these data show that CS(E) exposure, in particular acrolein, disrupts the molecular regulation of mitochondrial metabolism in cells of the human airways.

P17 – Reproductive toxicity

P17-01

Benzoyl-CoA conjugate accumulation as an initiating event for male reprotoxic effects in the rat: Structure-activity analysis, species specificity and *in vivo* relevance

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A number of *para* substituted benzoic acids (*p*-BA) and fragrance aldehydes such as 3-(4-*tert*-butylphenyl)-2-methylpropanal (BMHCA) metabolized to *p*-BA have been found to confer adverse effects in male rats on sperm viability, motility and morphology following oral gavage dose of >25 mg/kg body weight. These effects are putatively associated with the metabolism of *p*-BA to toxic intermediates. We had shown that *p*-BA lead to accumulation of high levels of benzoyl-CoA conjugates in plated rat hepatocytes (Laue *et al.* 2017). Here we further investigated relevance of this metabolic pathway for the reprotoxic effects in rats and rabbits. Intracellular formation of CoA conjugates from either *p*-alkyl-phenyl-propanals directly or from their benzoic acid metabolites was assessed for 19 chemicals in plated primary rat hepatocytes using high-resolution LC-MS. The nine reprotoxic compounds metabolizing to *p*-alkyl-benzoic acids led to accumulation of high and stable levels of *p*-alkyl-benzoyl-CoA con-

jugates in plated rat hepatocytes, whereas benzoyl-CoA conjugates of ten non-reprotoxic compounds were only transiently formed in this *in vitro* system which confirms the very strong correlation between benzoyl-CoA-accumulation in rat hepatocytes and the toxic outcome. Species specificity was probed by comparing rat, rabbit and human hepatocytes, and benzoyl-CoA accumulation was found to be specific to the rat hepatocytes, not occurring in human hepatocytes. There was also very limited accumulation in hepatocytes from rabbits that are a non-responder species in *in vivo* studies. Tissues of rats orally treated with 3-(4-isopropylphenyl)-2-methylpropanal were analyzed and *p*-isopropyl-benzoyl-CoA conjugates were detected in the liver and in the testes in animals at toxic doses indicating that the metabolism observed *in vitro* is relevant to the *in vivo* situation and the critical metabolite does also occur in the reproductive tissue. These multiple lines of evidence further support benzoyl-CoA accumulation as a key initiating event for a specific group of male reproductive toxicants, and indicate a species specific effect in the rat.

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P17-02

A species specific metabolism leading to male rat reprotoxicity of cyclamen aldehyde

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Cyclamen aldehyde (CA; 3-(4-isopropylphenyl)-2-methylpropanal) has been widely used for the last 100 years as a muguet olfactive note in perfumery. The safe use of this material, supported by a wide range of toxicology studies, is well established through the understanding of exposure and based on quantitative risk assessment confirmed by the RIFM Expert Panel. However, repeated dose studies in rats, mainly conducted for the purposes of hazard identification for the REACH registration, have revealed effects on sperm maturation at high doses leading to impaired fertility.

Purpose of the study: Here we investigate species specificity of the toxicity at the mechanistic and metabolic level.

Methods: Different metabolism studies were performed with suspended and plated hepatocytes from different species. *In vivo* metabolic data in rats were obtained from a repeated dose study. Several repeated dose studies in rats and rabbits are reviewed to characterize the fertility effects.

Results: The effect on spermatogenesis appears to be linked to the main circulating metabolite, 4-isopropyl-benzoic acid (iPBA). Metabolism studies in rat, rabbit and human primary cultures of suspended hepatocytes, indicated large metabolic differences with iPBA readily formed by rat hepatocytes but below detection limit in cells from rabbits and humans. In plated rat hepatocytes, iPBA is further conjugated to Coenzyme A and this conjugate (iPBA-CoA) accumulates to rather stable levels over 22 h. It has been shown, that *in vitro* accumulation of CoA conjugates is a metabolic hallmark strongly correlated to male rat reproductive toxicity for a number of related compounds. Indeed, iPBA-CoA was now also found to be formed *in vivo* both in the liver and in the testes of rats dosed with CA and a dose-dependent toxicity is observed. iPBA-CoA does not accumulate in

plated rabbit and human hepatocytes where it is largely cleared within 22 h. In a rabbit *in vivo* study, no effects of CA on spermatogenesis were observed. Thus, a species specific metabolic fate linked to CA toxicity in male rats can be postulated *in vitro* and *in vivo* in the liver, and in male reproductive tissue *in vivo*. Furthermore, the strong evidence indicates that this species specific metabolic fate in the rat is not relevant to the rabbit, which is a non-responder species. Finally, lack of accumulation of iPBA-CoA in human hepatocytes indicates that like the rabbit, humans are unlikely to be vulnerable to iPBA hepatic and testicular toxicity.

P17-03

Epigenomic analyses in sub-populations of spermatozoa from infertile men with short anogenital distance

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The key developmental steps in the male germ cell lineage include the epigenetic reprogramming of the primordial germ cells, the large-scale replacement of histone by protamine in spermatids and some degree of apoptosis. In past experiments, we prenatally exposed mice to di(2-ethylhexyl)phthalate (DEHP, CAS number 117-81-7) [1]. At adulthood, the exposed mice were characterized by reduced anogenital distance (AGD), poor semen quality, and epigenomic abnormalities in their gametes' DNA [2]. However, the phenotype heavily depended on the genetic background of the mouse strain. We identified SNP-dependent binding motifs for sex hormones signaling pathways varying between the susceptible and the resistant mouse strains. These SNP-dependent motifs were located in the genes that were the most affected one by DEHP, at both DNA methylation and RNA transcription levels [3]. We then tried to replicate these observations into human. We selected 6 infertile patients with short AGD and 5 fertile sperm donors. Testicular volume was significantly reduced in infertile men with shorter AGD. Sub-populations of spermatozoa were FACS-sorted using the chromomycin A3 dye (protamine vs. histone) and with the YO-PRO-1 dye (apoptotic vs. non-apoptotic). In the four sorted sperm populations, we analyzed the epigenomes through reduced-representation bisulfite sequencing (RRBS). The results demonstrated both lower CpG methylation and higher DNA fragmentation in infertile patients. Interestingly, numerous de-methylated regions localized within families of Alu repeats containing estrogen response elements (ERE). We are now in the process of replicating the entire study with a new cohort of five infertile patients and five fertile controls. The aim is to understand if there is a relationship between these epigenomic alterations within Alu repeats containing ERE and fertility, and whether exposures to anti-androgenic endocrine disruptors may alter methylation marks within these Alu-ERE repeats.

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P17-04

The effect of perfluorohexanesulfonate (PFHxS) on bovine preimplantation embryo development *in vitro*

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Perfluorohexanesulfonate (PFHxS) is an industrial chemical that belongs to the group of per- and polyfluorinated alkyl substances (PFAS). Due to its oil and stain repelling properties, it has been widely used in products as surfactant, for example in protective clothing. Because of its persistent nature, PFHxS is found widespread in drinking water, food contact materials and other consumer products. Consequently, humans are exposed to PFHxS, reflected by the fact that it is frequently measured not only in serum but also in similar concentrations in ovarian follicular fluid. This means that oocytes are constantly exposed to PFHxS, potentially affecting oocyte maturation and competence to form a viable embryo. The present study investigated the effect of PFHxS exposure during the final stage of oocyte maturation in a bovine model.

Cumulus oocyte-complexes (COCs, n=531) were collected from the abattoir. During the final maturation *in vitro*, the COCs were randomly divided in groups of 10 either exposed to PFHxS (0.01, 0.1, 1, 10, 20, 40 and 100 µg/mL) or vehicle only (water). After 22h maturation, the COCs were fertilized and cultured until day 8 of embryo development followed by staining to assess the number of blastomeres and neutral lipid content using confocal microscopy and computerized image analysis. The odds ratio (OR) of treatment effect on developmental parameters (cleavage stage at 44h after fertilization, proportion of embryos at day 7 and 8 after fertilization) were calculated using mixed effect logistic regression. For image analysis (n=60 embryos) average lipid volume per blastomere and total lipid volume were analyzed using linear regression with treatment in increasing concentration as fixed factor and stage of development, number of blastomeres and replicate taken into account.

After exposure to PFHxS, the chance of reaching cleaved stage was reduced in the 10 µg/mL (OR=0.49 (0.25-0.96), p=0.038) and 40 µg/mL (OR=0.42 (0.22-0.80), p=0.008) compared to the control. Similarly, the chance of reaching beyond two cell stage was reduced in concentrations ≥20 µg/mL (20 µg/mL OR=0.37 (0.17-0.75), p=0.007, 40 µg/mL OR=0.38 (0.20-0.70), p=0.003, 100 µg/mL OR=0.52 (0.27-0.99), p=0.04). The chance of reaching blastocysts stage was negatively affected only in the 40 µg/mL group (OR=0.12, p=0.014) 7 days after fertilization and no difference was seen at day 8 of development. In day 8 embryos, PFHxS increased the lipid volume per cell (p=0.005) and the total lipid volume (p=0.015) compared to the control.

In summary, we show that PFHxS affects preimplantation embryo development in a bovine *in vitro* model during a limited window of exposure. The altered lipid accumulation is in agreement with previous studies reporting altered lipid metabolism induced by PFAS, possibly due to activation of peroxisome proliferator-activated receptors or metabolic stress.

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P17-05

Assessment of the reproductive toxicity of *Methylococcus capsulatus* methanotrophic bacteria in an *in vivo* experiment

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Although Russia has more than 40-year experience in the use of microbial biosynthesis products, such products are classified as a novel food and are the subject of state registration. The state registration procedure involves comprehensive safety assessment, including studies of the effect on reproductive function and offspring development.

This publication presents the results of the methanotrophic bacteria *Methylococcus capsulatus* denucleated biomass evaluation in an *in vivo* reprotoxicological experiment.

The experiment was carried out on male and female Wistar rats: the animals were divided into two groups of 106 animals (34♂ and 72♀) each. The control group (C-group) rats received the AIN-93 diet, the experimental group (E-group) – a diet containing 7-12% denucleated bacterial biomass. Pregnant F0 females (7 and 9 in the C- and E-groups, respectively) were euthanized on day 20 of pregnancy, the number of ovarian corpora lutea, resorptions, implantation sites, the number of alive and dead fetuses, the pre-implantation loss (i.e. difference between the number of ovarian corpora lutea in ovaries and the number of implantation sites in uterus) as well as the post-implantation loss (i.e. difference between the number of implantation sites in uterus and the number of alive fetuses) were calculated. Fetuses were extracted, macroscopic examination was performed, fetal weight and craniocaudal size, absolute and relative weights of liver, kidneys, heart and lungs of fetuses were determined. A total of 35 and 45 fetuses were examined in the C- and E-groups, respectively.

Prenatal development of F1 offspring of the C- and E-groups had no significant differences, the values of all studied parameters were within the physiological fluctuations typical for Wistar rats. The number of alive fetuses, number of ovarian corpora lutea, resorption and implantation sites in uterus of C-group rats were 12.1, 15.0, 0.86 and 13.0, E-group rats – 11.1, 13.6, 0.75 and 12.3, respectively. Fetuses' body weight and craniocaudal size in the C-group were 4.34 g and 3.85 cm, in the E-group 3.82 g and 3.54 cm. The absolute weights of kidney, liver, heart and lung in the C-group were 0.032, 0.331, 0.023 and 0.117 g and the relative weights were 0.75, 7.69, 0.54 and 2.71 g/100 g, respectively; those in the E-group were 0.030, 0.328, 0.022 and 0.118 g, and 0.78, 8.58, 0.57 and 3.09 g/100 g, respectively. The values of the studied parameters varied within the physiological norm.

Thus, the results of the methanotrophic bacteria *Methylococcus capsulatus* denucleated biomass evaluation in an *in vivo* reprotoxicological experiment didn't reveal any effect on rats' reproductive function and prenatal development of the offspring.

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P17-06

Research of postnatal development of two generations rats' offspring fed with a diet containing a novel insect-derived food

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The reprotoxicological studies on several generations of Wistar rats (F0, F1, F2) were carried out as a part of a comprehensive evaluation of the novel insect-derived food. This publication presents the results of postnatal development of the F1 and F2 generations offspring that received a diet including Black soldier fly larvae biomass (*Hermetia illucens*) throughout the whole period of prenatal and 1st month of postnatal life.

A biomass of Black soldier fly (*Hermetia illucens*) larvae dried at 110-120°C and crushed to a homogeneous mass was used as a material for research. Dry larvae biomass was included into the diet of test group animals during the whole period of the study; control group animals received a basic semi-synthetic casein diet. For fertilization F0 and F1 females were co-housed with males in the ratio of 2:1. In the F0 generation 26 and 24 females were used; in the F1 generation 32 and 31 females in the control and test groups, respectively.

Postnatal F1 and F2 progeny development was being assessed during the 1st month of life by counting the number of alive and dead pups, dynamic of body weight and length, physical developmental landmarks (i.e. ear unfolding, first coat, incisor eruption, eye opening, testicle lowering, vagina opening). Pups' body weight and length were measured on post-natal days 2, 5, 10, 15, 20 and 25th. The average litter size, the male-to-female ratio and the postnatal survival index were calculated from 1st to the 25th day of life (i.e. the ratio between the number of pups being alive on the 25th day to the total number of pups born alive).

The total number of rats in the control and test groups of the F1 generation was 292 and 281, the F2 generation was 373 and 382; the average litter size was within normal limits: 11.23 ± 0.47 and 11.71 ± 0.55 , 11.66 ± 0.68 and 12.37 ± 0.39 , respectively. The male-to-female ratio regardless of group affiliation was close to 1:1 with a slight prevalence of females in both generations. Physical development of all rats was without deviations. The body weight and craniocaudal size dynamics didn't have any significant differences between the groups and corresponded to the norm, typical for rats of the 1st month of life. Survival rate of F1 and F2 offspring in the control and test groups was over 99%, which corresponded to the optimal level for the Wistar rats.

Therefore, the research of postnatal development of two generations rats' offspring fed with a diet containing a novel insect-derived food did not reveal any negative effect of the studied product. The values of all examined parameters were within the physiological norm, and the survival rate of offspring corresponded to the optimal level for rats of this line.

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P17-07

Cytotoxic effects of bisphenol A alternatives in TM3 mice Leydig cells *in vitro*

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Hazardous chemicals escape to the environment by a number of anthropogenic activities and may cause adverse effects on human health and the environment. A wide heterogeneous group of chemicals identified around the world are endocrine disruptors (EDs). One of the oldest and most studied synthetic substance is bisphenol A (BPA). BPA is frequently used as a component of paints, beverage containers or plastics packaging. Many experimental studies confirmed the adverse effect of BPA including neurotoxicity, genotoxicity and reproductive toxicity as well. Several reproductive changes have been

reported in males, including a decline in semen quality, an increase in the prevalence of cryptorchidism, a decrease in sex hormone secretion, and an increase in the incidence of testicular germ cell cancer. Due to many negative effects, using BPA has been partially banned in some consumer products, and new analogues such as bisphenol B, AM, Z, F or S have begun to use. Nowadays, toxicological data are scarce and experimental studies evaluating the effects of bisphenol analogues are unclear. The aim of the present *in vitro* study was to investigate the potential impact of bisphenol A, B, S, and F on mice Leydig cells. TM3 cell line was cultured in the presence of experimental doses of selected bisphenols start from 0.04 to 50 µg/mL during 48h exposure. The cytotoxic effect was evaluated by alamarblue and 5-carboxyfluorescein diacetate assay. The concentrations of steroid hormones were determined using enzyme-linked immunosorbent assay. In addition, the level of reactive oxygen species was assessed by the chemiluminescence method, while intercellular communication of exposed cells was evaluated by scalpel loading/dye transfer technique. The obtained data indicate a dose-dependent decrease in Leydig cell viability after bisphenols exposure. The highest cytotoxic effect was observed at 25 and 50 µg/mL of bisphenol B. Significant decreases in cell viability was observed at 10; 25 or 50 µg/mL of bisphenol A, S and F. Within the panel of bisphenols under investigations, the highest doses of bisphenol A and B (25 and 50 µg/mL) significantly reduced progesterone secretion. Similarly, at 10; 25; 50 µg/mL of bisphenol S and bisphenol F was recorded moderate decreased without significant changes. The level of reactive oxygen species was statistically increased at 10; 25 or 50 µg/mL of bisphenols, while the gap junctional intercellular communication was significantly inhibited. Considerably more detailed and systematic research in environmental toxicology is definitely required for a better understanding of risks associated with an endocrine and reproductive disruption in humans and wildlife.

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P17-08

Per- and polyfluoroalkyl substances (PFAS) during oocyte maturation and the effect on early porcine embryo development and apoptosis *in vitro*

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PFAS constitute a large and diverse group of man-made compounds used in e.g. clothing, packaging materials, and fire-fighting foams. The aims of this study were to evaluate the effects on early porcine embryo development *in vitro* and apoptosis rate after exposure to perfluorohexane sulfonic acid (PFHxS) and perfluorooctane sulfonic acid (PFOS) during oocyte maturation. Embryos were produced *in vitro* according to standard procedures with oocytes from abattoir ovaries. A total of 855 oocytes were run in 9 cycles of embryo production. During the 44 h maturation process, PFHxS (40 µg/mL) and PFOS (0.1 µg/mL), respectively, were added to the maturation medium, always with a control present. Since PFOS is known to have adverse effects on reproduction, a biologically relevant concentration was chosen for this compound. As the effects of PFHxS are incompletely understood, a higher concentration based on previous *in vitro* data was used. Cleavage rate was observed 48 hours post fertilization (pf). Developmental stages and grades of resulting blastocysts were assessed on day 5 and 6 pf. All presumed blastocysts were then stained

using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to detect apoptotic nuclei (*In Situ* Cell Death Detection Kit, TMR red, 12156792910, Roche, Mannheim, Germany). Positive and negative controls were included, and DAPI was used as counter stain. Images were collected by laser scanning confocal microscopy and evaluated by computerized image analysis. Logistic regression with binominal distribution was used to evaluate the effects of treatments on developmental parameters. Linear regression was used to evaluate the treatment effect on proportion of apoptotic cells. Neither PFOS nor PFHxS had any significant effect on cleavage rate, developmental stage, or grade. PFOS had no effect on blastocyst rate on day 5 or 6 pf, but PFHxS treatment tended to result in higher blastocyst rate on day 5 pf ($p=0.07$) and resulted in higher blastocyst rate on day 6 pf ($p=0.05$). Blastocysts with a higher total number of nuclei contained lower proportions of apoptotic nuclei ($p=0.01$). Treatment with PFHxS tended to result in higher total numbers of nuclei in blastocysts ($p=0.06$) and blastocysts in the PFHxS group tended to contain higher proportions of apoptotic nuclei than control blastocysts ($p=0.08$). Mean apoptosis proportions $\pm$ standard deviations were as follows: 0.105 ± 0.082 (control group), 0.126 ± 0.093 (PFOS group), and 0.131 ± 0.087 (PFHxS group). PFOS did not have any effect on total numbers of nuclei or apoptosis rate. This study suggests that PFHxS is likely to interfere with some parameters in the early development of porcine embryos. The relatively low concentration of PFOS used here had no effect on any parameter. More research is needed to obtain further knowledge on the effects of PFAS on early development and apoptosis in embryos.

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P17-09

Study of progeny prenatal development of Wistar rats fed with *Hermetia illucens* larvae dry biomass

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In the Russian Federation, the practice of novel food sources safety assessment, in particular, obtained with the use of insects, includes research of reproductive function and progeny development in the experiment on Wistar rats. This publication presents the data of prenatal development of F1 generation offspring.

Male and female rats 25–30 days old were randomly divided into control and test groups of 64 females and 32 males each. Rats of the control group received a standard semi-synthetic casein diet; the test group received the diet with inclusion of Black soldier fly (*Hermetia illucens*) larvae dried biomass. In order to impregnate females, they were housed together with males in 2:1 ratio for one estral cycle (7 days). During the mating period, the rats' age was 110–120 days. Pregnant F0 females (19 and 15 in the control and test groups, respectively) were euthanized on day 20 of pregnancy. The number of ovarian corpora lutea, resorptions, implantation sites, the number of alive and dead fetuses, the pre-implantation loss (i.e. difference between the number of ovarian corpora lutea in ovaries and the number of implantation sites in uterus) as well as the post-implantation loss (i.e. difference between the number of implantation sites in uterus and the number of alive fetuses) were determined. Fetuses were extracted for macroscopic examination, weight and craniocaudal size measuring, absolute and relative weights of internal organs (liver, kidneys, heart and lungs) determination. Also fetuses were examined according to Wilson J.G. and Dawson A.V. methods.

The comparison of F1 offsprings prenatal development didn't reveal any significant differences between the test and control groups, all studied parameters were within typical for Wistar rats physiological fluctuations. Zoometric parameters of fetuses and internal or-

gans weights varied within the normal range and had no significant differences at $p > 0.05$. During the examination of F1 fetuses according to Wilson J.G. method no abnormal development of internal organs was detected. The formation of fetuses' main anatomical systems ran without any peculiarities in both groups. The study of F1 fetuses skeletal development according to Dawson A.B. method did not reveal any congenital skeletal anomalies in the fetuses of control and test groups. The ossification sites length in the limb bone and skull tabs of F1 fetuses had no significant differences between the groups.

Thus, the study of progeny prenatal development of Wistar rats' fed with *Hermetia illucens* larvae dry biomass indicate the absence of any negative effect. The obtained data can be considered as a direct evidence of *Hermetia illucens* larvae safety in terms of influence on reproductive function. Fetuses' zoometric parameters in both groups corresponded to the physiological norm, and no fetal development abnormalities were detected.

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P17-10

Expression of xenobiotic transporters in the rat and rabbit kidney, liver and placenta during pre- and postnatal development

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Introduction: For the approval of drugs and plant protection products, their teratogenic effects, which can occur in prescribed animal experiments, play a decisive role. To correctly assess the relevance of such effects for men, it is crucial to know the molecular mechanisms underlying the teratogenic effect. This is very difficult due to the high complexity of the system (maternal and embryonic / fetal circulation) and many unknown parameters.

Transporters play an important role in ADME, especially for substances that require active transport in cells. Differences in developmental toxicity between species may be due to the different expression of xenobiotic transporters. The transporters of two superfamilies are known for their role in the distribution of xenobiotics and endogenous substances: the ATP-binding cassette transporter (ABC) and the solute carrier (SLC) familie. Regarding embryo-foetal developmental toxicity, the liver and kidneys of the embryo, the foetus, and the mother as well as the placenta are crucial organs for xenobiotic and endogenous transport. The data on the expression of xenobiotic transporters during ontogenesis are very limited and are almost completely absent, especially in rabbits.

The aim of the study was to investigate the gene expression of xenobiotic transporters in the liver, kidneys and placenta of rats and rabbits in different pre- and postnatal stages of development and to compare the received data with the results of human studies.

Methods: Rats and rabbits were scarified at different but pre- and postnatal time points, considering the species-dependent developmental timelines. Tissue samples of liver, kidney and placenta of embryos, foetus or offspring were collected, snap frozen, and stored at -80°C . mRNA was extracted and expression rates of ABCB1, ABCB11, ABCC1-6, ABCG2, SLC22A6-8 and several SLCOs were determined by 2-Step RT-qPCR.

Results: The expression of the transporters during ontogenesis mostly differed greatly between the species. However, there were also transporters whose expression was very similar in at least two of the species examined. Most of the transporters showed a significantly

lower expression in the embryo/foetus compared to the adult value, which increased with advancing development. Other transporters showed a similar expression during the entire ontogenesis or higher values at prenatal time points.

To be able to understand the molecular causes of the teratogenic effects of substances, knowledge of their ADME is essential. Xenobiotic transporters can play a decisive role here. Once possible xenobiotic transporters have been identified, knowledge of their expression during embryonic and foetal development in the relevant species is of enormous importance. The gene expression data generated can therefore be used to better assess the developmental toxicity results in non-clinical species and their relevance to humans.

P17-11

Investigation of pre and postnatal developmental toxicity of caffeine in rats

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Caffeine-based beverages and foods are widely consumed and considered a harmless habit. The study on pre- and postnatal development (PPND) in rats was performed to investigate the potential toxicity on pregnant/lactating female rats and on their offspring of caffeine following ICH guideline S5 (R2).

Sprague Dawley rats were received from Vital River Laboratory Animal Technology Co., Ltd., China. The animals were housed in a double barrier area. At initiation of dosing, F0 animal ages were approximately at 12 weeks old. Mated female rats, 20/group, were treated with caffeine at dosages of 0, 20, 40, and 80 mg/kg/day once daily during the period of organogenesis and lactation (from Gestation Day 6 to Lactation Day 21). Routine endpoints/evaluations. Test article-related decreases of maternal body weight and food consumption were observed at 40 and 80 mg/kg/day during gestation but recovered and were comparable to the controls late gestation and/or lactation period. Persistent and significant decreases in offspring body weight and food consumption were observed at 80 mg/kg/day during lactation period, or in puberty, and during the period from weaning to termination. Minor developmental retardation on eye opening, tooth eruption and learning and memory [the total distances traveled and escape latency were slightly higher than control only for males in the training trials for 5 consecutive days (three trials each day)] were also observed in offspring at 80 mg/kg/day. No caffeine-related changes on reflex and sensory development, automated motor activity test, functional observational battery test, accelerating rotarod test, and reproductive developmental toxicity, and macroscopic observations were observed in this study. No-Observed-Adverse-Effect-Level (NOAEL) of caffeine on maternal toxicity (reduction of body weight and consumption) was considered 80 mg/kg/day, the NOAEL on offspring pre- and postnatal developmental toxicity (reduction of body weight and consumption, developmental retardation on eye opening, tooth eruption and learning and memory) was considered 40 mg/kg/day.

P17-12

Exposure to Persistent Organic Pollutants in Danish pregnant women: hormone levels and fetal growth indices

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Objective: Previous studies have suggested that Persistent Organic Pollutants (POPs) decrease foetal growth, although to elucidate the possible mechanisms needs further research behind. This study examines possible associations of i) maternal POPs exposure in early pregnancy and effects on the homeostasis of endogenous hormones and ii) maternal hormone levels and their effect on fetal growth indices (FGI).

Method: During 1st trimester maternal hormones (thyroid-, androgen- and estrogen hormones) and POPs levels (lipophilic POPs and perfluoro-alkyl acid substances (PFAAs)) were measured in serum from 800 nulliparous women in the Aarhus Birth Cohort sampled from 2011–2013. FGI (weight, length, head circumference, ponderal index and gestational age at birth) were collected postpartum. Multivariate-linear regression models under adjustment for potential confounders were used to assess associations between exposure and outcomes. To explore the combined effect of POPs we used principal component analysis.

Results: Maternal POPs exposure affected maternal hormone levels. In overall, the analyses showed negative association between lipophilic POP exposure and androgen and estrogen levels in the continuous and categorical regression analysis. We found no strong evidence of association between lipophilic POP exposure and thyroid hormones. For PFAAs, results indicated a positive association between PFAAs exposure and maternal thyroid- and androgen hormones in the continuous and categorical regression analysis. PFAAs exposure did not affect estrogens in the continuous analysis, but categorical PFAAs exposure analyses elucidated some associations. The categorical data predominantly indicated a positive association with quartiles of most PFAAs congeners and estrogens, mainly estrogen sulphate.

Only estrone levels was weakly associated with FGI in the total study population in the continuous analysis. After stratification on gender, the thyroid-peroxidase-antibody levels were positively associated with male birth weight. The categorical exposure analyses indicated a positive association between estradiol and birth length and negatively in the 2nd quartile of free thyroxine for gestational age.

Conclusion: POP exposure significantly affected maternal thyroid and sex hormone levels in early pregnancy. Lipophilic POP exposure was mainly inversely associated with androgen and estrogen levels, whereas PFAAs exposure was predominantly positively associated with thyroid and androgen hormone levels. We did not find strong evidence of maternal hormone level and impact on FGI.

P17-13

Reproductive health of transgender people: development and improvement of animal models and evaluation of the impact of environmental chemicals

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Transgender (TG) is an umbrella term used to describe individuals whose gender identity and behaviour significantly differs from the social norms for males and females. For some TG people, social change is enough without further physical intervention that may not be opportune or appropriate. Many access health services to obtain gender affirming treatment in the form of hormone therapy (HT) and/or through gender affirming surgery. Masculinizing HT involves parenteral or transdermal testosterone (T) treatment. For TG women, guidelines suggest treatment with both estrogen and androgen-lowering drugs. HTs aims to modified physical characteristics and to

induce masculinization or feminization with T levels within the cis-gender range.

Up to now, scarce data on the potential impact on reproductive health, the long-lasting effects and the liver alterations due to HTs are available. In addition, TG people are daily exposed to food and environment contaminants but tools for hazard identification to be used for a more reliable risk assessment do not exist yet; indeed, TG people may be considered as a sub-group of population specifically susceptible to contaminants, especially Endocrine Disruptors for their target and mode of action.

Few *in vivo* studies intentionally mimicking gender-affirming HT are available. One study investigates the effect of T on bone health and atherosclerosis in ovariectomized adult female mice without analysis of reproductive effects. The second study proposes a mouse model to evaluate reproductive effects; adult female mice exhibit several reproductive perturbations also observed in TG man, but the persistence of these changes after HT cessation was not studied. Recently, a third study investigates the brain changes of TG women using a model of pharmacologically-estrogenized adult male rats, but the reproductive system was not evaluated.

In this respect, the development and the improvement of innovative TG animal models mimicking gender affirming HT are of paramount usefulness; they should be used to evaluate long-lasting effects on reproductive health and fertility of TG man and woman. Moreover, the TG models may help to investigate the role of log-life exposure to sex hormones in the liver giving that it is essential for reproduction in both sexes. Several approaches including 'omics' should be used in order to identify specific pathway and to improve and limit the undesired effects of gender affirming HTs.

Using the TG model to test ubiquitous chemicals as model compound in the experimental plans, it will be possible to explore the potential different susceptibility of TG to the exposure to chemicals and the main metabolic pathways potentially involved in the liver response. Thus, the TG models will provide tools for hazard identification of TG sub-groups of population potentially applicable to other contest such as the impact of medications use and/or life styles.

P17-14

Effects of long-term exposure of human granulosa cells to low-dose of DEHP

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Di-(2-ethylhexyl) phthalate (DEHP) is a powerful endocrine disruptor that belongs to the group of phthalates. DEHP can be found in human body fluids at nM concentrations. Exposure of women to DEHP adversely affects IVF outcome, while *in vivo* and *in vitro* studies demonstrate its harmful effects on granulosa cells' steroidogenesis in animals. The majority of *in vitro* studies examine the effects of DEHP using short-term exposure; however, this scenario is unlikely to reflect the consequences of long-term, real-life exposures that are common in humans. The aim of this study was to investigate the *in vitro* effects of long-term exposure to low dose of DEHP on the function of human granulosa cells (HGrC1). The cells were exposed for 4 weeks to DEHP at concentrations found in follicular fluid and serum (50 nM and 250 nM). The steroidogenesis in granulosa cells was evaluated every week. The results showed that 50 nM DEHP decreased the basal mRNA levels for CYP19A1 after 3 weeks, however, protein CYP19A1 levels and estradiol production remained at control levels during 4-week long exposure with 50 nM and 250 nM DEHP. The basal and forskolin-stimulated expression of steroidogenic acute regulatory protein mRNA and protein and production of progesterone were not affected during 4-week long treatment with 50 nM and

250 nM DEHP. These results indicate that long-term treatment with low doses of DEHP doesn't have detrimental effects on steroidogenesis in HGrC1 cells. Further research is needed to reveal other functions of human granulosa cell that might be affected by low dose and long-term exposure to DEHP.

P17-15

Characterization of the effects of the steroidogenesis inhibitor Ketoconazole on the human fetal ovary

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A woman's fertility is largely programmed early during embryonic and fetal development. An increasing body of evidence shows that exposure during specific developmental "windows of sensitivity" in her life to endocrine disrupting chemicals (EDCs) can induce life-long adverse effects. However, underlying mechanisms linking an early exposure to EDCs with reproductive dysfunction remain poorly understood. A serious concern is that the repercussions of such exposure are often studied only in germ cells thus missing the critical somatic lineages and the whole developing ovary. Our aim was to systematically characterize the cellular and molecular effects of a well-known EDC on the entire human fetal ovary. Ketoconazole (KTZ) was selected for its properties as potent inhibitor of the enzymes of the steroidogenic pathway.

Fragments of human fetal ovaries (6-12 developmental weeks (DW)) were cultivated with concentrations of KTZ across a broad concentration range (10^{-5} - 10^{-9} M) or vehicle (DMSO) for one week. We tested classical endpoints in conjunction with a range of molecular approaches to identify the most useful, relevant and sensitive assays that could be included in an experimental strategy toolbox for regulatory purposes. Cell proliferation, viability (apoptosis and necrosis) and the germ cell lineage were analyzed in the explants by flow cytometry and histology, while the culture medium was used for functional and endocrine assays.

Except for an obvious toxicity at 10^{-5} M, which was measurable as early as after 24h of exposure, neither cell viability nor proliferation were globally altered by KTZ. The impact on germ cells was similar to that on the somatic cell lineage. Assays targeting endocrine activities showed that KTZ decreased estradiol production from 10^{-7} M onwards. In contrast, inhibin B production increased after exposure to 10^{-6} M of KTZ before dropping after exposure to 10^{-5} M. To obtain insights into the metabolic effects of KTZ exposure, lactate released in the medium was measured. KTZ induced an increase in lactate production at 10^{-5} M KTZ, but not at lower concentrations. A better characterization of mechanistic effects will be further obtained by a wider screening of the cell metabolism and by the high throughput analysis of the ovarian transcriptomes.

The identification of relevant techniques and sensitive endpoints suitable to screen for direct effects of a given EDC on the cultured human fetal ovary will help in the global effort to screen for deleterious molecular and cellular initiating effects of EDCs. Our experimental approach will significantly contribute to defining key events at the cellular and molecular levels for the construction of novel Adverse Outcome Pathways.

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P17-16

Evaluation of the impact of cannabinoids used for recreational or therapeutic purposes on the male germ cell differentiation during development

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Cannabis is the most widely consumed drug worldwide. Its legalization for recreational use is actively debated in France and its therapeutic importance is under study. To date, the impact of cannabis exposure onto human health has not been well characterized. A recent meta-analysis highlighted a significant association between cannabis consumption and the risk of developing testicular germ cells tumors (TGCTs)^[1]. Recreational cannabis consumption was also associated with a disturbance in male hormones levels and sperm alterations^[2]. Δ -9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are the two main components of the cannabis plant used in therapies, either alone or in mixture. They mainly act on the cannabinoid receptor 1 (CB1) and 2 (CB2), which also respond to endogenous ligands called endocannabinoids^[3]. The expression and localization of the components of the endocannabinoid system (ECS) (receptors, synthesizing and degrading hormones) have been characterized in the adult human testis^[4] but never in the fetal testis. Since TGCTs arise from a disturbance in germ cell differentiation and cannabis increased the risk of TGCTs, we hypothesize that the ECS plays a role in germ cell differentiation during the testicular development. Cannabis being the drug the most consumed by pregnant woman, cannabis exposure during fetal life could potentially lead to TGCT by disrupting the differentiation of germ cells. To address these hypotheses, our project is to (1) investigate the expression and localization of the ECS in the fetal human testis and (2) characterize the impact of a CBD and/or THC treatment on the fetal testicular physiology, using both an *ex vivo* organotypic culture model of fetal human testis and an *in vivo* murine model. *Ex vivo* tissues were treated for 72h with CBD and/or THC, at a concentration of 10^{-8} to 10^{-5} M and collected along with supernatants at the end of the culture. *In vivo*, pregnant Swiss mice were treated with a mixture of CBD/THC (1:1) at the dose of 5mg/kg/day from the embryonic day (E) 6.5 to E15.5, which covers the testicular development period. The analysis of bulk and single-cell RNA seq data, confirmed by RT-qPCR, showed the presence of ECS components in the fetal human testis at 9 to 14 amenorrhea weeks. While the exposure of human fetal testis explants to 10^{-5} M of CBD/THC (1:1) did not affect tissular organization, cell proliferation and apoptosis nor AMH or inhibin B secretion, we observed a significant decrease in testosterone secretion. *In vivo* experiments revealed a teratogenic effect of CBD/THC treatment at a dose of 5mg/kg/day, but the developing germ cells were not affected. However, gestational exposure to these compounds affected morphological parameters and testis weight in adulthood. Altogether, our study highlights the importance of determining the potential adverse effects of cannabinoids on reproductive health before legislate on their recreational or therapeutic use.

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P17-17

Investigation of Selected Persistent Organic Pollutants (POP) on Human and Dog Placental Cholinergic System

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Purpose: Placental cholinergic system is known to be an important factor in many intracellular metabolic activities and placental development besides neuronal neural transmission. Persistent organic pollutants are known to effect these system causing changes in epigenetic and glucose metabolism that will form the basis for structural anomalies, functional disorders, congenital neoplasia, growth retardation, preterm birth, vitamin D deficiency, systemic inflammation, low birth weight, preterm birth, adult coronary artery and diabetes diseases. These alterations would eventually lead gestational hypertension, preeclampsia, abortion and fetal death. Even though, human and dog share the same environment, their response to pollutant toxicity are different. The aim of this study is to investigate the relationship between environmental pollutants and placental cholinergic system through a comparative toxicology perspective.

Methods: For this purpose, 20 human placenta with hemochorial structure and the 20 dog placenta with endothelial structure were used. Analysis of selected 28 POPs (PCB, PBDE, PAH and Organochlorine pesticides) were performed by GC-MS. Microarray transcriptome analysis was performed in these collected samples to evaluate cholinergic related genes and pathways. These results were later confirmed by *in silico* and *in vitro* analysis, to further evaluate the relation between the pollutant and receptor using HTR8/SVneo cell line and molecular docking analysis.

Results: 13 pollutants were detected in human and 7 were detected in dog placenta samples. A close correlation was found between pollutant detection and mother's smoking status/exposure history. Average PAH load was 877 ng/g, where samples also contain residues of PBDE 28, PCB 153 and 180. Residue of 4,4, DDT was the highest contaminant with a concentration of 54.82 ng/g. In dog samples average PAH values were 160 ng/g and DDT concentration were much higher with a concentration of 156 ng/g. The average total PCB load obtained was 16.6 ng/g, and the PBDE load was 89.71 ng/g. These values are 133 ng/g PCB and 488 ng/g PBDE load for canine placenta samples,

respectively. Microarray results revealed the cellular functions that could be associated with the non-neuronal cholinergic system such as cell proliferation, calcium signaling, acetylcholine receptor signaling, cytoskeleton and amino acid transfer were found to be affected with pollutants; which was mostly similar in both canine and human species. *In silico* screening with muscarinic, nicotinic receptors and acetylcholinesterase enzymes, reveal the high affinity of 4-4 DDEs and PCB153. qPCR results from the treated placenta cell line with pollutants revealed the increase of cholinergic genes in accordance to pollutant treatment.

P18 – Systemic toxicology

P18-01

Brain acetylcholinesterase activity in Wistar rats subacutely exposed to low doses of Pb

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Lead (Pb) is toxic metal which exposure can affect particularly all systems and organs. The neurotoxic effects of lead have been studied for many years and could be attributed to numerous mechanisms including disruption of calcium and iron homeostasis, blockade of the NMDA receptor, oxidative stress, etc. Pb can also alter brain enzymes activity, including acetylcholinesterase (AChE), which has function in brain neurotransmission process as well as learning and memory. In this study we investigate dose-dependent effect of Pb on the AChE in the brain of Wistar rats.

Forty-two male (150–160 g body weight) Wistar rats were randomized into seven groups. Control group received distilled water while other six groups were treated with Pb – acetate solutions by oral gavage every day for 28 days: Group 1 (0.1 mg Pb/kg); Group 2 (0.5 mg Pb/kg); Group 3 (1.0 mg Pb/kg); Group 4 (3.0 mg Pb/kg); Group 5 (7.0 mg Pb/kg); Group 6 (15.0 mg Pb/kg). After 28 days rats were sacrificed and brains were removed, washed and homogenized. The AChE activity was examined in brain homogenates by Elman's spectrophotometric method.

Subacute treatment with Pb – acetate led to lower activity/inhibition of AChE. Statistically significant differences were observed in the brain AChE activity in lower dosage groups (from 0.1 mg Pb/kg to 3 mg Pb/kg), while treatment with 7.0 mg Pb/kg and 15.0 mg Pb/kg did not change brain AChE activity compared to control group.

The results have shown that exposure to lower Pb doses inhibited AChE activity in the rat's brain and probably disrupted neurotransmission, while higher doses did not change it. Further studies need to be carried out to understand this biphasic AChE response and determine other related mechanisms by which Pb induced neurotoxic effects in the case of low dose exposure and/or activated some other mechanisms of detoxification in case of higher doses.

P18-02

Update on toxicokinetics of type II pyrethroids in central nervous system after oral exposure: involvement to dopaminergic neurodegeneration

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Type II pyrethroid insecticides (deltamethrin, lambda-cyhalothrin and cyfluthrin) are largely used in agriculture, in human and veterinary medicine, in textile industries and household spray formulations. Epidemiological and animal studies provide evidence to support the hypothesis that pyrethroid exposure is adversely associated with effects on the male reproductive system, neurobehavioral and neurodevelopment effects as well as endocrine disrupting effects. The primary mode of action of all pyrethroid insecticides is the disruption of the voltage gated sodium channel electrophysiology in neurons of target pests and non-target species. The present study aimed to explore deltamethrin, lambda-cyhalothrin and cyfluthrin oral absorption and disposition in CNS in male Wistar rats after single oral doses (26 mg/kg, 20 mg/kg and 20 mg/kg, respectively) determining toxicokinetics parameters in plasma and brain tissues. Deltamethrin, lambda-cyhalothrin and cyfluthrin concentrations in plasma and brain tissues were quantified by LC/MS. All experimental procedures involving animals were conducted in accordance with the ethics requirements and authorized by the Institutional Animal Care and Use Committee of the Complutense University of Madrid. Our results showed that deltamethrin, lambda-cyhalothrin and cyfluthrin crossed the blood-brain barrier, entered the brain and accumulated in significant quantity mainly in the hypothalamus, hippocampus and striatum. Nervous tissue accumulation was reflected by the elimination half-life ($T_{1/2\beta}$) and by the area under the concentration time curve (AUC) ratios of tissue/plasma. For deltamethrin, the $T_{1/2\beta}$ and the ratio of $AUC_{(0-24)}\text{tissue}/AUC_{(0-24)}\text{plasma}$ were 40.8 h and 295 for hypothalamus, 26.6 h and 8.84 for frontal cortex, and 38.5 h and 93 for hippocampus. For lambda-cyhalothrin, the $T_{1/2\beta}$ and the ratio of $AUC_{(0-24)}\text{tissue}/AUC_{(0-24)}\text{plasma}$ were 34.8 h and 1.99 for hypothalamus, 18.8 h and 1.26 for frontal cortex, and 18.3 h and 1.17 for striatum. For cyfluthrin, the $T_{1/2\beta}$ and the ratio of $AUC_{(0-24)}\text{tissue}/AUC_{(0-24)}\text{plasma}$ were 22.7 h and 3.17 for hypothalamus, 18.5 h and 1.48 for striatum, 19.3 h and 1.31 for hippocampus and 19.8 h and 1.27 for frontal cortex. Because the dopaminergic nerve terminals are localized in these brain regions and the striatum, which is considered as the beginning site of dopaminergic neurodegeneration, our results imply deltamethrin, lambda-cyhalothrin and cyfluthrin as dopamine neurotoxins and possible environmental risk factors for neurodegenerative diseases. Further research on Type II pyrethroid neurodegenerative effects by involving multiple biological pathways, including oxidative stress, inflammation, cellular energy, xenobiotic metabolism and microglial activation should be addressed.

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P18-03

This abstract has been withdrawn.

P18-04

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P18-05

Effect of a chronic exposure to arsenite on the intestinal epithelium

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Arsenic (As) is a toxic metalloid widely distributed in the environment. Chronic exposure to inorganic As [As(III) and As(V)] affects approximately 200 million people, and is linked to a greater incidence

of certain types of cancer and other pathologies such as diabetes type II and cardiovascular diseases. Drinking water is the main route of exposure, so, in the endemic areas, the gastrointestinal epithelium is continuously exposed to the metalloid. *In vitro* studies have shown an adverse effect of inorganic As on intestinal epithelial cells. Subchronic exposures to high concentrations (≥ 50 mg/L) of As(III) in mice affect intestinal microbiota and modify epithelial permeability. However, there are no studies aimed to evaluate the effects of a chronic exposure to inorganic arsenic at the intestinal level.

The objective of the study is to evaluate the toxicity of a chronic exposure to As(III) on the intestinal epithelium. BALB/c mice (9 animals/treatment) were exposed during 6 months through drinking water at concentrations of 0, 15 and 30 mg/L. Various toxicological parameters were determined in intestinal tissues and fecal samples.

Chronic exposure to As(III) generates oxidative stress in the intestinal tissue, with an increase of reactive oxygen and nitrogen species (ROS/RNS, 41-76% increase with respect to non-treated animals) and malondialdehyde (8-51%) along with a decrease in intracellular glutathione (13-34%). Higher concentrations of various pro-inflammatory cytokines were observed in the intestine of treated mice: IL1 β (101-201%), IL-2 (97-166%), IL-6 (67-95%) and TNF α (24-94%). In addition, morphological abnormalities of the epithelium were detected, consisting of elongated crypts and greater number of goblet cells. The inflammatory status was also confirmed by neutrophil infiltrations, evidenced by an increase of fecal lactoferrin (23-29%) and a greater myeloperoxidase activity (17-55%) in the intestine. Furthermore, increased fecal concentrations of albumin were detected in treated mice, indicating a disruption of the intestinal barrier.

The analysis of the fecal microbiota evidenced significant differences in α -diversity between the mice exposed to 30 mg/L As(III) and the animals of the other groups, whereas a significantly lower Simpson index (α diversity) was observed for the group exposed to 15 mg/L. These results suggest that exposure to As(III) also affected the intestinal microbiota.

Changes in the intestinal permeability and microbiota after chronic exposure to As(III) through drinking water could be one of the causes of the systemic diseases reported in endemic areas.

P18-06

In vitro evaluation of the effect of a subchronic exposure to mercury upon intestinal epithelium

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Human exposure to mercury (Hg) mostly occurs through the diet, where it is primarily found as inorganic Hg [Hg(II)] or methylmercury (MeHg). In populations of Asia, Africa and Latin America, near mining areas, situations of risk have been described due to the consumption of contaminated local fishery products. The European Food Safety Authority indicates that the intake of MeHg of the European population is close to or above the maximum recommended intake and identifies frequent consumers of fish products as a risk group. *In vivo* studies have linked continuous exposure to Hg through the diet with neurological and renal diseases. However, its possible harmful effects upon the gastrointestinal tract are largely unknown even though oral ingestion is the main route of exposure to this metal.

The aim of this study was to evaluate *in vitro* the effect of mercury species on intestinal epithelium. For this purpose, a bicameral system was set up, where co-cultures of Caco-2 and HT29-MTX intestinal epithelial cells were grown onto semipermeable inserts (Transwell®) into 12 well plates, and differentiated THP-1 macrophages in the basolateral compartment. Cells were subchronically ex-

posed to Hg(II) and MeHg at various concentrations (0.1, 0.5, 1 mg/L) during a period of 11 days from the moment of seeding.

The results show that the subchronic exposure to Hg(II) increases the release of the pro-inflammatory cytokine IL-8 at the highest concentrations tested (33 to 85% increase with respect to control cells) whereas no significant increase was observed under MeHg treatment. However, a continuous exposure to both Hg species increased oxidative stress. Cells were able to establish a intestinal monolayer although it was observed that Hg(II) and MeHg exposure increased the cellular volume and disrupted the barrier integrity, as shown by an increase in paracellular permeability at 10 days after seeding (6 to 178% increase with respect to untreated cells). From the data obtained, we can conclude that the co-culture of intestinal cells and macrophages is a suitable model to study the influence of both Hg species on intestinal epithelium. Using this cell model, a pro-inflammatory and pro-oxidant response, accompanied by structural and functional abnormalities have been observed on the intestinal monolayers.

P18-07

Retrospective analysis on the tolerability of non-standard used vehicles in non-clinical studies

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Vehicles, commonly used for the formulation of test articles in non-clinical studies, can cause significant clinical signs or toxic effects that might complicate the interpretation of study results and the evaluation of the potential toxicity of the active test article.

We have performed a retrospective analysis on vehicles used over the past 7 years in studies conducted in rats, dogs, Cynomolgus monkeys and minipigs. The most non-standard used vehicles were evaluated and included cyclodextrins (HPBCD and SBECDD), polyethylene glycol (PEG) 400, methylcellulose, carboxymethylcellulose, dimethyl sulfoxide and tween 80, used to solubilize or stabilize the active substance or as thickening agents. The vehicle-related effects on in-life observations and postmortem examination were analyzed from general toxicology studies, from dose range finding to 39 Week studies. The effects were evaluated based on the vehicle quantity, route of administration and species.

Intravenous administration of both cyclodextrins resulted in vacuolation in the kidneys and urinary bladder, histiocytosis in the lungs as well as inflammation and hemorrhage at the injection site in almost all studies conducted in rats, dogs and Cynomolgus monkeys. When administered orally, HPBCD was sometimes associated with transient hypersalivation and abnormal foraging in rats, Cynomolgus monkeys and minipigs. After injection of PEG 400, intravenously in rats and Cynomolgus monkeys and subcutaneously in minipigs, inflammation and necrosis at the injection site, along with vacuolation of the macrophages were occasionally observed. After oral administration of PEG 400, hypersalivation, abnormal foraging, soft and/or colored feces were observed in some of the rat, dog and Cynomolgus monkey studies. The use of methylcellulose and tween 80 resulted in rare clinical signs, respectively salivation and ploughing following oral administration in rats and inflammation and necrosis at the injection site following subcutaneous administration in minipigs. Carboxymethylcellulose, administered orally, was not associated with any adverse findings.

This retrospective analysis brings valuable information in our toxicology studies, such as acceptable ranges for the use of vehicles in different species. Knowledge on the findings induced by the vehicles allows us to better understand and distinguish these effects from those associated with test articles.

P18-08

Evaluation of toxic effects mediated by exposure to Chlorobutanol in SD rats using a single and 28-day feeding study

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Chlorobutanol is used as preservatives in cosmetics and has antibacterial activity. This study aimed to investigate the single and repeated dose 28-days oral toxicity of a Chlorobutanol suspension in Sprague-Dawley (SD) rats. For the single-dose toxicity study, dose of 62.5, 125, 250 mg per kg of body weight (mg/kg b.w.) of Chlorobutanol was given once orally to SD rats. Mortality and clinical signs were observed and recorded for the first 30 min after treatment, at 4h post-administration and at least once daily for 14 days after administration. For the repeated dose 28-day toxicity study, the high dose was set as 100mg/kg b.w. and the middle, middle-low, and low dose were set to 50 mg/kg b.w., 25 mg/kg b.w., and 12.5 mg/kg b.w., respectively. Clinical signs as well as body weight and food consumption were measured and recorded daily. Hematology and biochemistry parameters were examined. Gross pathologic and histopathologic examinations were done on selected tissues from all animals. Mortality was not observed in the single-dose toxicity and repeated dose 28-day toxicity study. Body weight was no significant change in the repeated dose 28-day toxicity study. Relative liver and kidney weight was significantly increased both sex of 100 mg/kg b.w treatment group. But, there was histopathological change in liver and kidney for female and male respectively. These data suggested that the approximate lethal dose (ALD) of Chlorobutanol was over 250 mg/kg b.w. in the single dose toxicity study and the no adverse effect level (NOAEL) for Chlorobutanol was over 50 and 12.5 mg/kg b.w. for female and male rats in the repeated dose 28-day toxicity study.

P18-09

Toxicological evaluation of Propyl-propane-thiosulfinate (PTS) by 90-days oral subchronic study in rats

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Propyl-propane-thiosulfinate (PTSO) is one of the main organosulfur compounds present in *Allium* essentials oil. Different applications in the Agri-food sector have been proposed for PTS, such as feed additive, limiting the use of antibiotics in the diet of animals to curb human resistance to antibiotics and decreasing chemical residues in the environment generated by their use. However, the authorization of this compound depends on its toxicity profile. Thus, as a part of its safety assessment, in this work a repeated dose 90-day oral toxicity study has been conducted for the first time in rats following the OECD guideline 408. PTS was administered to groups of 10 male and 10 female rats at dose levels of 0, 14, 28, and 55 mg/kg/day. No relevant clinical signs or mortality and no relevant changes in body weight, food consumption and feed conversion efficiency were detected through the study. Moreover, no treatment-related changes in hematological and biochemical parameters were observed, for either sex or dose groups. The histopathology study was performed in liver, kidney, spleen, heart, brain, pituitary, stomach, intestine, lung, testi-

cle/ovary and skeletal muscle. No differences were found in organ weights, and no morphological and histopathological changes were observed. Based on these results, the no-observed-adverse-effect level (NOAEL) of PTS was judged to be ≥ 55 mg/kg/day for both sexes.

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P18-10

Is there a difference in expression of chosen metabotropic receptors at protein and mRNA levels in the brain of rats with different vulnerability to the rewarding effects of cocaine?

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Abuse and addiction to psychoactive substances, including cocaine, is a chronic brain disease. Many psychoactive drugs, like cocaine, induce addiction relatively quickly [1]. While the initial effects of cocaine (feeling pleasure, euphoria) depend primarily on the activation of the dopamine system, numerous studies show that adaptation within the glutamatergic system in the brain is responsible for the development of substance use disorder [2]. It is worth noting that addiction with drug craving and relapses develops only in 20% of persons who began to abuse these substances (so-called addiction vulnerable phenotype) [3]. Molecular mechanisms underlying these phenotypic differences have not been discovered so far.

The aim of this study was to determine the changes in expression of chosen metabotropic glutamate receptors mGluR1, mGluR5, and mGluR2/3 (protein and mRNA level) in rats showing phenotype either vulnerable or resistant to cocaine rewarding effects.

In our experiment, we used Wistar rats (250–280g). Conditioned place preference (CPP) was used to obtain animals showing addiction vulnerable or resistant phenotype. The level of mGluR1, mGluR5, and mGluR2/3 was determined by the Western Blot (protein) or RT-qPCR (mRNA) in the nucleus accumbens (NAc), prefrontal cortex (PFC), dorsal striatum (DSTR), and hippocampus (HIP), immediately after CPP test or after CPP re-test (conducted 7-days after CPP test).

We observed a decrease in mGluR2/3 in the PFC in vulnerable phenotype and decreased mGluR1 and mGluR5 expression in the DSTR in the resistant phenotype after the CPP test. After the CPP re-test, a diminished level of mGluR2/3 in the PFC and mGluR5 in the NAc was observed. The results of mRNA analysis confirmed or excluded the involvement of transcriptional mechanisms in the observed changes at the protein level depending on the type of protein and brain structure.

Summing up, the results indicate that both phenotypes – vulnerable and resistant, are characterized by various neuroadaptations resulting from the administration of cocaine. Those changes can influence the activity of specific brain circuits and most likely can contribute to the development of specific traits of the vulnerable or resistant phenotype.

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P18-11

The long-term neurotoxicity of doxorubicin in the brain of adult CD-1 mice

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Long-term cognitive dysfunction has been observed in patients treated with systemic chemotherapy, a phenomenon known as 'chemo-brain'. Doxorubicin (DOX) is a topoisomerase II inhibitor used to treat a wide range of tumours. Nevertheless, it can cause neurotoxicity through hitherto poorly elucidated mechanisms. Our work aimed to evaluate the neurotoxicity of a clinically relevant cumulative dose of DOX in the brain of adult mice, mainly focusing on the long-term neurotoxic effects. Male 3-month-old CD-1 mice received bi-weekly intraperitoneal administrations of DOX for 3 weeks, until they reached a total cumulative dose of 9 mg/kg. Animals were euthanized one week after the last administration (short-term) or 5 months (long-term). In both the short- and long-term groups, coronal sections of fixed brains were used for immunofluorescent detection of proteins related to neuronal damage in pre-frontal cortex (PFC) and hippocampal formation (HF). Moreover, in the HF of the short-term group, the volume and the total number of glial fibrillary acid protein (GFAP)-immunoreactive (ir) astrocytes were determined. In the right-brain hemisphere of the short-term group, the expression of ATP synthase β , manganese superoxide dismutase (MnSOD), endothelial nitric oxide synthase (eNOS), nuclear factor kappa B subunit p50 (NF- κ B) and glycogen synthase kinase-3 (GSK-3) was determined through western blot (WB).

Regarding the apoptotic markers, in the short-term study, Bax increased in the PFC and CA3 region of DOX-9 whereas in the long-term Bax expression increased in the CA1 and CA3 regions and p53 increased in the dentate gyrus and CA3 region. The total estimated GFAP-ir astrocytes also increased with DOX in the CA3 region, while decreasing in the dentate gyrus region of the HF. In the short-term study, so far, there were no significant changes in the biomarkers analyzed through WB. In summary, DOX significantly increased apoptotic markers (Bax and p53) in the adult mice brain and those changes persisted even 5 months after treatment. These changes may be possible causes for the cognitive impairments observed in treated patients, but further studies are needed to evaluate the long-term neurotoxicity and elucidate if chemobrain is an irreversible effect.

P18-12**mRNA vaccines against SARS-CoV-2: review of non-clinical challenge studies and potential contagiousity in Non Human Primates**

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SARS-CoV-2 infects humans by the use of the extracellular Spike protein (S) to attach to specific cell surface receptors. Due to its indispensable role, the S protein is a major target of virus neutralizing antibodies and has become a key antigen for vaccine candidates development. Recent improvements in mRNA vaccines act to increase protein translation, modulate innate and adaptive immunogenicity and improve delivery. mRNA vaccines are a new type of vaccine to protect against infectious diseases and were the first COVID-19 vaccines authorized worldwide. Relevant animal models are critical for a successful development of all SARS-CoV-2 vaccine candidates and are necessary to characterize the immune response induced by a SARS-CoV-2 vaccine candidate before moving into larger clinical studies, so as to address the theoretical risk for SARS-CoV-2 vaccine-induced disease enhancement.

If vaccines are effective on disease severity and reduce hospitalizations, the question is whether vaccination reduces virus transmission. Even, protection against reinfection with SARS-CoV-2 has been observed in rhesus monkeys, who formed neutralising antibodies on initial exposure and a minimum neutralising antibody titre has been proposed, no formal studies have been conducted from a non-clinical or clinical point of view, assessing the effect of the vaccination on a potential reduction in transmission nor the transmission between individuals.

This review provides an overview of the non-clinical data of the two different covid-19 RNA vaccines approved on the market, the different animal models studied as well as the virus transmission between individuals.

The objective is to make a comparison of the results of the challenge study on Non Human Primates (NHP), analysing all criteria that can approach this activity on the transmission of the infection (i.e. Viral RNA in nasal and oropharyngeal swabs and bronchoalveolar lavage after vaccination, clinical signs, ...)

However, since SARS-CoV-2 is a novel pathogen, any surrogate endpoints identified in animal studies would ideally need validation in clinical trials to ensure that they adequately predict efficacy in humans.

P19 – Ecotoxicology**P19-01**

This abstract has been withdrawn.

P19-02**Development of a battery of Atlantic cod receptor-based reporter bioassays for assessing environmental toxicity of sediments and chemicals**

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In the Adverse Outcome Pathway (AOP) concept, the molecular initiating event (MIE) represents the binding of the toxicant to its target, often a receptor inside a cell or on the cellular surface. MIEs lead to a

cascade of downstream key events (KE), which can result in an adverse outcome, i.e. toxicity. The aryl hydrocarbon receptor (Ahr) and the nuclear receptors (NRs) comprise important MIE targets for environmental pollutants. These receptors are able to bind and be activated by a wide range of xenobiotic chemical compounds, acting as xenosensors, and as transcription factors their modulation may lead to physiological perturbation representing endocrine and metabolic disruption.

Knowledge about the specificity and sensitivity of xenosensors in wildlife in general, and in key indicator species in particular, is an important aspect of ecotoxicological research. This knowledge is also the basis for developing receptor-based bioassays for use in environmental monitoring and toxicity testing. The Atlantic cod (*Gadus morhua*) is a fish species of high importance in pelagic and coastal ecosystems of the North Atlantic, as well as in Norwegian fisheries. It is also a widely used indicator species in environmental monitoring programs in Europe.

In the dCod 1.0 project (NFR 2016–2021) we have cloned and characterized the two cod AhRs as well as a number of cod NRs. For each receptor, we have established a luciferase gene reporter assay (LRA), where single compounds, mixtures, and environmental extracts have been tested. Using this battery of LRAs, we have shown how 11 different bisphenol A (BPA) analogs present similar, and sometimes stronger, endocrine disrupting properties, by activation of the cod Er. Using extracts from sediments representing a gradient of pollution from the inner parts of Byfjorden in Bergen to relatively pristine reference sites, we observe strong receptor activation at the more polluted sites. In the PW-exposed project, we are characterizing the toxicity of produced water from petroleum production using fractionated extracts, pinpointing specific fractions with the highest Ahr activating potency.

Together, our results demonstrate that a battery of receptor-based bioassays are useful in environmental monitoring and toxicity testing, at the same time advancing our knowledge of environmental risk from various complex pollution sources.

The studies were funded by the Norwegian Research Council through the projects dCod 1.0 (248840), iCod 2.0 (244564), and PW-exposed (280511).

P19-03**Splitting species sensitivity distributions (SSD) to improve accuracy of ecotoxicity results***S.A. Oginah¹, L. Posthuma^{2,3}, M. Hauschild¹, P. Fantke¹

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Species sensitivity distributions (SSDs) describe variation in sensitivity of multiple species exposed to a hazardous compound or mixture, as input to ecological risk assessment and deriving environmental quality criteria. Since chemicals have different toxic modes of action (MoA), they affect specific species groups more than others. For example, algae and aquatic plant species are on average more sensitive to exposure to chemicals with herbicidal MoA as compared to other aquatic species groups, such as invertebrates or fish. Current SSDs usually treat all species groups equally and focus on the variation in individual species sensitivities. However, for chemicals with specific MoA, this might lead to underestimation of ecosystem-level sensitivity. We propose to develop SSDs at the level of sensitive species groups versus less-sensitive species groups for chemicals with specific MoA, in order to improve the representativeness of SSDs in terms

of ecosystem-level sensitivity. We demonstrate that splitting SSDs into distributions for specific species groups for selected chemicals is feasible, and propose to apply average SSD slopes from data-rich chemicals as proxy SSD slopes for data-poor chemicals. With this approach, we will demonstrate and develop criteria for splitting SSDs, which will complement the current approach of constructing the SSDs for use in environmental protection, assessment, and management of freshwater aquatic ecosystems.

P19-04

Ecotoxicological influence of single-walled carbon nanotubes, graphene nanoribbons, and graphene quantum dots on marine microalgae

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The number of modifications of graphene-based nanomaterials is rapidly growing due to their extraordinary electronic, mechanical, optical, and antibacterial properties [1–3]. The environmental safety assessment of new materials required ecotoxicological bioassays with various endpoints [4,5]. In this study, we compared the aquatic toxicity of three types of graphene-based nanoparticles (NPs) (Table 1) in two marine microalgae, namely, *Attheya ussuriensis* and *Porphyridium purpureum*. Changes in microalgae cell conditions were measured by flow cytometer CytoFLEX (Beckman Coulter, USA) (Table 2). Table 1. Characteristics of the nanoparticles

Nanoparticles	Codename	Mean Size, nm	Impurity, %
Single-walled carbon nanotubes	SWCNT	Diameter 1.6, length >5	Total metals 14%wt.
Graphene nanoribbons	GNR	Width 1, length >100 nm, formation of nanobelts with length of >1 µm	–
Graphene quantum dots	GQD	Diameter 167.3	– Table 2. Endpoints

Endpoint	Fluorescent dye
Growth-rate inhibition	Propidium iodide

Nanoparticles	Codename	Mean Size, nm	Impurity, %
Single-walled carbon nanotubes	SWCNT	Diameter 1.6, length >5	Total metals 14%wt.
Esterase activity	Fluorescein diacetate		
ROS generation	2',7'-dichlorodihydrofluorescein diacetate		
Membrane potential	3,3'-dihexyloxa-carbocyanine iodide		

The one-way ANOVA test was used with GraphPad Prism 8.0.2 (GraphPad Software, USA). A value of ≤ 0.05 was considered statistically significant. All the measurements were performed in triplicate after 24 h, 96 h, and 7 days of exposure to the concentrations of NPs of 1, 5, 10, 25, 50, 100, and 200 mg/L.

The growth rate test demonstrated no dose and time-dependent toxicity in all the experimental series. Interestingly that all the tested NPs inhibited microalgal growth at the range of concentrations between 1 and 25 mg/L, and further increase of the concentration either mitigate the toxic influence or stimulate the growth of microalgae. The highest growth inhibition of both microalgae species (up to 25%) was caused by GQDs after 24 and 96 h of exposure at the concentration of 10 mg/L. GNR inhibited the growth of *P. purpureum* only at the highest concentrations. SWNTs demonstrated dose and time-dependent growth stimulation of *A. ussuriensis* (4 times after 7 days compared to control) and dose-dependent growth stimulation of *P. purpureum* (up to 1.5 times).

Among the registered biochemical changes, all the NPs caused membrane depolarization of microalgae. However, only SWCNTs caused dose and time-dependent esterase activity inhibition, which can be caused by metal impurities in this sample. This observation correlates with our previous results [6]. All the tested NPs increased ROS generation in *P. purpureum* cells.

In general, graphene-based NPs demonstrated low toxicity in used microalgae and even stimulated their growth. Registered biochemical alterations varied depend on microalgae species and types of NPs.

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P19-05**Impact of silver nanoparticles on the biouptake, physiological responses and metabolic perturbations in freshwater alga *Poterioochromonas malhamensis****W. Liu¹, S. Majumdar², W.W. Li², A.A. Keller², V.I. Slaveykova¹¹ University of Geneva, Environmental biogeochemistry and ecotoxicology, Geneva, Switzerland;² University of California, Bren School of Environmental Science & Management, Santa Barbara, USA

Given widespread use of silver nanoparticles (AgNPs), environmental risk assessment of these nanoparticles is of great importance. The toxicity of AgNPs was shown to be a result of an interplay of the particles uptake, dissolution and induction of the oxidative stress in aquatic organisms. Despite the progress in assessing their freshwater system implications, but little information exists concerning the metabolic perturbations induced by AgNPs on phytoplankton. Thus, the overall goal of the present study is to combine biouptake, physiological response and targeted metabolomics studies to elucidate metabolic perturbations in alga induced by AgNPs. We examine the interactions of citrate-coated 20 nm AgNPs compared with those induced by the dissolved Ag with the freshwater alga *Poterioochromonas malhamensis*. This golden alga often dominates mixotrophic phytoplankton population, and without cell wall can internalize these NPs.

Results of metabolomics evidenced that the exposure to AgNPs and dissolved Ag ions released by AgNPs resulted in time-dependent perturbation (exposure duration from 2 to 24h) of the concentration of metabolites involved in various metabolic pathways involving amino acids, nucleotides, fatty acids, TCA, antioxidants, photosynthesis and photorespiration. These perturbations of AgNPs toward *p. malhamensis* was attributed to the dissolved Ag ions. The exposure to AgNPs induced a significant accumulation of Ag in alga cell and increased with time. AgNPs internalized and accumulated in the vacuoles of *P. malhamensis* forming the agglomerates of 100 nm size. AgNPs internalized in food vacuoles contributed to the perturbation of amino acid metabolism, TCA cycle and oxidative stress. The physiological responses including ROS generation, lipid peroxidation, photosynthesis efficiency was also affected by AgNPs and dissolved Ag, particularly at 24 h exposure. These physiological responses confirm the metabolic perturbations observation. In this study, we demonstrate the integrated assessment of metabolomic and physiological changes for early detection of stress on phytoplankton induced by AgNP.

P19-06**Effect of PET and PVC microplastics on rainbow trout cell lines RTgill-W1, RTG-2 and RTL-W1***J. Boháčková^{1,2}, T. Cajthaml^{1,2}¹ Charles University, Faculty of Science, Institute for Environmental Studies, Prague 2, Czech Republic;² Czech Academy of Sciences, Institute of Microbiology of the Czech Academy of Sciences, Prague 4, Czech Republic

The contamination of the aquatic environment by microplastics is an emerging issue requiring a thorough research of the potential effects microplastics can have on living organisms. One of the organisms commonly used in toxicological studies is the freshwater fish rainbow trout (*Oncorhynchus mykiss*). It has been shown that *in vitro* toxicity assays on rainbow trout cell lines are a suitable alternative to whole-fish assays, providing comparable results for many tested pollutants and offering a more ethical approach. Polyethylene terephthalate (PET) and polyvinyl chloride (PVC) are among the six most produced polymers, but their representation in toxicological studies is rather low. Therefore, the toxicity of PET and PVC particles was tested using three cell lines from rainbow trout, the RTgill-W1 (gill),

RTL-W1 (liver) and RTG-2 (gonad) cell line. Multiple assays were applied to assess the changes in the metabolism of the exposed cells. The changes in cell viability were analyzed using three fluorescent dyes – alamarBlue, 5-carboxyfluorescein diacetate, acetoxymethyl ester and neutral red. In these assays, membrane integrity is evaluated as a marker of the general cell viability. The cells were also analyzed for changes in reactive oxygen species (ROS) generation and 7-ethoxyresorufin-O-deethylase (EROD) activity. The EROD assay enables to monitor the metabolism of xenobiotics by measuring cytochrome P450 1A induction. The ROS generation was studied using the 2',7'-dichlorofluorescein diacetate assay. After the exposure to PET particles, no significant changes were observed. On the other hand, PVC exposure did induce a significant increase in ROS generation in all tested cell lines. When studying the source of this effect, chemical additives leaching from the plastics were identified as the major contributor. Due to the ongoing leaching process, the age of the tested suspensions also proved to influence the observed toxicity, suggesting that the assessment of this variable should not be omitted in future toxicological studies related to microplastics.

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P19-07**Ecotoxicity effects of ZnCl₂ and ZnO nanoparticles on *Daphnia magna* and *Tubifex tubifex***

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Toxicological properties of nanoparticles (NPs) are currently under investigation because of their large applications in industry and common-use products. Due to their well-known chemico-physical characteristics, ZnO NPs are among the most used NPs and consequently subjected to be dispersed in the environment. The chronic toxicity of ZnO nanoparticles is less well documented including some ZnO nanoparticle toxicity studies on soil species but studies on aquatic species are still very limited.

We focused on acute and reproductive effects of ZnCl₂ and ZnO NPs (<50 nm, <100 nm), in particular. Assessment of the impact of selected NPs on important representatives of the aquatic environment. The aim of this study was to investigate the chronic toxicity of the nanoparticles to *Daphnia magna* and *Tubifex tubifex*. Selected representatives are an important part of the fish food chain. The effect of the ZnO NPs and ZnCl₂ salt was tested on the growth and reproduction of *D. magna* in a chronic test scenario according to OECD guidelines 211. Tests with *Tubifex tubifex* were performed according to method ASTM E1706-04. The concentrations of ZnCl₂ and ZnO NPs tested were as follows: 0.01, 0.05, 0.1, 0.5, 1, 5, 10 mg.l⁻¹. Graphpad Prism was used for data visualization and statistics. Based on the chronic toxicity data, time to first brood, time between broods and number of broods per *D. magna* female were calculated. Dose-response curves were constructed and EC10,20,50 reproduction values were calculated.

Daphnia magna has been shown to be a better and more sensitive bioindicators than *Tubifex tubifex*. The unexposed daphnids started reproduction faster and had more broods per female than the ones exposed to the highest concentrations of nanoparticles and salts. It is obvious that the combined dissolution and aquatic species sensitivity distributions results indicate that the toxicity of these nanoparticles is mainly caused by dissolved metal ions. Based on the available information, no current risk of these nanoparticles to the aquatic environment is expected.

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P19-08**Assessment of the toxic effects of 2-dodecanone in the insect model species *Drosophila melanogaster****M. Aquilino¹, R. Planelló¹, L. Lorente¹, D. Siauxat², Ó. Herrero¹¹ *Universidad Nacional de Educación a Distancia (UNED), Departamento de Física Matemática y de Fluidos/Grupo de Biología y Toxicología Ambiental, Madrid, Spain;*² *Institute of Ecology and Environmental Sciences of Paris, Department of Sensory Ecology, Paris, France*

Among the defence mechanisms against insects, plants synthesise many secondary metabolic compounds that are a deterrent to pathogens and phytophagous insects. In this sense, methyl ketones constitute a group of metabolites produced by some plants to defend themselves against the attack of phytophagous arthropods. This fact has raised considerable interest in recent years for evaluating the effects of natural compounds on different pest species. However, information about their mode of action and molecular effects are still very scarce. Few models offer the opportunity to perform an integrated study with multiple approaches, from molecular variations to physiological consequences. In this sense, *Drosophila melanogaster* is a proven model organism for research in genetics and biology. The fruit fly has also been considered a suitable model in ecotoxicology in recent years due to its short developmental time and its extensive and well-described genome information.

This work analysed the effect of 2-dodecanone on different developmental stages of *D. melanogaster*. Specifically, transcriptional alterations induced by sub-lethal concentrations (5 µg/L and 500 µg/L) of this methyl ketone were evaluated in third instar larvae (acute 24-hour exposures), as well as in adult males and females (chronic full life-cycle exposures). Fertility (average number of eggs) in exposed adults was also analysed. Quantitative real-time PCR (qPCR) was used to measure the expression levels of selected genes related to the endocrine system (*EcR*, *ERR*, *HR3*, and *BR-C*), the cell-stress signalling pathway (*Tl*, *def*, *p38*, *hsf*, *hsp22*, *hsp40*, *hsp70*, and *hsc70*), and detoxification mechanisms (*cat*, *sod*, and *phgpx*). Our results showed that 2-dodecanone caused significant alterations in the transcriptional activity of most of the genes tested even after 24-hour exposures and that these toxic effects at the molecular level ultimately translated into a dose-dependent decrease in fertility.

This study provides for the first time in *D. melanogaster* novel and interesting results on the toxic effects of an isolated secondary metabolite naturally present in plants and highlights the potential suitability of this organism to delve into the molecular effects of plant defences in insects.

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P19-09**Organochlorine pesticides and PCBs in European eel (*Anguilla anguilla*) from Spanish Balearic coastal lagoons***A. Pérez-Vegas¹, M. Pérez-López², E. Barcala⁴, E. Trofimova³, D. Romero³, P. Muñoz¹¹ *Universidad de Murcia, Animal Health Department, Murcia, Spain;*² *Universidad de Extremadura, Toxicology Unit. Veterinary School, Cáceres, Spain;*³ *Universidad de Murcia, Toxicology Department, Murcia, Spain;*⁴ *Centro Nacional Instituto Español de Oceanografía, CSIC, San Pedro del Pinatar, Spain*

Certain anthropological activities are known to have a direct impact in the environment and ecosystem, therefore in wildlife. In fish, pollutants can incorporate through gills, skin or contaminated food. Organochlorine compounds are known as important pollutants, which have been proved to produce several adverse effects on wildlife. In this study, the concentration of twenty organochlorine pesticides (OCPs) and seven polychlorinated biphenyl (PCBs) in European eel (*Anguilla anguilla*) was determined. The samples were obtained in two Spanish Mediterranean coastal lagoons from two Balearic Islands: the S'Albufera Natural Reserve (Mallorca) and the Natural Park l'Albufera des Grau (Menorca). Muscle tissue was collected to do the analysis, the extraction protocol was adapted from a procedure used by Mateo *et al.*^[1] and the analysis were performed using a Bruker Scion 456 triple quadrupole gas chromatograph mass spectrometer. Among the twenty analyzed OCPs, eleven of them were detectable in at least one sample, being remarkable that endrin and DDT 2,4' were those with the highest percentage of individuals with concentrations above the detection limit (53.4% and 39.7% respectively), DDD 2,4' was also frequent, being detected in 20.7% of the samples. Four PCBs were detected, being the most frequent PCB 101 and PCB 138, positive in 15.5% and 10.3% of the samples. Lindane, HCH α , HCH β , chlordane trans, endosulfan, heptachlor, heptachlor epoxide A, heptachlor epoxide B, dieldrin, PCB 28, PCB 52 and PCB 18 were not detected in any of the samples analyzed. This study provides information about the European eel status regarding exposure and concentrations of organochlorine compounds in the Balearic Islands aquatic ecosystem, which could be useful to improve management programs of this endangered species.

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P19-10**Characterization of *in vitro* models suitable for the analysis of relevant endpoints disruption in thyroid hormone system***O. Brózman, R. Liu, K. Puja, J. Novák, K. Hilscherová*Masaryk University, RECETOX, Brno, Czech Republic*

Anthropogenic and environmental endocrine disruptive pollutants have been studied for a long time. However, the potential of pollutants to interfere with thyroid hormone system (THS) has been rather neglected so far, even though the dysregulation of THS had been linked with adverse health effects such as carcinoma development and (neuro)developmental changes. One of the reasons is the lack of suitable high throughput-compatible bioassays. Nowadays, several initiatives are trying to close this gap. The H2020 ERGO project aims to identify, develop, and align thyroid-related biomarkers and endpoints for a linkage of effects between different vertebrate classes. This includes a development of a battery of *in vitro* bioassays targeting prioritized molecular initiating events and/or alternative approaches respecting the “3R” principle. An important step in such process is to characterize the relevance of *in vitro* systems and evaluate their suitability for studying different endpoints. By using methods of quantitative PCR and Western blotting, we therefore focused on identifying key genes expression and subsequent protein products detection in THS-relevant *in vitro* models. The gene profiling endpoints associated with THS have been selected with reference to Adverse Outcome Pathway (AOP) network and follow the set of 18 prioritized genes representing both molecular initiating as well as key

events. These endpoints correspond to crucial protein-encoding genes included in hormone synthesis (e.g., TPO, TSHR, IYD, DUOX and NIS), transport (e.g., MCT-8 and -10 and OATP1C1), peripheral metabolism (e.g., DIO-1, -2 and -3) and receptor (trans-)activation (e.g., TR-a and -b) with a special focus on TPO, NIS and DIO-1, -2, 3-. Since thyroid hormone regulation is a complex system with an involvement of multiple organs, several human THS-relevant *in vitro* systems, i.e., thyroid Nthy-ori 3-1 and neural NSC-H9, were used for thorough profiling and/or identification of changes in gene expression to address the usability and applicability of these models to study the disrupting effects in a variety of AOP-relevant events. Since the Nthy-ori 3-1 *in vitro* cell line, a model for human thyroid, did not provide the sufficient level of TPO or NIS expression to monitor the effect of thyroid-disrupting agents, additional *in vitro* transfected constructs of HEK293 cells carrying the corresponding target genes were created and sorted. Additionally, multiple profiles of NSC-H9 varying in the degree of *in vitro* differentiation and exposure statuses were compared. The resulting gene expression profiles were subsequently compared with profiles of commercially available human pooled thyroid and brain *ex vivo* samples to provide a background for the new *in vitro*-based battery of bioassays being developed within the ERGO project.

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P20 – Environmental toxicology

P20-01

Constructed wetlands to face water pollution: effective role of water hyacinth and the potential effects of this process on plant toxicity

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The presence of pollutants in water bodies has increased exponentially, both traditional and emerging pollutants. One of the causes that have led to this high pollution is the strong pressure that human activities generate on natural resources, with wastewater (WW) discharges being a specific source of pollution. Constructed wetlands are widely used technology for the treatment of wastewater which involves biological, chemical and, physical steps simulating the natural process^[1]. This wastewater treatment lets reduce heavy metals, organic matter, nutrient removal, and emerging pollutants content in WW^[2]. In this study, it was designed a Constructed wetland in a small scale *in situ* treatment of domestic wastewater. It was tested the ability of water hyacinth (WH) and a bacterial consortium system to reduce Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Nitrogen compounds, Settleable solids (SSed), and pH according to international water quality standards. At the same time, it was tested the relative growth (RG) rate in plants as well as the regular morphologies features during the degradation process in order to assessed toxicity effects on plants.

The results showed a high performance in the elimination of organic matter, COD was reduced by an average of 80%, BOD was reduced by about 70% at the outlet of the biological processes. The parameters temperature, conductivity, pH, and dissolved oxygen

(DO) are within the expected for potential agricultural use with average values at the exit of the biological treatments of conductivity 678.75 µS/cm, pH 6.9±0.2 pH units, DO 6.1675 mg/L O₂. Turbidity decreased to 14.7 NTU, and SSed was reduced by an average of 0.15 mL/L. In addition, a significant reduction was observed in the compounds associated with the nitrogen chain (Nitrates, Nitrites, and Ammoniacal Nitrogen). As well as orthophosphates and iron. Regarding the microbiological analyses, the data at the end of the biological processes showed a reduction of 94% for total coliforms and 99% for Fecal coliforms. Once the treated water is subjected to the chlorination process, coliforms were reduced to 0 CFU/100mL. On the other hand, the plants exposed to the wastewater showed no severe toxic effects, the relative growth rate of WH increased by 1% per day, and the average RG was 2.05. No anatomical deformities were observed. 67% percent of the plants generated new shoots. Bacterial biomass maintained a high removal rate, with a low loss of viability during the process. These results show that biological treatments are a suitable eco-technology. No severe toxic effects were observed during the degradation of organic and inorganic compounds in the treatment, the process had a minimum energy expenditure, this strategy allows the reduction of the human footprint in the water and significantly improves the quality of the effluent.

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P20-02

Short-term effects of some *Fusarium* mycotoxins on the lipid peroxidation processes and the glutathione redox system of broiler chicken

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Trichothecene mycotoxins are secondary metabolites of *Fusarium* moulds. Deoxynivalenol (DON), fumonisin B1 (FB1) and T-2 toxin are the most relevant members of this mycotoxin family, based on their economic significance and occurrence. In temperate climates, these mycotoxins are frequent contaminants of cereal grains. Earlier it was described that *Fusarium* mycotoxins in single application induce reactive oxygen substances (ROS) formation and cause oxidative stress. However, there is rare information about the response to the multi-mycotoxin exposure, which might be additive, synergistic, or antagonistic toxic effects.

In the present study, the intracellular biochemical changes in the case of multi-mycotoxin exposure, with attention to some components of the glutathione system and initiation and terminal phase of the lipid peroxidation process was investigated. A 3-day broiler chicken feeding trial was performed with the low (0.25mg T-2 toxin; 5 mg DON and 20 mg FB1 kg⁻¹ feed, according to the EU recommended limit) and high (1 mg T-2 toxin; 20 mg DON and 80 mg FB1 kg⁻¹ feed, as quadruple of the EU recommended limit) doses of multi-mycotoxin exposure. The markers of the glutathione system and lipid peroxidation in the liver and the blood were determined with biochemical methods: the amount of reduced glutathione (GSH), the activity of glutathione peroxidase (GPx) and the concentration of

malondialdehyde (MDA) and the conjugated dienes (CD) and trienes (CT). Based on our results, there was no induction of lipid peroxidation in the liver, and significant differences were observed only in the blood plasma but not in the liver as compared to the untreated group. It means that the well-known oxidative stress-inducing effect of the investigated mycotoxins did not occur in multi-mycotoxin exposure; therefore, an antagonistic effect among them would be suggested.

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P20-03

The influence of subacute poisoning with lambda cyhalothrin on nuclear factor kappa B and vascular endothelial growth factor receptor 2 in mice livers

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Lambda cyhalothrin (LCH) is a pyrethroid (insecticide) that has widely applied to agricultural plants, such as almond, apple, and cherry. Nuclear factor kappa B (NFkB) is a protein complex that controls transcription of DNA, cytokine production and cell survival. NFkB is involved in cellular responses to stimuli such as stress, cytokines, heavy metals and free radicals. Vascular endothelial growth factor receptor 2 (VEGFR2) is a type V receptor tyrosine kinase mainly known to be expressed in vascular endothelial cells. VEGFR2 is expressed in many but not all vascular endothelial cells from early fetal development, and it is more highly expressed in neovascular tumor endothelia than in normal endothelia.

The aim of the study was to check influence LCH on NFkB and VEGFR2 in a model of subacute poisoning in mice.

A total of 32 mice were divided into 4 groups of 8 animals:

1. control females;
2. control males;
3. females receiving 2mg/kg LCH during 7 days per os;
4. males receiving 2mg/kg LCH during 7 days per os.

On the eighth day of the experiment, the animals were weighed and then sacrificed, and their livers were removed. The determinations were made by Enzyme Linked Immunoabsorbent Assay (ELISA) kit from Cloud-Clone Corp., Assembled by Usen Life Science INC.: ELISA kit for VEGF2 and ELISA kit for Nuclear Factor Kappa B (NFkB).

The mean body mass ($\pm$ SD) on day 8 was: for control females 25.62 ± 2.62 g, for control males 27.83 ± 2.55 g, for LCH females 22.52 ± 1.52 g, for LCH males 27.21 ± 4.18 g. Females from the LCH group had statistically significant lower body mass than animals from other groups $p=0.0117$. The mean NFkB ($\pm$ SD) on day 8 was: for control females 5.02 ± 1.36 ng/ml, for control males 5.54 ± 0.61 ng/ml, for LCH females 6.12 ± 1.88 ng/ml, for LCH males 4.89 ± 1.27 ng/ml. The mean VEGFR2 ($\pm$ SD) on day 8 was: for control females 170.12 ± 37.33 ng/ml, for control males 165.87 ± 37.89 ng/ml, for LCH females 173.25 ± 36.09 ng/ml, for LCH males 169.62 ± 31.59 ng/ml. NFkB and VEGFR2 levels were similar in all groups and the differences were not statistically significant.

To sum up: low doses of LCH (2 mg/kg body mass per os for 7 days) do not affect NFkB and VEGFR2 levels. The study has shown that despite exposure to LCH in water and food, people should not be afraid of large side effects in this aspect.

P20-04

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P20-05

Histopathologic changes in rat tissues induced by phthalate and bisphenol A mixture after subacute exposure: more pronounced effect of the mixture in comparison with the single substances

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In day to day life, general population is simultaneously exposed to a great number of chemicals from many different sources. In most cases, the joint chemical toxicity is higher than the toxic effect of each single compound. Thus, this study was conducted to compare the histopathologic changes in rat tissues after 28-day exposure to single endocrine disruptors (bis (2 -ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), and bisphenol A (BPA)) with their mixture. Male rats were randomly divided into 5 groups (6 animals): Control group (corn oil), DEHP group (50 mg/kg b.w./day), DBP group (50 mg/kg b.w./day), BPA group (25 mg/kg b.w./day) and MIX group (50 mg/kg b.w./day DEHP + 50 mg/kg b.w./day DBP + 25 mg/kg b.w./day BPA). At the end of the experiment all animals were weighted and organ samples (liver, spleen and kidney) were extracted. Relative organ weights were calculated by dividing final organ weights with the final body weights. After haematoxylin and eosin staining of the organ tissue, histological assessments were made under the light microscope (Olympus, Olympus BX43, Japan). The statistical analyses were performed with GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, California, USA). One-way ANOVA with LSD post-hoc test was used to analyse the differences between the experimental groups. There was a significant increase in liver relative weight only in DEHP and MIX group in comparison with the control, while liver relative weight was significantly lower in both DBP and BPA groups in comparison with MIX. However, all three substances and their mixture led to pathological changes in liver tissue. Moderate sinusoid dilatation in lobules was observed in all treated groups, predominantly around the central vein. In the MIX group, additional changes included light expansion of port spaces due to the increased number of inflammatory cells (predominantly lymphocytes, in a smaller number eosinophils), indicating port hepatitis. Spleen relative weight was significantly lower only in the MIX group compared to the control. Nevertheless, changes in spleen were noted in all the treated groups apart from DBP. Extramedullary hematopoiesis (primarily erythroid and myeloid lineage) was easily increased in the red pulp. The white pulp exhibited a very slight reduction in PALS size, while the marginal zone was of preserved size but easily reduced cellularity. Kidney relative weight was higher in the MIX group compared to the control, as well as compared to the DBP and BPA groups. In kidneys, focal loss of brush tubulocyte border in renal tubules was observed in DEHP and MIX group. The obtained results demonstrated that DEHP, DBP and BPA mixture lead to more pronounced histopathological changes in comparison with the single substances in all the investigated tissues. However, further study is needed to explore the in-depth mechanisms underlying the possible additive toxic effects of this mixture on liver, spleen and kidney.

P20-06

This abstract has been withdrawn.

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P20-08

2-month exposure to a low dose of bisphenol A does not alter femur growth in young rams (*Ovis aries*)

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Humans are exposed daily to bisphenol A (BPA), one of the most commonly used man-made chemicals, and more than 90% of people of all ages excrete it in urine, saliva, and sweat^[1,2]. Studies suggest a connection between BPA exposure and increased risk of obesity, diabetes, and heart disease^[3]. This xenoestrogen has an affinity for binding to estrogen receptors, which could disrupt the function of sexual hormone-regulated processes, although the concentration to induce effects comparable to estrogens is supposed to be more than 1000-fold higher^[4]. Estrogens play an important role at the epiphyseal growth plate (EGP) of long bones during the pubertal intensive growth phase^[5]. Recent developmental studies showed that rats exposed to low doses or environmentally relevant concentrations of BPA had reduced bone size and increased number of fibrotic lesions^[6]. Therefore, the purpose of our study was to evaluate the effect of a 2-month exposure to 25 µg BPA/kg bw/day (which corresponds to 50% of the US Food and Drug Administration reference dose) orally on femur geometry, density, and EGP architecture in growing rams.

Two groups (BPA-treated and control, n = 7 rams/group) of the Slovenian sheep breed Istrian Pramenka, 9–10 months of age, were group-housed in indoor pens and administered BPA (dissolved in ethanol) in commercial pellets individually each morning for 64 days. Special care was taken to ensure that the rams did not come into contact with products containing BPA. The rams were anesthetized i/v with xylazine and euthanized with pentobarbital. Left femurs were dissected, and their geometric dimensions were obtained from computed tomography (CT) and analyzed using RadiAnt Dicom Viewer and ImageJ. Distal EGP of right femurs were collected, decalcified, and stained with hematoxylin-eosin for histomorphometric analysis (NIS-Elements BR). An independent samples t-test was used to compare the data between the two groups.

The mass, length, width, total and medullary cross-sectional area (CSA) of the left femur, including the density of cortical and trabecular bone tissue, and the areas of reserve cartilage, proliferation zone, hypertrophy zone and calcified cartilage in the distal EGP of the right femur were not significantly altered between the BPA-treated and the control group (p > 0.05). Cortical bone occupied approximately 50% of the total bone CSA. The area of reserve cartilage, proliferation zone, hypertrophy zone, and calcified cartilage accounted for 14%, 43%, 36%, and 7% of EGP, respectively.

Our results indicate that a 2-month low-dose dietary exposure to BPA does not disrupt endochondral ossification during long bone growth in growing rams. This supports the assumption that, compared with estrogens, a much higher concentration of BPA would be required to interfere with the longitudinal bone growth in large animals^[4].

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P20-09

Protein corona mitigated the toxicity of CdTe QDs to macrophages by targeting on mitochondria

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Toxic effects of cadmium telluride quantum dots (CdTe QDs) have been documented for their application in biomedicine. It is known that the immunotoxicity of them targeting macrophages is one of the adverse effects, and the protein corona (PC) adsorbed on the surface of nanoparticles will affect their biological effects. Here, we further investigated the impact of the protein corona formed in fetal bovine serum (FBS) on their toxicity on mitochondria. RAW264.7 mouse leukemic monocyte-macrophage cells were as the model to study the effects of CdTe QDs and PC-CdTe QDs complexes on the structure, function, quantity, morphology and mitochondrial quality control of mitochondria. As result, the protein corona alleviated the inhibition of CdTe QDs on the mitochondrial activity, the damage to the mitochondrial membrane, the level of cellular reactive oxygen species, and the reduction of ATP levels. Also, CdTe QDs increased the number of mitochondria in macrophages, while the PC-CdTe QDs complexes did not. In line with this, the mitochondria of macrophages being exposed to CdTe QDs and PC-CdTe QDs complexes showed different morphology of mitochondrial network, CdTe QDs transformed the network into fragmented, punctate and short rods, while PC-CdTe QDs complexes altered the mitochondrial network to a highly branched state, which is related to the balance of mitochondrial dynamic (mitochondrial fission and fusion). Mechanically, CdTe QDs promoted mitochondrial fission and inhibited mitochondrial fusion, while protein corona reversed the trend of these two processes. In addition to mitochondrial dynamics, mitochondrial biogenesis and mitophagy were also affected. CdTe QDs promoted the expression of signaling molecules related to mitochondrial biogenesis including PGC-1 α -NRF1/NRF2-TFAM, while PC-CdTe QDs complexes played the opposite role; with regard to mitophagy, the two materials showed the same promoting effect. In conclusion, the formation of protein corona moderated the toxic effect of CdTe QDs on the mitochondria of macrophages to a great extent and affected mitochondrial quality control. These findings shed light on and provided useful insight into reducing the toxicity of CdTe QDs from two perspectives: protein corona and mitochondria, and shared valuable information for the safe use of QDs in the future.

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P20-10

This abstract has been withdrawn.

P20-11

Micro-sized polyethylene particles decrease cell viability and increase mitochondrial superoxide production in human colorectal adenocarcinoma Caco-2 cells

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Background: Microplastics are small plastic particles (size <5 mm) which are produced to be in a micro-size range or results from fragmentation of larger plastic pieces in the environment^[1]. Plastics are inert materials but during fragmentation they can adsorb different types of hazardous substances from the environment. In addition, in the manufacturing process different additives e.g. phthalates and flame-retardants are added to plastics. However, there is a little reliable information on how microplastics and plastic associated chemicals affect the human health. Majority of the human exposure to microplastics occurs via ingestion route, and one of the most abundant microplastics is polyethylene (PE)^[2]. Therefore, the aim of this study was to evaluate the ability of micro-sized PE to cause harmful effects in human colorectal adenocarcinoma cells.

Methods: Raw ultra-high molecular-weight PE (Sigma-Aldrich, particle size 40–48 µm) was used. In addition, to investigate the effects of possible dissolved compounds from plastics, extraction with ethanol as solvent was studied. Ethanol extracted PE samples (PE-EtOH) were prepared by extracting 0.5 g raw ultra-high molecular-weight PE in 10 ml of ethanol in shaker (+37°C) for three days. After three days, the solution was filtrated and ethanol was evaporated, leaving a PE residue in a glass test tube. PE residue was mixed with 3 ml cell culture medium (100%) and diluted to 75%, 50%, 25% and 10% solutions.

In the experiments, human colorectal adenocarcinoma Caco-2 cells were exposed to PE (0.25–1 mg/ml) or PE-EtOH (10–100%) for 48 h. Immediately after exposure, cell viability and cytotoxicity were assessed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and lactate dehydrogenase (LDH) assay using commercial CytoTox assays (Promega).^[3] Reactive oxygen species (ROS) production was measured with DCFDA (2',7'–dichlorofluorescein diacetate) and cytoplasmic production of superoxide (O₂^{•−}) with dihydroethidium and mitochondrial O₂^{•−} production with MitoSOXTM (Invitrogen, Molecular Probes).^[3,4]

Results: The 48 h-exposure to PE or PE-EtOH decreased cell viability and both exposures also increased cytotoxicity on Caco-2 cells. Cell

viability was decreased in a dose dependent manner with both microplastics used in this study. Exposure to PE-EtOH increased ROS production in the highest dose (100%), but pure PE had no effect on ROS production on Caco-2 cells. Furthermore, mitochondrial superoxide production was elevated after both type of exposures in all doses. However, effects on cytosolic superoxide production was not observed. In conclusion, both micro-sized pure PE and ethanol extracted PE decreased cell viability on Caco-2 cells and increased cytotoxicity and ROS production, especially mitochondrial superoxide levels.

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P20-12

Localization and impact of PFOA *in vivo* and *in vitro* by high-resolution chemical imaging

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Perfluoroalkylated substances (PFASs), such as perfluorooctanoic acid (PFOA), have been widely used for many industrial purposes and consumer-related applications and are therefore omnipresent in our environment. However, these chemicals are persistent and accumulate in human and animal bodies, leading to major health issues. The gastrointestinal tract is the first physical barrier against these toxicants upon oral uptake. Surprisingly, their impact on the intestinal wall is largely unknown. This study aims to achieve a breakthrough in the knowledge of PFASs uptake and effects using *in vivo* and state-of-the-art *in vitro* assays combined with novel analytical imaging techniques. We correlate light and electron microscopy (LM & EM) that reveal inner morphology of the specimen with mass spectrometry (MS) which provides analytical information. In this last respect, we use Secondary Ion Mass Spectrometry (SIMS) which has both an excellent spatial resolution and an excellent detection limit, allowing sub-cellular localization of low-level toxicants.

For the *in vivo* assay that was approved by the relevant ethical committee, adult male mice were exposed to PFOA by oral gavage for acute exposure (0–100 mg/kg bw/day for 3 days) and via drinking water for sub-chronic exposure (0–15000 ng/kg bw/day for 90 days). Next to weekly follow-ups and key biomarkers analyses, the intestines (jejunum and colon) and the liver were cryo-fixed for molecular imaging and chemically fixed for high-resolution elemental imaging. The samples were respectively analyzed using a Time-of-Flight SIMS (TOF-SIMS, 1µm lateral resolution) and a Helium Ion Microscope combined with a SIMS (HIM-SIMS, sub-20nm lateral resolution). HIM-SIMS images were further correlated to Scanning Electron Microscope (SEM) images. For the *in vitro* assay, an intestinal cell line (Caco-2) was grown on Transwells and acutely (24h) or chronically (every two

days) exposed to PFOA (0–100 µM). Cells were either prepared for the toxicity assays or for high-resolution elemental imaging by chemical fixation. The samples were analyzed using HIM-SIMS and SEM.

We successfully localized PFOA inside tissues and cells. At tissue level, PFOA is localized in some specific areas in the jejunum and in the colon while it is homogeneously distributed in the liver. In the latter, we observed colocalization of PFOA with fatty acids, probably due to their structural similarities. At cellular level, PFOA is only localized in the cytosol of intestinal and hepatic cells, but not in cytosolic lipid droplets (observed both *in vivo* and *in vitro*). This new visualization methodology provides insightful information to better understand potential negative impacts of PFASs on the gastrointestinal tract and the liver and opens new opportunities in toxicology and life science in general.

P20-13

Research of microplastics in the gastrointestinal contents of sea turtles by the method of artificial digestion

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Purpose: Due to improper management and low biodegradability, plastic waste has become one of the most serious environmental issues and microplastics (MPs), defined as particles from 0.1 to 5 mm, are now ubiquitous^[1]. Marine habitats are especially sensitive to plastic pollution: several studies have demonstrated that over 500 marine species can take up marine litter and microplastics, often with physical, chemical and biological consequences^[2]. MPs can accumulate in tissues, serve as vehicles for transport of pathogens and adsorb toxic pollutants, inducing a variety of biological effects, such as disturbances in energy metabolism, oxidative balance and antioxidative capacity; DNA, immunological, neurological, reproductive and histological damage^[3]. Loggerhead turtle, *Caretta caretta*, is the official European bioindicator of marine litter in the Mediterranean Sea. The shallow water of the northern and central Adriatic Sea is one of the most important neritic feeding areas^[4]. Along its coasts human activity is intense, with heavy marine traffic, intensive mussel aquaculture, fish farming and seasonal tourism. In addition, the Po River provides the largest riverine influx to the north Adriatic Sea that, consequently, is one of the most polluted areas of the Mediterranean's basin. Although much research has been conducted, the overall knowledge of the issue of MPs in the Adriatic Sea is still limited and fragmented^[5]. The aim of this study was to evaluate the prevalence and the type of MPs ingested by loggerhead sea turtles found stranded along the coast of Emilia Romagna region, north-western Adriatic Sea, between June and December 2020.

Methods: A total of 35 loggerhead *C. caretta* with Curved Carapace Length from 18 to 83 cm was examined. A general necropsy was performed and then 20 grams of gastrointestinal content of each turtle was subject to enzymatic digestion, which is quick, safe, inexpensive and does not alter the physical characteristics of the MPs. Each sample was digested with pepsin and hydrochloric acid and then

filtered using a 1 mm mesh sieve. Lastly, each filter was examined under an optical microscope.

Results: MPs were found in 15 turtles, 43% of the samples. In total, 38 MPs were identified, with an average of 2.5 fragments in each sample. The dominant types were foam (53%) and pellets (47%). According to the color, the following percentages were obtained: white (53%), brown (5%), yellow (24%) and transparent (18%). The last analysis was about the length of microplastics: the most abundant was 1 mm (55%) followed by 2 mm (23%), 3 mm (11%), 4 mm (8%) and 5 mm (3%).

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P20-14

A binary mixture of PFOS and PFOA elicits a potentiating effect on the peroxisome proliferator-activated receptor alpha 1 (Ppara1) from Atlantic cod (*Gadus morhua*)

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Per- and polyfluoroalkyl substances (PFAS) are a group of man-made compounds that have been widely used in consumer and industrial products. Perfluoroalkane sulfonic acids (PFSA) and perfluoroalkyl carboxylic acids (PFCA) are two major classes of PFAS, which include perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), respectively. Many PFAS molecules possess both bioaccumulating and biomagnifying properties, and have recently been detected in marine teleosts, including Baltic cod and Atlantic cod. Furthermore, it has been shown that some PFAS can bind and activate peroxisome proliferator-activated receptors (PPARs), which have a major role in the regulation of lipid- and carbohydrate metabolism in vertebrates. Using an *in vitro* luciferase reporter gene assay, we have found that Atlantic cod PPARa1 was transactivated by the carboxylic acids PFHxA, PFOA, PFNA, as well as the sulfonic acid PFHxS, while PPARa2 was not activated by any of these compounds. Homology modeling, molecular docking and molecular dynamics simulations (MD) of PFOS, PFOA, PFNA and PFHxA complexed to the PPARa1 and PPARa2 ligand-binding domains (LBD) supported the experimental data, suggesting that PFHxA, PFOA and PFNA more significantly stabilized the omega loop region of the LBD in PPARa1 than in PPARa2, while PFOS did not stabilize this region in neither subtype. Intriguingly, a binary mixture of PFOS and PFOA produced both a higher activation of PPARa1 and lowered the EC₅₀ compared to the activation by PFOA alone. Ligand docking analyses of double-ligand complexes identified a putative allosteric binding site situated near the ligand binding pocket and the omega loop in the AF-2 region.

Subsequent MD simulations of the gmPPARa1 complex revealed that the hydrophobic fluorocarbon chain locates to a hydrophobic pocket, and that binding of PFOS in this alternative binding site leads to further stabilization of the omega loop region, leading to a more structurally stable ligand binding domain and, in particular, the region near the coactivator binding site. Thus, although not active by itself, binding of PFOS to this second allosteric binding site gives rise to an unforeseen interaction effect that potentiates gmPPARa1 activity, putatively by stabilizing an active conformation of the receptor. Thus, exposure of Atlantic cod to PFAS individually, or in mixtures, could potentially modulate the lipid- and carbohydrate metabolism by directly interfering with PPARa1. Importantly, also PFAS that do not act as PPARa agonists may modulate receptor activity via binding to allosteric binding sites.

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P20-15

MCPA-based herbicides and their effect on urease activity of soil

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Research objective: To study in a model experiment the effect of MCPA-based herbicide on the activity of urease, a key enzyme of urea biodegradation in soil.

Materials and methods: Herbicides based on active substance MCPA (IUPAC- (4-chloro-2-methylphenoxy) acetic acid) are highly dangerous compounds by carcinogenic action (hazard class: by hygienic classification in RF – 2C, by classification of IARC – 2B). They are used in agriculture on cereals, vegetables, legumes, pastures and meadow herbs. Preparation in the form of aqueous solution was introduced into sandy soil (pH-aqueous extract 6.1–6.5) in concentrations 1.04 mg/kg, 0.104 mg/kg, 0.0104 mg/kg in terms of active substance in triplicate, control – soil without preparation. The test was carried out at stable (230°C) temperature and humidity (60%). Sampling was carried out by conventional methods on days 3, 7, 10, 14, 20, 30, 45, 60. Urease activity was determined by photometric method and calculated using the formula based on control measurements in sterile soil.

Results of researches: All test samples showed a significant, compared to control, different-directional change in the urease activity of the soil. On day 10, inactivation of enzyme, mg NH₃ 10g/24h: control – 1.73; 0.104 mg/kg – 0.65, 1.04 mg/kg – 0.71; 10.4 mg/kg – 0.91. On day 30 enzyme activation: control – 0.12; 0.104 mg/kg – 0.92, 1.04 mg/kg – 0.49; 10.4 mg/kg – 0.46. At the end of the observation, no differences in test samples and control were found.

Conclusions: The obtained results related to the change of urease activity of the soil reflect a set of processes taking place in the soil (binding of cations and anions of xenobiotic with soil particles, suppression of the microbiological fraction of the soil, etc.). High rate of urea decomposition and considerable resistance to MCPA indicate activation of self-purification processes in soil following primary enzyme response.

P20-16**Soil phytotoxicity by using MCPA-based pesticides***T.A. Sinitskaya¹, I.P. Gromova¹

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The aim: study the impact of the herbicide MCPA (a derivative of phenoxypropionic acid) on the growth and development of test cultures in experimental conditions.

Materials and methods: MCPA (IUPAC–(4-chloro-2-methylphenoxy) acetic acid) is selective herbicide of systemic action – a highly dangerous compound with a carcinogenic effect (hazard class 2C in the Russian Federation), according to the IARC – 2B classification. Certified seeds of agricultural plants: barley, oats, peas, radishes, squash, lettuce were sown in Petri dishes filled with sandy model soil (pH of water extract 6.6–7.3) with the addition of Pryanishnikov nutrient medium and water solutions including of the active substance (MCPA) and the preparative form. In experiments five different concentrations of active substance (ranged from 0.052 to 5.2 mg/kg) and five concentrations of preparative form (0.104–10.4 mg/kg) were tested (in three independent series of experiment), the control was soil with distilled water. The treatment was conducted in a chamber during 7 days at 23°C and 50–70% humidity. Throughout the experiment germination of seeds and the length of the roots were recorded.

Results: MCPA had an effect on soil phytotoxicity in maximum concentrations. It was fixed in form of decreasing in the growth and development of the root system in all test plants (the range was 84–100%). The others concentrations caused similar changes but to a lesser extent: 30–50% compared to the control in barley, radish, zucchini and lettuce seedlings. The length of the roots of pea and oat seedlings was slightly lower compare with the control but this lag in the growth of experimental plants was within 20% (relative to the control group). Germination of seeds in all tested plants by different concentrations of the active substance was at control level (≤20%)

Conclusions: The established dose-response relationship between the use of the MCPA and the parameters of soil phytotoxicity are among the fundamental parameters in the development of necessary hygiene standards for this class of herbicides.

P20-17**Blood concentration of selected trace elements in horses and its relation to biochemical parameters: A correlation study**

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Environmental pollution is global environmental problem, and it can result in serious health hazards to humans and animals. Typical toxic elements such as mercury, lead, cadmium, and others are well investigated in human and animals. However, at elevated levels, relatively “safe” or essential elements can also be toxic to the organism. Based on the above the aim of the present study was to investigate possible interactions between selected microelements (Al, Ba, Mo, and Sr) content in the blood serum/blood clot and serum chemistry parameters (total proteins – TP, glucose, AST, ALT, ALP, urea, cholesterol, triglycerides – TG, and creatine kinase – CK) in horse.

Blood samples from *vena jugularis* were taken from 24 animals and placed into collection tubes. Serum chemistry parameters concentrations were measured using DiaSys (Diagnostic Systems GmbH, Germany) commercial kits and the semi-automated clinical chemis-

try analyzer Randox RX Monza (Randox Laboratories, UK). For detection of microelements, samples were prepared as follows. The blood serum (1 ml) and blood clot (1 g) samples were diluted with 5 ml HNO₃ (TraceSELECT®, Honeywell Fluka, USA) and 1 ml H₂O₂ (30%, for trace analysis, Merck Suprapur®). Furthermore, the samples were mineralized with microwave digestion system (Milestone Srl, Italy). The content of selected microelements was determined in blood serum and blood clot by inductively coupled plasma optical emission spectrometry (ICP OES, iCAP7600 Thermo Fisher Scientific, Waltham, Massachusetts, USA).

The general scheme of descending concentrations of trace elements was the same for blood serum and blood clot: Mo > Al > Ba > Sr. Pearson correlations were used to assess the relationships between the serum markers and the trace elements concentrations. Statistically significant positive correlations were found between the concentration of all microelements in serum and AST levels (P < 0.01). Statistically significant negative correlations were found between the concentration of all microelements in serum and ALP levels (P < 0.05). Weak and insignificant correlations were detected between trace elements in serum and ALT concentrations. On the other hand, the weak and insignificant correlations were detected between trace elements in blood clot and AST and ALP concentrations. However, concentrations of Ba, Mo, and Sr in blood clot negatively correlated with content of ALT (P < 0.05). Weak and insignificant correlations were detected between Al in blood clot and ALT concentration and between all trace elements and other investigated parameters (TP, glucose, urea, cholesterol, TG, and CK).

Research activities about effects of heavy metals in human and animals are relatively well investigated. Monitoring of other trace elements (Al, Ba, Be, Li, Mn, Mo, Sr, Se) in animals will be worthy of further investigation.

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P20-18**The endocrine-disrupting potential and a sex-specific effect of a generic pyrimethanil, as explored in a subchronic study *in vivo***

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The many possible negative impacts of endocrine-disrupting chemicals (EDCs) on normal human development on a large scale is a growing public health concern. Much spotlight has been given to plant-protection products (PPTs) ever since the endocrine effects of insecticide dichlorodiphenyltrichloroethane (DDT) became known^[1]. A wide-scale study of PPTs operators in the United States had demonstrated that fungicides are the most harmful pesticide group in terms of their ability to affect the thyroid gland^[2]. Likewise, the European Food Safety Authority (EFSA, 2019) has designated multiple fungicides as potential endocrine-disruptors based on the lack of sufficient evidence that would confidently suggest otherwise^[3]. One of such PPTs is an anilino-pyrimidine fungicide pyrimethanil (PYR), as only a handful of studies have explored its action on the endocrine system^[4–9].

The present study aimed to assess the potential influence of PYR on the thyroid and generate data to assist in its classification of PYR as either EDC or non-EDC. A generic PYR technical product was tested in a subchronic 90-day study according to the OECD Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption. Concentrations of thyroid-stim-

ulating hormone (total TSH), triiodothyronine (total T3), and thyroxine (total T4) were determined in blood serum of adult outbred rats of both sexes that were administered PYR in the diet in doses 2; 20; 240 mg/kg bw. Pre-made hormone kits (Vector Best, JSC, Russia) with a StatFax 2100 spectrophotometer (Awareness Technologies Inc., USA) were used in the study. 6 animals per group were subjected to the morphological examination of the thyroid, ovaries, and gonads. Statistical analysis was performed in IBM SPSS Statistics v.22 (IBM Corporation, USA) at $\alpha=0.05$.

The tested generic PYR did not significantly influence hormone concentrations in the study: neither dose group differed from the control ($p>0.05$); no trend was observed (Spearman rank correlation). The morphological analysis demonstrated significant dose-dependent ($p<0.05$) desquamation of thyrocytes and the loss of follicles in the thyroid of female rats only, suggesting a sex-dependent effect.

To conclude, no biologically significant effect on the endocrine system (thyroid, gonads, ovaries) and thyroid hormones was observed for the generic pyrimethanil in the 90-day study. This result contributes to the debate on the endocrine-disrupting potential of pyrimethanil, indicating the relative safety of the tested plant-protection product.

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P20-19

Elucidating the role of DNA methylation in DNA damage and repair in mutagen-treated *Brachypodium distachyon* cells

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In response to stress conditions living organisms have generated epigenetic mechanisms, associated with chemical modifications of DNA and histones, arisen independently of nucleotide sequences. Stress factors, which include mutagenic agents cause damage in the form of single- and double-stranded DNA breaks that can lead to chromosomal aberrations. As a result of the action of mutagenic factors, micronuclei may be formed, due to the elimination of the entire chromosome(s) or its acentric fragment(s). A number of studies on the effects of mutagens on the level of epigenetic modifications have been conducted in plant cells, but no studies describing the role of epigenetic modifications in micronucleus formation are known so

far. This study focused on understanding the role of DNA methylation in the response of model species- *Brachypodium distachyon* (Brachypodium) cells to mutagenic agents. Selected chemical mutagens, characterized by different mechanisms of action: maleic hydrazide (MH) and N-nitroso-N-methylurea (MNU) to induce micronuclei in Brachypodium cells were used. Immunodetection technique using an antibody against 5-methylcytosine to examine a global DNA methylation in control and mutagens-treated cells was used. Also, the level of DNA methylation in micronuclei, together with their parental nuclei was analyzed. Studies have shown that DNA methylation plays an important role in the response of the Brachypodium genome to mutagenic treatment, in particular in the formation of chromosomal aberrations.

P20-20

The endocrine-disrupting potential of a generic carbamate insecticide methomyl, as explored in a 90-day study in vivo

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The endocrine-disrupting potential of pesticides has been a topic of great importance for public health ever since the dangers of organophosphate compound use were first explored. This issue is actively investigated at both the worldwide (OECD, WHO/FAO) and national levels. The unyielding global interest stems from the fact that the availability of *in vivo* and *in vitro* data does not always lend itself to extrapolation to risk assessment for generic pesticides, which can contain newly added understudied impurities. Also, for a number of reasons, epidemiological data do not always allow for a causal relationship to be established between pesticides' action and symptoms of endocrine dysregulation in studied populations. Therefore, it is important to conduct standardized, verified, and reliable tests to assess the endocrine-disrupting potential of pesticides in non-clinical studies prior to them entering the market.

A generic carbamate insecticide methomyl (MET), a suspected endocrine-disruptor, was tested in a subchronic 90-day study (OECD Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption) on male and female adult outbred rats. MET was administered with diet in doses 2.5; 5; 20 mg/kg bw. The negative control group received vehicle. The animals were weighed weekly, their general condition, appearance, and behavior were assessed daily. Blood samples were collected for enzyme immunoassay at study termination. Concentrations of total thyroid-stimulating hormone (total TSH), triiodothyronine (total T3), and thyroxine (total T4) were determined in blood serum using pre-made kits (Vector Best, JSC, Russia) with a StatFax 2100 spectrophotometer (Awareness Technologies Inc., USA). Statistical analysis was performed with IBM SPSS Statistics v.22 (IBM Corporation, USA) at $\alpha=0.05$.

The results showed that MET caused a statistically significant (Kruskal-Wallis test, $p=0.046$) dose-dependent (Spearman rank correlation, $\rho=0.454$, $p=0.009$) increase in the level of T3 when administered at the highest dose (20 mg/kg bw). It is known that the biosynthesis of T3 from T4 occurs via deiodination by type II deiodinase (D2), which is highly expressed in the thyroid. Research shows that a high level of circulating T3 in the absence of changes in T4 is sometimes observed due to the inhibition of the functional activity of thyrocytes by acetylcholine (ACh). Subsequently, this results in overexpression of type II deiodinase (D2) and thyrotoxicosis. MET is an acetylcholinesterase inhibitor, which could account for the observed effect.

In conclusion, an effect on the endocrine system was observed for the carbamate insecticide methomyl in the study, which outlines the need to continue worldwide efforts in identifying possible endocrine-disrupting pesticides in order to ensure public health safety.

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P20-21

Evaluation of the oxidative potential of pyrotechnic smoke particles and *in vitro* toxic assessment on a 3D model of primary pulmonary cells at Air-Liquid Interface

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Pyrotechnic smokes are widely used for civilian and military applications: protection, security, festivity, obscuring or signaling. Particulate air pollution is a major public health concern and smokes combustion induces intense pollution episodes. There are multiple types of smokes with various initial compositions, but there is a lack of data on chemicals produced after combustion and their toxic effects.

In this study, we evaluated the toxicity of particles of two different smokes, a red signaling smoke (RSS) and an hexachloroethane-based obscuring smoke (HC-OS) by measuring their oxidative potential (OP) and by exposing a 3D model of primary human pulmonary cells (NHBE) grown at the Air-Liquid-Interface to suspended particles. OP was examined with the dithiothreitol (DTT) and the antioxidant (acid ascorbic (AA)) depletion assays. Cytotoxicity (MTT, cell cycle) and genes expression (RT-qPCR) were explored after 24h of exposure (RSS and HC-OS) and 24h of recovery (RSS).

Physico-chemical characterization of particles revealed that particles were smaller than 0.5 µm diameter; therefore particles are capable to penetrate deep into the airways after inhalation. Furthermore, RSS particles were more organic (quinones and polycyclic aromatic hydrocarbons) than HC-OS particles that were mainly metallic. Indeed, we noticed a higher metal content (133 mg/g) especially in Al and Fe compared to RSS particles (12 mg/g). Results showed that DTT was significantly depleted by RSS (74%) and HC-OS (42%) particles at the highest dose of exposure (50 µg/cm²) but AA was only depleted by HC-OS (42%). Both particles were not cytotoxic but genes expression was modified and depending on particles type. Particles from RSS (50 µg/cm²) but not from HC-OS significantly increased superoxide dismutase 1 (SOD1) and 2 (SOD2) by 1.5-fold and heme oxygenase-1 expression (HO-1) by 8.3-fold. Both particles significantly induced NADPH quinone oxidoreductase-1 (NQO-1) (RSS: 6.4-fold; HC-OS: 1.7-fold) and interleukine-8 (IL-8) expressions (RSS: 2.1-fold; HC-OS: 1.8-fold) whereas the catalase expression was unchanged. After 24h of recovery, genes expression were back to normal (SOD1, SOD2, HO-1), stayed significantly increased (NQO-1, IL-8) or remained unchanged (catalase).

Because of their different chemical composition, smoke particles produced many different oxidative reactive species, which can be detectable by the two OP assays. Indeed, the reaction of DTT assay is mainly associated with organic components, which may explain the higher depletion by RSS particles. On the contrary, AA depletion is generally attributed to metals corresponding to HC-OS depletion. Our study demonstrated that NHBE exposure to different military smoke particles triggered an adaptive antioxidant response that was reversible for RSS particles and lead to inflammatory response. This study improves the knowledge of the toxicity of pyrotechnic mixtures like smoke particles to assess human health risk.

P20-22

Childhood Leukemia – Influence of Extremely Low Frequency Magnetic Fields on the Development of the Disease

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Precursor B-cell acute lymphoblastic leukemia (pB-ALL) in children is associated with an exposure to extremely low frequency magnetic fields (ELF-MF), which were classified as “possibly carcinogenic to humans” by IARC in 2001. Former experiments in two different mouse strains (CD1 and Sca1-ETV6-RUNX1) point towards a cytotoxic effect of ELF-MF on CD8+ T-lymphocytes or a modulation of the maturation of the T-cell compartment in the juvenile organism.^[1,2] The goal of this project is to evaluate the potential effects of pre- and postnatal ELF-MF exposure on the developing immune system of juvenile Sca1-ETV6-RUNX1 mice and to point out a possible role of ELF-MF on pB-ALL development.

Pregnant Sca1-ETV6-RUNX1 mice were exposed from the first day post coitum to a continuous linear polarized sine-shaped 50 Hz magnetic field, following a blinded, sham-exposure controlled experimental setup. A total number of 978 pups, consisting of 427 (44%) Sca1-ETV6-RUNX1 mice with a balanced male to female ratio were born in the exposure systems. On postnatal day (PND) 7, 14 and 28, offsprings were sacrificed for a comprehensive analysis of their hematopoietic system. Therefore, a deep immunophenotyping of lymphocytes from peripheral blood, thymus, spleen, and bone marrow was performed by flow cytometry.

A first statistical evaluation of B-cell development in the bone marrow revealed an interesting insight into a possible hematopoietic anomaly, potentially linked to an ELF-MF exposure at European basic restriction levels. Investigating the composition of developing B-cell subpopulations in the bone marrow, no alterations have been found between exposure groups. However, significant changes in CD127 (IL-7Rα) expression between exposure groups in Pre-Pro-B, Pro-B and Pre-B cells in male and female PND14 mice have been observed. Taken into account the highly complex mechanisms behind the described alterations, as well as the fact that these changes were only evident in PND14 but not in PND7 and PND28 animals, a profound interrelated statistical analysis of the entirety of the generated data sets will be required to obtain a complete picture.

The IL-7R fulfills a crucial role in the development of the B-lymphocyte arm of the immune system. As an aberrant expression of IL-7R is suggested to contribute to B-cell oncogenesis^[3], our findings possibly could lead towards an indication of the underlying role of ELF-MF in the development of childhood leukemia. In summary, with our study we hope to contribute to elucidate a possible relevance of ELF-MF as an environmental trigger for adverse health effects and help to improve disease prevention in the future.

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P20-23

Toxic effects of methyl ketones in the insect model species *Nasonia vitripennis*: potential alternatives to synthetic insecticides?

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Current agriculture faces the challenge of moving towards more sustainable models where production and profitability do not affect human or environment health. Plants synthesise secondary metabolic compounds, not directly related to photosynthesis and primary metabolic routes, that are a deterrent of pathogens and phytophagous insects. Among all secondary metabolites characterised in different plant taxa, only few have been evaluated as biocides which has raised considerable interest recently for evaluating the effects of natural compounds on different pest species.

Methyl ketones constitute a group of metabolites produced by some plants to defend themselves against the attack of phytophagous arthropods. These compounds are commonly found on leaves of tomato plants, constituting up to 90% of secondary metabolites secreted by glandular trichomes.

This work analysed the effect of 2-dodecanone on the pupal stage of *Nasonia vitripennis*. Transcriptional alterations induced by sublethal concentrations (5 and 500 µg/L) of this methyl ketone as well as ecdysteroids levels were evaluated (acute 48-hour exposures). Quantitative real-time PCR (qPCR) was used to measure the expression levels of genes related to the endocrine system (*Ecr*, *usp*, *E78*, *HR4*, *HR38*, *dronc*), detoxification mechanism (*gst1*, *gpx*) and homeostasis (*l(2)efl*). Results showed that 2-dodecanone caused significant alterations in the transcriptional activity of many ecdysone-related genes tested after 48-hour exposures, these effects were not concomitant with changes in ecdysteroids levels, which remain constant. Moreover, *l(2)efl* gene that encodes a member of the heat shock 20 protein (HSP20) family, was repressed dramatically under the highest concentration tested.

These data show the ability of 2-dodecanone to interfere with endocrine marker genes in insects. It provides novel and interesting results on the toxic effects of an isolated secondary metabolite, naturally present in plants, in the ecologically relevant parasitoid wasp, *N. vitripennis*, and highlights the potential suitability of this organism

for deep into the molecular effects of plant defences in insects. These findings provide new insights into the insecticidal efficacy of 2-dodecanone, which might be explored under field conditions for plant protection and pest management to reduce reliance on synthetic pesticides.

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P20-24

Experimental studies of the effect of the pesticide active substance, a derivate of cinnamic acid, on the nitrogen cycle in soils

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This article presents the results of experimental studies of the effect of the pesticide active substance, a derivative of cinnamic acid, on the nitrogen cycle in the soil, the disruption of the functioning of which can lead to serious consequences for both human health and the environment. The threshold concentration of the pesticide was established, which guarantees a change in the ammonifying and nitrifying capacity of the soil on the 5-7th day of the experiment by no more than 25% compared to the same indicators of the control samples.

The studies were carried out considering the physicochemical properties of the active substance under study, as well as taking into account the requirements for conducting experimental studies in standard, comparable soil and microclimatic conditions, and in compliance with the principle of extremality, which contribute to the preservation, growth, reproduction of microorganisms and the formation of the greatest number when exposed to the pesticide active ingredient. To create standard soil conditions, the studies were carried out using a unified, imitating, standard model soil standard No.1 (MSS No.1), based on a constant particle size and physicochemical properties of sandy soil. MSS No.1 consists of a mixture of clean medium-grained and fine-grained quarry sand, free from coarse fractions, with a grain diameter of ≤ 1 mm.

Three doses of the active substance in triplicate were tested and control conducted. The first dose of the active substance corresponded to the maximum recommended application rate in agriculture in the Russian Federation-3 mg/kg. The second dose was 10 times less than the recommended dose-0.3 mg/kg. The third dose was 10 times higher than the maximum application rate of 30 mg/kg. The need to carry out studies of the pesticide in concentrations 10 times higher than the maximum recommended application rate is due to the possibility of accumulation of the active substance in the soil with repeated use.

On the first day of the experiment, in all doses, the content of ammonium, nitrite and nitrate nitrogen was 30-47% lower than the control values. On the 5th day, there was a decrease in the content of ammonium, nitrite and nitrate nitrogen in the control samples and at a concentration of 0.3 mg/kg of the active substance. At the same time, the active substance at concentrations of 3 mg/kg and 30 mg/kg on the 5th day of the experiment had a weak stimulating effect on the processes of ammonification and nitrification. On the 7th day, in all the studied samples, the content of ammonium, nitrite and nitrate

nitrogen did not differ from the control samples by more than 25%. This indicates that the tested concentrations of the pesticide active substance have no effect on the mechanisms of soil self-cleaning. Based on the experimental studies carried out, the threshold value for the pesticide active substance, a derivative of cinnamic acid, for the nitrogen cycle was established at a level of 30 mg/kg.

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P20-25

Evaluation of plasticizers from protective gloves: determination of the content of plasticizers in vinyl gloves and *in vitro* toxicity studies on microvascular endothelial cells

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Over the past few years, exposure to plasticizers have been linked with multiple effects on human health, including endocrine disruption and cardiovascular diseases. Plasticizers are wide use additives which are incorporated in many products including protective gloves. The frequent use of gloves is a source of dermal exposure to plasticizers because it favours the occlusion of skin barrier. It can contribute to their transcutaneous migration since they are not covalently bound. Thus, they may affect dermal microvascular endothelial cells. Despite the large use of gloves in the workplace, their plasticizers content have been poorly studied. This study aims to investigate the toxicity of plasticizers found in gloves on microvascular endothelial cells by evaluating their effects on mitochondrial activity. To that purpose, the plasticizers content of five vinyl gloves was analyzed using a GC-MS method previously developed in our laboratory for the simultaneous determination of 11 plasticizers. The effects of the main plasticizers found in gloves were evaluated on a human dermal microvascular endothelial cell line (HMEC-1) after single and repeated exposures performed on four consecutive days. Plasticizers were assessed at a concentration of 15 mg/L individually and in the mixture. Their effects on mitochondrial metabolic activity was measured by using MTT assay and the cellular levels of adenosine nucleotides (ATP, ADP and AMP) was measured by HPLC-DAD. The GC-MS analysis of the vinyl gloves revealed the presence of three main plasticizers, namely di-(2-ethylhexyl) phthalate (DEHP), di-(2-ethylhexyl) terephthalate (DEHT) and di-isononyl phthalate (DINP). Amounts were reported up to 44.44% (w/w). From the experiments conducted on cells exposed to the found plasticizers, results showed significant alterations on the mitochondrial activity. Following single exposure, results showed a higher cell viability after 24 hours of exposure to DEHP, DINP and the mixture. It was followed by a decreased viability observed for DEHP and DINP at 48 hours and for the mixture at 72 hours whereas DEHT reduced cell viability after 96 hours of exposure. Repeated exposures affected cell viability in a time-dependent manner for the individual DEHP and the mixture. For the cellular levels of adenosine nucleotides, ATP and ADP were detected with all exposure conditions but AMP was not detected. First results showed modifications after three hours of exposure with all three plasticizers individually but not in combination. Further experiments are in progress to complete the results. This study throw light on plasticizers in vinyl gloves with reported amounts that raise some concerns regarding health. *In vitro* studies provide new knowledge on the endothelial toxicity of individual and mixture effects of DEHP, DINP and DEHT at the microvascular level. These findings contribute to fill knowledge gaps on the subject.

P20-26

Polybrominated diphenyl ethers in indoor dust of different microenvironments in Zagreb, Croatia and assessment of toddlers' exposure

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Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants used as additives in a wide range of consumer products, such as polyurethane foam in furniture and car seats, textile, building and plastic materials, as well as electrical and electronic equipment from which they are easily released and bonded to dust particles. Growing evidence on their persistency and adverse health effects (thyroid hormone and endocrine disruption, neurotoxicity) has led to a global ban of their production and use.

Given the dominantly indoor lifestyle in developed cities, dust ingestion and dermal absorption are considered the main routes of human exposure to PBDEs. Toddlers are identified as being the most exposed and vulnerable population group due to their frequent hand-to-mouth activity and extensive contact with floors, carpets, and other dusty surfaces, in combination with their lower body weight.

The levels of seven PBDE congeners (BDE-28, -47, -99, -100, 153, -154, and -183) were determined in dusts collected in different microenvironments in Zagreb, Croatia. Although comparable median levels of Σ_7 PBDEs were measured (4.34, 6.08, and 8.26 ng g⁻¹ dust) significantly different maximum values of 200.09, 14.42 and 5666.98 ng g⁻¹ dust were detected in dust from homes, kindergartens, and cars, respectively. Based on the obtained data, toddlers' exposure to PBDEs via dust ingestion and dermal absorption was estimated.

Total estimated daily intake (EDI) values for the Σ_7 PBDEs in each microenvironment calculated as the sum of two individual intake pathways ranged from 1.27×10^{-3} (cars) to 1.07×10^{-2} ng kg⁻¹ bw day⁻¹ (homes) at central, and from 2.37×10^{-2} (kindergartens) to 1.69 ng kg⁻¹ bw day⁻¹ (cars) at worst case scenarios. Intake via dust ingestion is the dominant pathway, with contributions of over 94%. All EDI values, even at worst case scenarios were several orders of magnitude lower than the reference dose (RfD) of 2000 ng kg⁻¹ bw day⁻¹ set for pentabDEs. We calculated the EDI based on a time-weighted model that integrates the time fraction spent in each microenvironment, which is 4%, 22% and 64% for cars, kindergartens, and homes. It was observed that daily intake of dust from homes contributes to overall exposure with the largest proportion of 63% at central case scenarios, while at worst case scenarios the highest contribution was from intake of car dust, because of the higher PBDE levels found. Calculated values of hazard indexes (HI) presenting non-carcinogenic risk of investigated exposure pathways was several orders of magnitude below 1 indicating that PBDE uptake via ingestion and dermal absorption of dust originating from various microenvironments poses a low risk for toddlers. Nevertheless, care should be taken considering that dust ingestion is not the only exposure pathway to PBDEs as well as that these are not the only contaminants present in dust.

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P20-27

This abstract has been withdrawn.

P20-28

Investigation of the biological effects of anti-inflammatory drugs on *C. elegans* and their cost-effective degradation of problematic substances using UV-C radiation

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The aim of the project is to develop an innovative process for removing pharmaceutical residues from municipal and industrial wastewater and to test its efficiency in model organisms of Environmental Toxicology. Drugs, like anti-inflammatory drugs, and their metabolites are difficult to remove during the wastewater treatment process due to their persistence and high water-solubility. In previous investigations the anti-inflammatory drugs Diclofenac and Acetaminophen (0.5; 1; 2 mM) as well as Ibuprofen and Naproxen (2.5; 10; 25 µM) did not lead to any acute toxic effects in *C. elegans*. This means Diclofenac shows low toxicity, despite the selection of a concentration approximately 600-fold higher than in previously analyzed wastewater. For the new investigations Ibuprofen is used, because of its high consumption within the population of Germany (334,880 kg/a in 2001).

To investigate possible biological effects, the model organism *C. elegans* was cultivated in NGM (Nematode Growth Medium) with different concentrations of anti-inflammatory drugs (Diclofenac, Ibuprofen, Naproxen and Acetaminophen). Subsequently, studies of acute and chronic toxicity (life span assay, locomotion) and studies of reproductive toxicity (progeny assay) were established and conducted.

The other integral parts of our project were tests to investigate efficiency of adsorption of drugs on a catalytically active membrane followed by degradation using UV-C radiation. Results show that UV-C irradiation can reduce the amount of Ibuprofen in water by 22.5% within 5 minutes and by 61.1% in 10 minutes.

In studies on the chronic toxicity of Ibuprofen, the life span of *C. elegans* is not shortened at any concentration compared to the untreated control. During the locomotion assay we could not observe any differences between groups exposed to Ibuprofen and controls. Moreover, Ibuprofen in NGM did not show effects in the progeny assay.

In an initial assessment, Ibuprofen shows no toxicity, despite the selection of approximately 1000-fold higher concentrations than in wastewater treatment plants drain waters previously analyzed. Further investigations will be carried out on the biological effects of mixtures of the drugs investigated and their metabolites that arise after applying UV-C radiation.

P20-29

Renal toxicity and biokinetic models after repeated uranium instillations

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Uranium kidney toxicity is well documented after acute uranium intake with a threshold of around 1–3 µg/g of kidney. Results of chronic exposure studies are controversial even after long-term exposure via drinking water during 9 months reaching 6 µg/g of kidney. During nuclear fuel processing, workers can potentially be exposed to repeated inhalations of uranium compounds.

A biokinetic model after uranium intake has been recently updated by the International Commission on Radiological Protection to include recent data. However most of experimental data consider either acute inhalation, chronic ingestion via drinking water, or wound contamination (shrapnels). To evaluate uranium kidney concentration after protracted inhalations, assumptions of repeated acute intake are made, but no experimental data are available to support them. The objectives of this work is therefore to verify 1) if the uranium biokinetic model developed from the data obtained for acute inhalation is consistent with the data obtained for repeated contaminations of animals; 2) if nephrotoxicity modifies uranium retention and excretion; 3) if nephrotoxicity threshold can be predicted by the models.

Mice (C57BL6/J) were exposed to different uranyl nitrate hexahydrate concentrations (0.03–3 mg/kg/day) via intranasal instillation four times a week during two weeks. Concentrations of uranium in urine and in tissues were measured at several time points during exposure and up to 42 days post exposure. In parallel, kidney toxicity was evaluated in urine using Clusterin and Kim-1 kidney biomarkers and confirmed by *in situ* kidney mRNA levels of early and late nephrotoxicity markers.

For the lower uranium concentrations, the experimental retention in kidney was well predicted by the standard rat specific biokinetic model when accounting for the exposure profile. For every concentration tested, the amount of activity retained in the kidney reflected the two consecutive weeks of instillation. Uranium biokinetics was modified for higher doses, probably due to tissue alteration and organ dysfunction not considered in biokinetic models. A clear nephrotoxicity and renal impairment was detected for the highest concentration of 3 mg/kg/day and moderate nephrotoxicity was observed at 1 mg/kg/day.

Our results suggest that specified biokinetic models should be developed to take into account the alteration of excretion and retention due to organ toxicity. The threshold of uranium nephrotoxicity should be adapted according to the contamination mode and its duration (acute, chronic, and repeated).

P20-30

Health risk assessment of Bisphenol A and its alternatives (BPS, BPF) using a pregnancy PBPK model

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Bisphenol A (BPA) is an endocrine disruptor found in commonly used plastics causing adverse health outcomes. Maternal exposure to BPA can affect the fetus by crossing the placental barrier^[1]. It causes metabolic and immune disorders and affects neurological development in children^[2,3]. Other bisphenols (PBs) such as BPS and BPF have been increasingly used as alternatives to BPA, although some adverse human health effects have also been reported^[4]. The aim of this study

was to develop a pregnancy-physiologically based pharmacokinetic (P-PBPK) model to assess the health risk of prenatal BPA, BPS and BPF exposure in fetuses using available biomonitoring data. First, the PBPK model was optimized using an adult model incorporating pharmacokinetic parameters. Then, this optimized model was extended to develop a P-PBPK model, including physiological changes during pregnancy and BPs transplacental kinetic parameters. The expected outcome of this model is to understand the toxicokinetics profile of BPs and dosimetry assessment in humans from different routes of exposure. Such a model could be used to address the concern regarding health effects in fetuses prenatally exposed to BPA and its alternatives.

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P20-31

Anthropogenic pollution in the tributaries rivers of the Atoyac river in the metropolitan area of Puebla, Mexico

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In the last 30 years the Metropolitan Area of Puebla and Tlaxcala has reached a high degree of urbanization, in an area of 1867 km² there are 2 million 120 thousand people, becoming it the fourth most important metropolitan area at the national level. It currently presents a complex problem characterized by: poverty, marginalization and deterioration of water resources that put the present and future of the area at risk. The objective was to analyze the impact of urbanization on water quality in the Rabanillo and Zapatero rivers in the Puebla Metropolitan Area (ZMP). A database of the industries was created, due to their activity and wastes, that they probably generate dangerous pollutants to the health of the ecosystem and the population; the physicochemical parameters, heavy metals (HM) and volatile organic compounds (VOCs) were evaluated and the integrated pollution index (PI) was determined using the classification model of the Bayesian Network (BN) to correlate the effect of the industry on the water quality in the study areas. The area covered by both rivers is equivalent to ~62 km² where a total of 501 industries are located, among which stand out: metallurgy, construction (brick and cement),

metalworking, plastic, textile, chemical, food, pharmaceutical and paint. The physicochemical parameters showed that the rivers are in anoxic conditions and the results of pH (9.32–9.45) dissolved oxygen (OD 1.86–1.98 mg/L), chemical oxygen demand (COD 406–414 mg/L), biological demand oxygen (BOD₅ 129–138 mg/L) and electrical conductivity (EC 1.79–2.54 µS) exceeded the maximum permissible limits (MPL) of the Official Mexican Standard (PROY-NOM-001-SEMAR-NAT-2017), Ecological Criteria for Water and the Atoyac-Xochiac Declaration. HM and VOCs are products of the metallurgical, construction, textile and paint industries. The HM exceeded the MPL and presented the same trend in both rivers (Pb > Cu > Cr > Ni > Cd). 11 VOCs were detected, in which protrude trichlorethylene > chloroform > vinyl chloride > 1,4-dichlorobenzene and exceeded the MPL of the NMX-AA-103-SCFI-2006 in the Rabanillo river, the trichlorethylene in both rivers exceeded the MPL (0.08 µg/L). The correlation indices between the industrial growth rate and the water quality in both rivers presented high industrial densities respectively in the Rabanillo and Zapatero rivers (14.64 and 3.08 industries/km²). The PI is determined by the following factors: increase in population, industry by area and the number of physicochemical parameters; in this regard, the highest IP was presented by the Rabanillo River (15.48) in relation to the Zapatero River (12.63). It is concluded that both rivers are anoxic, have HM and VOCs that exceed the MPL as a result of anthropogenic activities and are little studied.

P20-32

Polystyrene nanoparticles interference in human colon adenocarcinoma cell line HT29

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Nanoplastic (NP) pollution has been gaining interest in the scientific community due to their widespread dissemination in the environment and their potential consequences to human health^[1,2,3]. This study evaluates NP toxicity and their potential to interfere with inflammation-related pathways in the human colon adenocarcinoma cell line HT29.

Cytotoxicity assays (MTT and TrypanBlue assay) were carried by exposing cells to five different concentrations (25, 50, 100, 250 and 500 µg.ml⁻¹) and tree distinct sizes (25, 50 and 100 nm) of polystyrene nanoparticles (PSNP) during 24, 48 and 72 hours.

For analysis of effects over inflammatory pathways, cells were exposed to 25 and 500 µg.ml⁻¹ of PSNP (25, 50 and 100 nm) during 6, 24 and 48 hours. Expression of p50 and p38 was analyzed in total lysates and nuclear fractions by Western Blot.

In general, the effect of PSNP exposure on cell viability was modest. Cells exposed to 500 µg.ml⁻¹ with 100 nm PSNP during 48h showed the biggest decrease in cellular viability (less 15% comparatively to control). A significant decreased also occurred after 72h for cells exposed to 25 nm PSNP (less 20% comparatively to control). Overall, the total cellular expression of p50 and p38 was slightly increased after 24h of exposure (all exposure conditions) to PSNP, but no increased phosphorylation of p38 was observed.

Interestingly, only p50 was significantly increased in the nuclear fraction of HT29 cells in particular when PSNP concentration was highest (500 µg.ml⁻¹) for all particle sizes. This is of significance since the NF-κB pathway has been considered a prototypical proinflammatory signal and a key transcriptional factor family that is implicated in multiple physiological and pathological processes, including cell proliferation and differentiation, inflammatory and immune response, cell survival and apoptosis, cellular stress reactions and

tumorigenesis^[4]. These results suggest that PSNP causes activation of NF- κ B pathway, and currently studies are underway to further dissect the consequences of such activation.

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P20-33

Toxicity of Vegetable Extracts in Biological Indicators of the Aquatic and Terrestrial Environment

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The European Union (EU) countries where the highest amounts of pesticides were sold in 2014 were Spain, France and Italy (Eurostat, 2016). The main objective of the European Research Project WASTE-4GREEN “Sustainable and green agri-waste based biopesticides” is to mitigate the adverse effects on the Environment and Human Health of the pesticides of chemical origin that are currently used in the protection of stone fruits crops.

It is intended to implement the use of 3 formulations whose active materials come from agroindustrial residual biomass extracts, with the aim of replacing the use of pesticides of chemical origin in the cultivation of stone fruits.

Initially, 8 extracts of vegetable origin are obtained that are effective against the most frequent pests in stone fruit trees.

A second aspect to consider when selecting the extracts is the assessment of their toxicological potential for the aquatic and terrestrial environment since they are the final destination, after their application on fruit trees in agricultural soils.

In this phase, a pre-screening with biomarkers of exposure and effect was carried out, with biological indicators of the aquatic environment (*Daphnia magna*) and the terrestrial environment, earthworm (*Eisenia foetida*).

The toxicological test methodology is based on the OECD guidelines for the control of chemical products (Acute Toxicity Test on Earthworm *Eisenia foetida*. Contact toxicity on filter paper. OECD #207) and the ISO standards of water quality (*Daphnia magna* acute toxicity test, ISO 6341: 2012).

We evaluated the toxicity effects of the following 8 vegetable extracts: Savory (Samo), Ditrachia (Olivardilla), Degreased Alperujo,

Eucalyptus leaves, Artichoke water (FO Acetate), Asparagus water (FO Acetate), Artichoke petals (EtOH extract) and Asparagus stem peelings (EtOH extract).

The results obtained show that none of the 8 extracts tested presents Acute Toxicity effects for *Daphnia magna*. In the Earthworm, *Eisenia foetida*, 4 of the extracts are classified as Moderately Toxic (Roberts, B.L. & Dorough, H.W., 1984) and are: artichoke petals, asparagus leaves, eucalyptus leaves and the defatted alperujo.

The following classification of extracts is established, depending on their acute toxicity, for *Eisenia foetida* (earthworm), from highest to lowest toxicity:

- Ethanolic extract of asparagus leaves > eucalyptus leaves (*Eucalyptus globulus*) > samo (*Ajedreavar. Martín*) > defatted alperujo > ethanolic extract of artichoke petals > olivardilla (*Olivardilla* or *Inula graveolens*) > artichoke water // asparagus water.

With these results, it is decided which are the effective and safest active principles for the development of the new Biopesticide formulations.

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This abstract has been withdrawn.

P20-35

Investigation of nanoplastic cytotoxicity using SH-SY5Y human neuroblastoma cells and polystyrene nanoparticles

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Nanoplastics have spread widely throughout not only the oceans but also the atmosphere, and recently created great concern about human health relevant to ingestion and accumulation of the nanoparticles by aquatic organisms in the human food-chain. However, how the nanoplastics have an affect on actual human body remains largely unknown, and in particular, little knowledge about nanoplastic exposure to the nervous system in human has been obtained *in vitro* and still less *vivo*. As for not human but aquatic organisms, Mattsson *et al.* reported that nanoplastics, polystyrene nanoparticles with 53 nm size, penetrated the BBB in fish and were incorporated in the brain tissue through the food chain, and caused the behavioural disorders, the decrease in predatory activity^[1]. In this background, this study evaluated how much concentration of nanoplastics had a direct impact on cells in the human nervous system as the fundamental information.

The cytotoxicity was investigated by exposure of polystyrene (PS) nanoparticles, characterized by the spherical shape, the average size of 50 nm, and the negatively discharged sulfonated surface, to cultured neural cells, human neuroblastoma cells (SH-SY5Y). The cells were cultured at 37°C in a 5% CO₂ incubator for 24 h using the mediums

prepared by adding the PS aqueous suspension at concentrations of 2, 10 and 50 µl/ml, or acrylamide (AA) at concentrations of 0.8, 20 and 500 µg/ml, respectively, and the cytotoxicity was evaluated using a Live/Dead Cell Staining Kit. More, after 24 h exposure of PS and AA to cells, immunofluorescent staining evaluation was performed, and the expressions of neuronal markers, Tuj1 and MAP2, neuronal progenitor cell marker, nestin, and glial specific marker, GFAP, were investigated.

The results of cytotoxicity testing revealed that the addition of PS nanoparticles into the medium and the exposure for 24 h strongly induced the death of human neuroblastoma cells promoted differentiation into neuronal phenotype, and the cytotoxicity effect of PS nanoparticle was comparable to or exceed that of acrylamide, recognized as one of neurotoxic chemicals. Also, the cells exposed to relatively higher concentration of PS nanoparticles exhibited shrinkage of neurite outgrowth, morphology alternation and swelling of the nuclei, and spilling of intracellular components. The adverse impact on the cells significantly appeared over the level of about 100 µg/ml of the PS concentration, which is likely to be much higher than those predicted from the marine environment [2].

Concerning the invasion of nanoplastics into actual human central nervous system, the following questions have to be further explored: the adverse effects of low dose exposure of nanoplastics repeatedly for a long period, the cases of several environmental polymers with different sizes and surface discharges, and the adverse impacts of various chemicals adsorbed on and included in nanoplastics in the environment.

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P20-36

Oxidative stress and inflammation induced by air pollution-derived PM_{2.5} persist in the lungs of mice after cessation of their subchronic exposure

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Purpose: More than 8 million early deaths/year are attributable to air pollution. Current health concerns are especially focused on air pollution-derived particulate matter (PM), and mostly fine particles (i.e., aerodynamic diameter ≤ 2.5 µm, PM_{2.5}), because of their ability to migrate deeply in the lungs. Despite oxidative stress-induced airway inflammation is one of the main adverse outcome pathways of PM_{2.5}, the persistence of this underlying mechanisms, even after the exposure stops, remains poorly studied. Here, we sought to better evaluate the oxidative and inflammatory responses in the lungs of mice exposed to urban PM_{2.5} (Lille, France) 24 h after their acute exposure, and 24 h and 2 months after their subchronic exposure.

Methods: Chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate, dithiothreitol, ascorbic acid, and glutathione assays provided the intrinsic oxidative potential of PM_{2.5}. A/J mice (male, specific and opportunistic pathogen free, 10 weeks, n=6/group) were exposed either for 24 h by one intranasal instillation, or for 28 days by 3 intranasal instillations/week, to 0, 10, 50 or 100 µg of urban PM_{2.5}

suspended in 30 µl of sterile saline. Mice were sacrificed either 24 h after their acute exposure, or 24 h or 2 months after their subchronic exposure. Oxidative stress and inflammation were studied in lung tissues by nuclear factor erythroid 2-related factor 2 (NRF2) and nuclear factor-kappa B (NFkB) signaling pathway activation, antioxidant enzyme activities (i.e., superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase), glutathione status, oxidative damage (i.e., 8-hydroxy-2'-deoxyguanosine, carbonylated protein, 8-isoprostane, and 4-hydroxynonenal), and cytokine secretion (i.e., tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-6) by TransAM, RT-qPCR, and ELISA/Luminex. Non-parametric Mann-Whitney U-test was used to look at statistical differences versus controls ($p < 0.05$).

Results: All the tests we used revealed the high intrinsic oxidative potential of the PM_{2.5}. Dose-dependent activation of NRF2 and NFkB signaling pathways, enzymatic and non-enzymatic antioxidant defense alterations, DNA and protein oxidative damage, and cytokine secretion were reported in mice acutely exposed ($p < 0.05$). All these features were reinforced and others appeared (i.e., lipid peroxidation) 24 h after the subchronic exposure ($p < 0.05$). Most of them persisted, at a lesser extent, 2 months after exposure cessation ($p < 0.05$). Taken together, these results confirmed that any exposure to PM_{2.5} induced significant oxidative and inflammatory responses within the lungs. Longer the exposure duration was, highest the harmful lung effects were. Interestingly, their persistence despite exposure cessation supported a high retention and slow elimination of these particles, which can also continue to exert their toxicity in the lungs.

P20-37

Impact of pesticides on ocular surface using an innovative *in vitro* design

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Background: Nowadays, agricultural exposure to plant protection products (PPP) poses a major threat to human health. Indeed, this exposure is associated with certain pathologies such as Parkinson disease, prostate and hematopoietic cancer. In order to quantify the exposures, a global approach is required to protect the users and reduce the exposure levels including biological surveillance of agricultural workers. However, we are still lacking the tools to precisely evaluate both exposure and effect biomarkers. Although inhalation is the main route of exposure to PPP there are other routes and in particular eye contact, which is too often neglected, and which can be interesting for the research of biomarkers. Indeed, the eye is continuously exposed to environmental pollutants, including PPP, and the impact on workers' lives can be significant. The main ocular syndrome linked to exposure to these pollutants is dry eye.

Objective: This study, supported by the "Agence Française pour le Biodiversité (AFB)", aims to develop new surveillance tools of professional exposure to PPP focusing on researching ocular biomarkers of effect, using an innovative *in vitro* approach.

Methods: Human corneal epithelial cells (HCE) were cultured then exposed, at the air-liquid interface by nebulisation using the Cloud-Max™ (Vitrocell®) system, to Captan, Folpet and Chlorpyrifos at different concentrations and times, with single or repeated exposures. After exposure, culture medium was collected for inflammatory response evaluation and cells were harvested for inflammatory and oxidative stress gene expression assessment.

Results: Captan and Folpet presented a dose dependent cytotoxicity greater than Chlorpyrifos. However, the most important result was the depletion of total RNA and the increase of ARNr18S after cells were treated with Captan or Folpet only suggesting an inhibition of the RNA polymerase for no more than 24h causing an immunotoxic effect. Concerning the assessment of potential biomarkers of effect, IL-8 appears to be the most sensitive and early inflammatory biomarker of effect.

P20-38

Inhalation of tungsten particles: impact on the central nervous system, an unsuspected target organ

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Being the main cause of contamination in nuclear facilities, aerosols represent a health risk factor raising serious concerns. The brain represents a direct target for inhaled aerosols, in particular through the involvement of the olfactory nerve entry pathway. There is a lack of knowledge about the toxicity of tungsten (W) in the central nervous system, although it is considered as an emerging contaminant. To study the potential effects of exposure to W airborne particles, preliminary metrological work was carried out to optimize and control the generation of the aerosol at concentrations in the order of the Occupational Exposure Limit Value, and to characterize its size distribution.

Adults rats were exposed to optimized and controlled W aerosols via inhalation at two different concentrations (5 and 80 mg.m⁻³) in a single acute or fractionated mode (4X daily exposures). Various assays were used to examine biological processes involved in brain toxicity, such as neuroinflammation, oxidative stress, cell death and neurogenesis.

Results obtained 24 hours after exposure suggest site-specific and concentration-dependent modifications of the transcripts of genes involved in these target processes. The impact of oxidative stress is currently being further evaluated using the Dihydroethidium probe sensitive to reactive oxygen species, complemented by immunohistochemistry and protein expression of 4HNE and Nrf2, which respectively reflect lipid peroxidation and antioxidant signalling. Immunohistochemistry experiments suggest a potential effect on the density and morphology of microglial cells involved in neuroinflammation, as well as an abnormal phenotype of the nuclei of some mature neurons indicating neuronal damage. At the cellular level, the effects were also site-specific, dependent on the mode of exposure and the concentration and could involve several signalling pathways, recently investigated by transcriptomics. ICP-MS assays show an increase in the concentration of W in brain structures in groups exposed to high concentrations, which could contribute to site-specificity of observed effects. Current work aims to investigate whether these effects are related to adaptive mechanisms or are neurotoxic and can ultimately alter neuronal integrity depending on exposure patterns. Co-exposure to W aerosols and ionizing radiation is another focus for our future research.

These results can help close knowledge gaps in understanding neurological health effects due to aerosols exposure, especially metallic ones, and thus help improve the system of human (radio)protection.

P20-39

An insight into the toxic effects of imidacloprid through measurements of cholinesterase activities and markers of oxidative stress in the blood and brain of male Wistar rats

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Imidacloprid is the first neonicotinoid insecticide that acts as a selective toxicant on nicotinic acetylcholine receptors in insects^[1,2]. It is used for crop protection worldwide, as well as for non-agricultural uses^[1]. Food is one of the main routes of human imidacloprid exposure^[3]. Due to the ever-growing evidence for toxic effects of imidacloprid in animals and humans^[4,5,6] and need to investigate them in more detail, we evaluated the effects of subchronic oral exposure to environmentally relevant doses of imidacloprid on relevant biochemical markers of toxicity in the blood and brain of adult male Wistar rats. Following a 28-day oral treatment with imidacloprid at 0.06 mg/kg b. w./day, 0.8 mg/kg b. w./day and 2.25 mg/kg b. w./day we measured total cholinesterase, acetylcholinesterase and butyrylcholinesterase activities as markers of cholinergic toxicity, and levels of lipid peroxidation, glutathione and reactive oxygen species as well as activities of glutathione peroxidase, superoxide dismutase and catalase as markers of oxidative stress. Exposure to low doses of imidacloprid did not cause significant changes in total cholinesterase, acetylcholinesterase and butyrylcholinesterase activity in rat blood and brain tissue. Concerning the influence on biomarkers of oxidative stress, treatment with the lowest dose of imidacloprid (which corresponds to the acceptable daily intake for imidacloprid) resulted with increased levels of lipid peroxidation and reactive oxygen species and decreased activities of antioxidative enzymes superoxide dismutase and catalase. The most pronounced effect was noticed in plasma. The highest levels of lipid peroxidation were detected in both plasma and brain tissue after treatment with the lowest dose of imidacloprid, being significantly elevated in plasma. Reactive oxygen species levels were also highest and significantly increased at the lowest dose in plasma. Superoxide dismutase activity in erythrocytes increased depending on the dose, being statistically significant at the highest dose. Treatment with the highest applied dose of imidacloprid also caused significantly increased activities of glutathione peroxidase in both plasma and brain tissue. Decreased superoxide dismutase and catalase activities were found in the plasma of rats treated with the lowest dose of imidacloprid. No changes in glutathione levels were found in the treated rats. Taken together, our findings suggest that oral 28-day exposure to low doses of imidacloprid caused a disturbance of redox homeostasis, i.e. the regulation of formation and removal of reactive oxygen species from the body system. These results call for further studies at molecular level to explain the toxicity of neonicotinoids after long-term exposure to environmentally relevant doses.

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Toxicity of combustion-derived particles from Ethiopian household solid fuels in human bronchial epithelial cells

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Purpose: Human exposure to emissions derived from combustion of solid fuels is very high in low- and middle-income countries. Indoor air pollution from traditional fuels such as biomass has been associated with an increase in the incidence of respiratory infections and chronic respiratory diseases, cardiovascular diseases and all-cause mortality both in adults and children. It is therefore crucial to decipher the health impact of exposure to solid fuel emissions and their mechanisms of toxicity. The aim of this study was to evaluate the toxicity of combustion-derived particles relevant for indoor Sub-Saharan environments using human lung cells.

Methods: Particles were collected using a high-volume sampler during combustion in laboratory settings of three solid fuels commonly used in Ethiopia: cow dung, eucalyptus wood and charcoal derived from eucalyptus wood. The charcoal smoke was generated using a traditional Sub-Saharan clay stove, and the wood smoke and dung smoke was generated in a 3-stone fire. Beas-2B bronchial epithelial cells were exposed for 24 and 48 h to particle suspensions from the dung, wood, and charcoal and to diesel exhaust particles (DEP, NIST SRM 2975) as reference. Cytotoxicity was evaluated as a function of metabolic activity (Alamar Blue assay) and membrane damage (LDH assay), and ROS generation was measured with the DCFH-DA assay in acellular and cellular environments.

Results: The smoke generated by the three fuels indicated high emissions of particles of variable chemical composition. Cow dung was the most polluting solid fuel in terms of PM_{2.5} concentrations, followed by wood and charcoal. The chemical composition is dominated by organics. The ratio of organics to (equivalent) black carbon was 31 for the cow dung, 15 for the wood, and 2.3 for the charcoal smoke. No ROS production could be detected for particles from the solid fuels in acellular conditions while DEP produced ROS in a dose-dependent manner. All solid fuel particles caused a dose- and time-dependent decrease of metabolic activity and increase of cell membrane damage in Beas-2B cells, with dung and wood particles inducing the highest toxicity. IC50 values (in µg/ml) for viability at 24h and 48h were 35,1 and 15,9 for dung, 28,0 and 10,9 for wood, while for charcoal and DEP, IC50 were higher than 150µg/ml at both time points.

Conclusion: Our results indicate that solid fuel combustion-derived particles are cytotoxic to bronchial epithelial cells with the particles emitted from the less energy dense solid fuels, such as dung, causing the highest effects. In further studies, the secretion of cytokines and chemokines of bronchial cells exposed to non- or low- cytotoxic doses of solid fuel particles will be measured by Mesoscale and their cellular uptake will be evaluated by TEM. Ongoing analysis will shed light on the chemical composition of the particles in order to correlate the cellular effects of the particles with their physico-chemical properties.

P20-41

Developing a quick, affordable and effective screening multi-method for most commonly used veterinary antibiotics in livestock products

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Introduction: The growing population demands higher volumes of food production, and strict regulations for food quality mean, that most of the production is dependant of intensive breeding methods. This brings the need to add a myriad of supplements and pharmaceuticals in the feed to ensure the health and fast growth of livestock. (1,2) Antimicrobials are used for treatment and prevention of various diseases, but also as growth promoters. (3–6) Although many of the antimicrobials are metabolized from the muscle within 4 days after withdrawal, the indiscriminate use of these pharmaceuticals may lead to adverse effects for consumers due to residues still present in the products. (4,7) LC-MS techniques have been widely adopted in EU for monitoring food quality and detecting pharmaceutical residues as it allows to separate analytes and quantify the substances. (2,8) Monitoring of antibiotics residues in livestock products sold in Estonia is not regularly done and therefore it is necessary to develop a quick, affordable and effective method for identifying substances simultaneously.

The aim of the study is to validate a multi-method for determination of most commonly used antibiotics residues in livestock products.

Methods: Commercially available reference antibiotics were used for the preparation of calibration solutions. Samples were analysed using the HPLC-DAD method. The Chromatographic column EC 250/4.6 Nucleodur C18 Gravity, 5 µm, length: 250 mm, ID: 4.6 mm was used. Potassium hydrogen phosphate and acetonitrile gradient was used as a mobile phase.

Results: The first stage of the experiments shows the possibility of using a multi-method as rapid and cheap method for the determination of antibiotics in meat products

Discussion: Food safety is a critical issue as pharmaceutical residues have become more commonly found in different products and associated with multiple health risks and detrimental effects on humans. It is paramount to ensure the minimum requirements for food safety are met. This method can be used for primary screening to sort out the samples that need additional expertise. This allows to reserve more expensive methods for samples that are actually contaminated.

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P20-42

Effect-based analysis of endocrine activity in sediment samples from lakes in Taihu Basin, China

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Within the last four decades, Taihu Basin in the East of China has transformed into a highly populated and industrialized area. While Lake Taihu is serving as drinking water source, tourism and for fishery, anthropogenic influences from agriculture, industrialization and municipal wastewaters led to drastic environmental changes. To ensure safe drinking water, the water quality is in focus of several investigations. Toxicological effects like endocrine disruption have been identified. This effect is mainly linked to the distribution of organic contaminants which are also likely to bind to sediment. However, sediment does not only act as a natural “sink” for contaminants but also as a “source”. In the present study, the endocrine potential of sediments from Taihu Basin was investigated.

Sediment samples were taken from a closed crab farm and an inlet of a drinking water treatment plant. Another sample was taken from the adjacent Lake Yangcheng, which is affected by industrial and residential areas. The samples were divided into vertical layer to identify changes over time. A pressurized liquid extraction (PLE) was performed to extract pollutants. The extracts were tested *in vitro* on U2-OS cells for cell viability (MTT test) and endocrine disruption including agonistic and antagonistic effects on both, the androgen and estrogen receptor (CALUX bioassay).

The results showed effects on the cell viability in the top layer of sediment from Lake Yangcheng. Endocrine effects generally decreased from upper to lower layer of sediment. Only the effects determined at the former crab farm increased to a depth of 18 cm before reducing in deeper layer. Anti-androgenic effects were determined in all samples in the range of 1.3 ± 0.6 to 13.8 ± 9.7 mg flutamide equivalents/kg, whereas highest concentrations were determined in Lake Yangcheng. The same sampling location showed also the highest estrogenic effect of 551.9 ± 119 ng 17β -estradiol equivalents/kg, while the lowest effect of 10.6 ± 1.6 ng 17β -estradiol equivalents/kg was found at the drinking water inlet in Lake Taihu. Neither androgenic nor anti-estrogenic effects were revealed in the bioassays. Lowest observable effects in the deepest sediment layer may be explained by a historically smaller release of endocrine active chemicals during the sedimentation process and degradation of endocrine active substances over time. However, higher endocrine activity in the upper

sediment layer in Lake Yangcheng and at the drinking water inlet may also suggest an ongoing introduction of contaminants by human activities. As wind and currents have a major impact on sediment resuspension the water quality in these shallow lakes may be affected. Positively, the operation stop of crab farms at Lake Taihu reduced sustainably the endocrine potential posed by the sediment.

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P20-43

Environmental presence and exposure to platinum, palladium and rhodium by teenagers living in Alcalá de Henares (Spain)

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The use of automobile catalytic converters has led to concerning concentrations of platinum group elements (PGEs), specifically platinum (Pt), palladium (Pd) and rhodium (Rh), in the soil, street dust and vegetation of urban environments. However, little is known about their toxicological effects in humans. As a result, a human and environmental monitoring programme was performed to determine the environmental presence and exposure to these pollutants in Alcalá de Henares (Spain). Scalp hair was collected from 96 adolescents (13–16 years-old; 28 boys and 68 girls) living in Alcalá, who were recruited following strict selection criteria to minimise the effect of confounding factors and suggest potential reference ranges for these elements. Pt, Pd and Rh was monitored in scalp hair by ICP-MS following previous methods; limits of detection (LoD) were 0.001, 0.006 and 0.005 ng/g, respectively. Additionally, Pt and Rh were monitored in 97 topsoil samples randomly sampled from different urban parks across the city after appropriate mineral digestion; LoDs were 0.38 and 0.22 mg/kg, respectively. Pd and Rh were detected in only a few of the scalp samples collected, specifically in 7.2% and 6.2%, respectively, which would be logical as Pd and Rh are known to have higher mobility and biological uptake rates compared with Pt. Data was processed using statistical methods applied to censored data available in the 'NADA' statistical package, specifically the regression on order statistics (ROS). Concentrations in ng/g were as follows (values provided as 95 percentiles and ranges): Pd (0.001, <0.001–0.002) and Rh (0.001, <0.0005–0.001). In contrast, Pt and Rh were detected in 41.24% [median and percentiles are provided in mg/kg; 0.27 (0.26–0.81)] and 15.46% [0.15 (0.15–0.16)] of the soil samples monitored respectively, which may suggest an early stage of accumulation of these two metals in topsoils from Alcalá de Henares. Our results could also suggest that the teenage population of Alcalá de Henares would have not been significantly exposed to these contaminants, as hair has been suggested as an appropriate tool to measure exposure to PGEs. Thus, although Pt was detected in almost half of the soil samples collected across Alcalá de Henares, its presence was below the limit of detection (0.006 ng/g) for all the hair samples studied in a relevant teenage population sample living in this city. We could conclude that, in general, there are no strong indications for the presence of Pt and Rh detected in soils from Alcalá to be a potential health risk for the population. Human hair would be a safe and non-invasive tool to follow-up exposure to PGEs in urban settings and identify potential future risks from these contaminants, as the use of automobile catalytic converters is relatively recent.

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P20-44

In vitro characterization of genotoxic damage induced by various PM sources on the bronchial epithelial cell line BEAS-2B

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Particulate matter (PM) is a complex air pollutant, comprising both particles and gases, whose presence negatively affects individuals' life quality and constitutes a major risk factor for health worldwide.

It is composed by a particles' population characterized by a variety of physiochemical characteristics (e.g. size, composition, aerodynamic behaviour) and sources.

The wide spectrum of adverse health effects occurring after PM exposure is also reflected at cellular level in the activation of many different toxicity mechanisms. The variety of the latter can suggest that each source may be responsible for specific kinds of cellular damage. To validate this hypothesis, 5 different sources of PM were evaluated in the present study: a positive control DEP NIES no.8 (D), coke dust (C), pellet ashes (PA), incinerator ashes (IA), and brake dust (BD); these sources were previously characterized and are all comprised in the PM₁₀ class.

To deeply investigate the genotoxic potential of the different PMs, an *in vitro* investigation was performed using the human bronchial epithelial cell line BEAS-2B. Cells were treated for 24 hours with increasing concentrations of PM (25, 50, 100, and 150 µg/mL). Previous analyses confirmed the absence of cytotoxicity at all concentrations tested. The modified alkaline Comet assay was used in combination with three endonuclease enzymes (ENDOIII, FPG, and ENDOV) to recognize oxidative or direct damage on the DNA strand. PM treatments induced an increase of DNA damage at all concentrations tested, compared to controls. The characterization of the genotoxic damage revealed that all the sources of PM are particularly active in inducing oxidation of the DNA bases. Analysing each source allowed to highlight some peculiarities as the oxidation of purines (adenine and guanine) identified by FPG treatments, that results to be particularly increased in samples treated with D, C, and BD; while ENDOIII highlighted the presence of oxidised pyrimidines in samples treated with PA and IA. Moreover, the involvement of direct damage, in the form of cyclobutane pyrimidine dimers, at the higher dose of treatment with D was recognized by ENDOV.

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P20-45

Toxicological assessment of leachates and leached substances from degraded plastic species using in-vitro bioassays

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The broad use of plastics and the persistence of the material result in plastic residues being found practically everywhere in the environment. Increasing contamination of rivers and lakes by plastics has already been demonstrated, as has their presence in drinking water and food. Substances added to plastic can leach into their surroundings, but little is known about degradation processes of plastics under environmentally relevant conditions. Also, there is a lack of information on these additives, their toxicity and effects on humans and the ecosystem.

As part of the PLASTRAT project, "Strategies for the reduction of urban plastic emissions into limnic systems," different types of UV irradiation (UV-C, UV-A/B), and a subsequent or simultaneous water bath were used to simulate weathering processes of different plastic material containing virgin as well as recycle material and biopolymers. After an enrichment step, the leached substances or their degradation products so formed were investigated toxicologically in bioassays *in-vitro*. Cytotoxicity was determined in the MTT assay, genotoxicity in the p53 Calux and umu test, and estrogenic effects in the ERα Calux. In addition, single substances identified in the leachates by chemical analysis were further investigated.

Genotoxic as well as estrogenic effects were detected in the samples depending on the material and the irradiation type. In leachates from 10 out of 13 plastic species estrogenic potential was detected, in four of them the estrogenic effect was above the recommended

safety level of 0.4 ng EEQ/L for surface water samples. In the umu test leachates from 11 out of 13 plastics were potentially genotoxic, which was confirmed in the p53 Calux only for three samples. Several compounds with suspected carcinogenic, mutagenic, reprotoxic (CMR) or endocrine disrupting (ED) function were identified via chemical analysis. An estrogenic potential was detected in the ER Calux for 17 out of 26 suspected single substances. Genotoxicity was detected in three single substances in the p53 Calux.

The results show that plastics release a variety of known and unknown substances, leading to a complex mixture with potentially harmful effects. A correlation between individual substances and the measured effects has not been confirmed yet. In order to investigate these aspects further and to be able to give recommendations for the use of additives in plastics, further effect-related investigations are advisable.

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P20-46

Air Liquid Interface (ALI) system: genotoxic and inflammatory profile of BEAS-2B exposed in Bologna, Italy

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The study of the detrimental health effects caused by the exposure to air pollutants has mainly been carried out through epidemiological studies or toxicological *in vitro* techniques. Limitation of *in vitro* model resides in the challenge to use a highly realistic *in vitro* exposure set of conditions that mimics what really happens in the respiratory system, following particulate matter (PM) exposure.

In order to obtain a higher similarity with a real cellular response, in recent years a new methodology has been developed. The Air Liquid Interface (ALI) exposure allows *in vitro* models treatment directly to ambient air pollutants for the evaluation of their effects, bypassing the steps of filter collection, extraction, and solubilisation in cellular medium. This study was performed in an urban site of Bologna (Italy) in 10 different days in the framework of the PRIN-RHAPS project. The human bronchial epithelial cell line BEAS-2B was exposed at the ALI for 24 hours. Three inserts out of six were exposed to clean filtered air, while the others were exposed directly to air pollutant passing by a cyclone selecting PM₁ fraction.

The genotoxic evaluation was carried out via Comet assay, and to have a complete picture of the damage induced by the air exposure, a modified test, relying on the properties of endonuclease enzymes (ENDO III, FPG, ENDO V), was applied. In parallel, the release of the pro-inflammatory cytokine interleukin 8 (IL-8) was evaluated by a commercially available ELISA kit.

Results showed differences in the biological responses both in the DNA damage and in IL-8 secretion. The results revealed the presence of DNA damage in most of the days evaluated. The use of ENDOIII and FPG enzymes in the Comet assay highlighted also the presence of oxidative damage. Although the analysis is still on-going, results in-

dicating that changes in sources and/or components of air pollution may determine specific biological responses.

In agreement with literature, the methodology here applied permits to study relevant biological responses in *in vitro* models directly exposed to air pollutants, under conditions relevant for human exposure. The use of ALI also allows to evaluate the day-by-day variability of air pollution, and has proved useful to assess health effects associated to the exposure to aerosol particles, down to the ultrafine particle size range.

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P20-47

Involvement of oxidative stress and calcium signalling in NiONPs – induced toxic effects on eel hepatocytes and endothelial cells

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Anthropic activities in New Caledonia, such as mining, amplify the natural erosion of nickel mines soils leading to atmospheric emission of oxide nickel nanoparticles (NiONPs). These particles produced during extraction, by soil erosion and atmospheric transport will: (i) end up by deposition or leaching in aquatic ecosystems and impact living organisms in the watercourses downstream of these activities, and (ii) after inhalation reach the respiratory tract and the pulmonary circulation of persons working or living near the mine. Despite alarming freshwaters and atmospheric nickel concentrations, today only few studies explain the cellular and molecular mechanisms of toxicity of NiONPs (i) on cells of aquatic organisms living under mining influence, particularly eels, fish known to be sensitive to water contamination by metals, and (ii) on pulmonary artery endothelial cells (HPAEC) known to be a direct target of NPs after inhalation. The aim of this *in vitro* study, was thus to assess, the cytotoxic effects of NiONPs on both eel hepatocytes (HEPA-E1) and HPAEC, with special attention to oxidative stress and calcium signalling. Cells were exposed for 4 or 24 h to NiONPs (0.5 to 5 µg/cm²). The following different endpoints were studied (i) cell viability by spectrophotometry (WST-1 assay), (ii) ROS production by spectrofluorimetry (CM-H₂DCF-DA probe) (iii) intracellular and mitochondrial superoxide anion production by electronic paramagnetic resonance and MitoSOX probe and (iii) calcium signalling by confocal microscopy (Fluo4-AM probe). In both cell types (HEPA-E1 and HPAEC), NiONPs induced, on the one hand, a decrease of cell viability and, on the other hand, an oxidative stress, evidenced by an increase in ROS production especially in intracellular and mitochondria superoxide anion production. In addition, our results provide evidence that NiONPs exposure altered calcium homeostasis with an disruption in cytosolic calcium concentration. It's interesting to note that mechanisms of action of NiONPs are different on HEPA-E1 and on HPAEC.

These results underline the potential toxic impact of NiONPs on eels living under the influence of mining activities and on workers and population living near mines in New Caledonia.

This study should allow us to define in an integrated way (i) to what extent the contributions of metals related to the mining activities impact the aquatic organisms and the human health, (ii) what would be the levels in natural environment tolerable to preserve the wild fauna and flora and (iii) the recommendations as for the limitation of the exposure of the populations so as to preserve them from cardiovascular pathologies.

P20-48

Effects of PCB28 and PCB153 on Antimicrobial Resistance in E.coli: A preliminary experiment

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Purpose: Persistent organic pollutants (POPs) are considered of global concern, due to transport to long distances persistence in environment, bioaccumulation and biomagnification in the ecosystem. Emerging contaminants including Polychlorinated biphenyls (PCBs) were found to alter intestinal microbiota including bacteria. *Escherichia coli* is common to human intestinal flora, prime indicator of fecal contamination and survive open environments; which is also the common pathogen in humans and representative indicator of antimicrobial resistance of Gram-negative bacteria. Antimicrobial resistance (AMR) represents a major global health threat and the global efforts to struggle with the antibiotic resistance are mainly focused on the antimicrobial prescription and overuse of antibiotics. Industrial burden and its pollution impact on the prevalence of environmental AMR have commonly been disregarded. The aim of this study was to evaluate the potential role of PCBs on antimicrobial resistance in *E. coli* *in vitro*.

Methods: Minimum inhibitory concentrations MIC50 concentrations of PCB28 and 153 were evaluated in *E. coli* (ATCC 25922) suspension adjusted 10⁵ bacteria/ml McFarland using microbroth dilution test. *E. coli* were later incubated with the MIC50/4, MIC50/8 and MIC50/16 concentrations, which were also environmentally relevant shown in previous studies, to test the potential shift of kanamycin MIC50.

Results: MIC50 value of PCB28, PCB 153 and kanamycin were 2.39 µg/ml ± 0.18, 0.985 µg/ml ± 0.24 and 1.25 µg/ml ± 0.12. Coincubation with PCBs were would to increase the MIC50 value of kanamycin by 21.6% (MIC50/4), 39.2% (MIC50/8), 48.8% (MIC50/16) for PCB28 and 30.4% (MIC50/4), 34.4% (MIC50/8), 56% (MIC50/16) for PCB153, respectively. As environmental pollutants are ubiquitous where low concentration exposure is inevitable, changes in intestinal microflora related to PCBs would contribute to decreased immunity and several resistance related diseases.

P20-49

Sustainable Solutions in Agriculture: Use of Biopesticides from Vegetable Extracts

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In order to establish an adequate management of phytosanitary products, which controls their risks in the environment and, consequently, on human health, a response must be made to the negative impacts of some current agricultural practices, developing sustainable and innovative actions.

The European Union has promoted the “From farm to table” strategy that seeks a 50% reduction in the use of xenobiotic phytosanitary products by 2030 and one of its objectives is to implement new innovative techniques, including the development of bioproducts.

In this sense, The GO VITINNAT Project is an innovative initiative to develop three naturally-based, stable and effective biopesticides for the control of vine wood diseases that can be used in Organic Agriculture.

In the project, 10 extracts of vegetable origin are initially selected: 6 Winemaking extracts from the same winery but from different vine strains; 3 Alperujo Extracts of the variety Picual, Arbequina, and Ext.; and 1 Extract of a vivacious plant *Ditrichia graveolens*, we evaluated its environmental toxicity in indicator organisms of the aquatic and terrestrial environment (Díaz-López *et al.*, 2021), of which the ones with the lowest toxicity and greater efficacy are chosen: Extract of *Ditrichia viscosa*, Winemaking extract M2 and Mix of *Ditrichia viscosa* + Winemaking extract M2, which will be the active substances that will participate in the development of these three new formulations.

In these three formulations with biopesticide capacity, we determined their short-term toxicity in two indicators of the terrestrial environment, the earthworm *Eisenia foetida* and the vegetable, *Lactuca sativa*. The methods used were “Acute Toxicity Test on Earthworm *Eisenia foetida*. Filter Paper Contact Test” (OECD 207), “Earthworm, Acute Toxicity Test on Artificial Soil” (OECD 207) and the “Lettuce Seed Germination Test (*Lactuca sativa*). Seed emergence test in terrestrial plants” (EPA 600/3-88/029, 1988)

The results of the three biopesticide formulations with the Contact Toxicity Test on Earthworm show an LC50–48h between 50 mg/mL and 8 mg/mL for all samples. The results in the tests with *Eisenia foetida* on Artificial Soil show LC50–14 days above 1000 mg/kg. In the results obtained in the Seed Germination Test with *Lactuca sativa*, all the samples show low toxicity.

Toxicity tests on biological indicators of the environment can be considered a very useful tool in the technological sector since they help during the development of new products, from the beginning of the process to the end of it, directing the obtaining of safer products, minimizing thus the risk that its application may pose, both for humans and for the environment.

Therefore, this action to develop phytosanitary products of natural origin will preserve natural resources and improve the health standards and well-being of society.

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P20-50

Hypercoagulability and cardiac injury are mitigated after cessation of waterpipe smoke exposure in mice

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Waterpipe tobacco smoking has gained worldwide popularity in particular among youths. Several clinical and experimental studies have reported that waterpipe smoking (WPS) induces injurious effects to the cardiovascular system. However, the impact of smoking cessation (CS) on the cardiovascular toxicity induced by WPS received scant attention. Hence, we assessed, in C57BL/6 mice, the cardiovascular effects of WPS exposure for 3 months followed by 3 months of SC compared with mice exposed for either 3 months to WPS or air (control). WPS exposure induced hypertension, prothrombotic events both *in vivo* and *in vitro* and increased the plasma concentrations of endothelin-1, tissue factor, fibrinogen and plasminogen activator inhibitor-1 (PAI-1). These effects were significantly alleviated by SC. In heart tissue, the levels of troponin I, creatine kinase, lipid peroxidation, 8-isoprostane, tumor necrosis factor α , interleukin IL-6, DNA damage and cleaved caspase-3 were significantly increased by WPS exposure for 3 months. These effects were significantly reduced in the group of mice exposed to WPS for 3 months followed by 3 months of SC. Similarly, the increase in the level of nuclear factor κ - β (NF κ - β) induced by WPS exposure was significantly mitigated by SC. Immunohistochemical analysis of the hearts showed that WPS exposure for 3 months increased the expression of nuclear factor erythroid-derived 2-like 2 by cardiomyocytes. The latter effect was significantly reduced by SC. Taken together, our data show that SC is associated with amelioration of WPS induced hypertension, prothrombotic events and cardiac oxidative stress, inflammation, DNA damage and apoptosis.

P20-51

Strontium, Rubidium and Titanium in three marine species for human consumption: blue crab, sea bass & mussel

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Some chemicals, like mercury (Hg), lead (Pb) and cadmium (Cd), are clearly toxic and very popular in scientific publications. However, very few information is shared about other elements, even when they are considered as potentially toxic. Such is the case of Rubidium (Rb), Strontium (Sr) and Titanium (Ti), which can be relatively abundant in the environment. Current analysis methods can detect these elements quickly and efficiently in biological samples, which allows them to be quantified in tissues of different animal species. Some of these elements are present in the human body, being their functional role and adverse effects still unknown (Maynar *et al.*, 2017; Winkler *et al.*, 2018), despite they may reach the environment from numerous sources, such as several industries and nanomaterials.

For the present study, samples from three different species were analysed by inductively coupled plasma optical emission spectrometry (ICP-OES): (i) muscle from 48 blue crabs (*Callinectes sapidus*) and (ii) muscle from 83 seabass (*Dicentrarchus labrax*), (iii) as well as the soft tissues from 132 wild mussel (*Mytilus galloprovincialis*) collected from different locations in Murcia coast's areas (South-Eastern Spain).

The three chosen elements: Rb, Sr and Ti are detected in 100% of the samples, regardless of the species and sampling area. Similarly, the Rb concentrations are very similar in the three species and locations, while in the case of Sr, crab meat has the highest concentrations (58,340 $\pm$ 16,859¹ mg kg⁻¹, wet weight -ww-) and seabass meat the lowest (0,431 $\pm$ 0,356 mg kg⁻¹, ww). In the case of Ti, the invertebrates' concentrations (crabs and mussels, meat and soft tissue respectively) were very similar (crab: 0,234 $\pm$ 0,060 mg kg⁻¹, ww; mussel: 0,228 $\pm$ 0,049 mg kg⁻¹, ww), being almost 10 times higher than the concentrations found in the seabass meat (0,033 $\pm$ 0,012 mg kg⁻¹, ww).

According to our results, there seems to be a different tissue accumulation profile of Strontium and Titanium between the species. Given the ubiquitous presence of these elements in the environment (from industrial and natural sources) and the ignorance regarding their role and their functional or hazard profiles, we consider of interest the knowledge of these elements' concentrations in seafood.

¹ Geometric mean $\pm$ standard error

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P21 – Occupational toxicology

P21-01

Salt and Potash Miners exposed to Nitrogen Oxides and Diesel Exhaust – Assessment of Health Effects

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In Germany, Occupational Exposure Limits (OEL) for NO₂ and NO have been significantly reduced to 0.5 ppm and 2.0 ppm by the regulatory authorities in 2016. For Diesel Particulate Matter (EC-DPM) a new OEL has been set to 50 µg/m³ in 2017. While workplace conditions in underground salt and potash mining couldn't be adjusted easily and in short-term, this industrial sector is tremendously affected.

While regulatory guidance suggests that all industrial sectors must comply with the new OELs starting in January 2021, the timeline was not considered feasible in underground salt mining. Instead, a German mining company developed a 5-year transitional action plan to reduce exposures. To investigate if the delay would negatively affect workers' health, an epidemiological health study was conducted in collaboration with the mining company's Liability Insurance Association and a panel of occupational health scientists, epidemiologists and biostatisticians.

For each study participant, personal exposure measurements were collected during a single shift. Biomarkers of acute and chronic health effects (cardiovascular health, inflammation and immune response and respiratory health) were assessed before and after the shift through blood samples, lung function measurements and measurements of NO in exhaled air (FeNO).

Underground workers were divided into two subgroups, miners (n=801) and maintenance workers (n=202), and their exposure levels and health measures were compared to above ground workers (n=243). Separately for miners and for underground maintenance workers, associations between exposure to NO₂, NO and EC-DPM (divided into quintiles) and biomarkers of acute and chronic health effects were evaluated graphically and statistically in a series of Analyses of Covariance (ANCOVA). ANCOVAs were adjusted for age, height, weight and smoking status, and model assumptions were assessed.

Personal exposure measurements among underground workers exceed OELs: 19%, 33% and 56% of miners exceeded the OELs for NO₂, NO and EC-DPM, respectively. Still, the majority of biomarker measurements was within the reference ranges and the proportion of those were similar among all exposure groups (~80% to 100% depending on the biomarker). In addition, acute effect biomarkers were not statistically significantly different post- versus pre-shift in any of the three exposure groups and between these three groups. Further, graphical evaluations and ANCOVA results did not identify consistent associations between exposure quintiles and biomarkers of health effects for underground maintenance workers or miners.

The results of the present study did not indicate any hazardous acute effects on underground salt mining workers due to the exposure to nitrogen oxides or EC-DPM, although German OELs were currently exceeded in some areas of the mine. Hence, the study results suggest that underground exposures don't have a negative effect on the health of underground salt and potash miners.

P21-02

This abstract has been withdrawn.

P21-03

This abstract has been withdrawn.

P21-04

Influence of co-exposure to methylparaben and dibutyl phthalate used in chemical/cosmetic industry on caspase-3/7 activity in A431 cells

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Purpose: The aim of the study was to assess whether there are differences between caspases 3/7 activity in A431 cells after single and join exposure to methyl paraben and dibutyl phthalate which can co-exist in the work environment of the cosmetic or chemical industry.

Methods: The tests were conducted on the A-431 (ATCC® CRL-1555™) cell line, derived from *Homo sapiens* skin/epidermis. The cells were exposed to methyl paraben (MePB) (CAS 99-76-3) and dibutyl phthalate (DBP) (CAS 84-74-2) separately or in binary equitoxic mixtures in 1:3; 1:1 and 3:1 ratio. Concentrations of the test compound/mixture corresponded to experimentally determined IC₅₀ values (aliquots 1/4 and 1/10 IC₅₀). Caspases activity was evaluated using the fluorescence image cytometer NucleoCounter® NC-3000™. Based on fluorescence measurements, the percentage of cells without apoptosis, early- and late apoptotic, and necrotic features was calculated. The negative control were unexposed cells, while the positive control were cells exposed to 0.1 nM staurosporin (the concentration of the compound was predetermined experimentally before starting the essential experiments). Apoptosis rates were calculated for all examined groups according to the formula: apoptosis rate = (percentage of apoptotic cells / total cells) x 100%.

Results: The apoptosis rates obtained for the compounds/mixtures tested at concentrations corresponding to ¼ of the IC₅₀ values were significantly higher than for combinations at concentrations corresponding to 1/10 of the IC₅₀ values. Additionally, the rates of apoptosis calculated on the basis of the caspase activity in A431 cells after exposure to equitoxic mixtures of MePB and DBP turned out to be much higher compared with those obtained in the cells exposed to the tested compounds individually and higher than the predicted (theoretical) values calculated on the grounds of the composition of tested mix-

tures. This phenomenon was clearly observed especially after the cell exposure to the mixtures in the higher concentration tested.

Conclusions: The results showed that in A431 cells exposed simultaneously to MePB and DBP a synergistic effect in effector caspases activity could be observed by comparing the apoptosis rates calculated for single MePB and DBP administration and their equitoxic mixtures in 1:3; 1:1 and 3:1 ratio.

This paper has been based on the results of a research task II.PB.11 carried out within the scope of the fifth stage of the National Programme “Improvement of safety and working conditions” partly supported in 2020–2022 – within the scope of research and development – by the Ministry of Science and Higher Education /National Centre for Research and Development. The Central Institute for Labour Protection – National Research Institute is the Programme's main coordinator.

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Pesticides are a broad set of compounds used to control, repel or destroy pests, whose exposure is especially relevant among workers in different agricultural sectors. Oxidative stress associated with pesticides has recently been linked to nephrotoxicity in some studies. Therefore, the objective of this work was to determine if the degree of exposure to pesticides could be related to early kidney damage.

Blood and urine samples were collected at four time points throughout a year from volunteer worker women residing in Marinaleda (Seville), who were divided into two groups: Farmers (n=22), workers in charge of collecting agricultural products; and NOE (non-occupational exposed, n=17), workers in a cannery. A database was created with its main anthropometric and biochemical blood parameters, including their cholinesterase activities and classic renal function biomarkers (plasma creatinine and urea). Likewise, urinary protein levels and biomarkers of early kidney damage were quantified: N-acetyl-beta-D-glucosaminidase (NAG), kidney injury molecule 1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL). These urinary levels were compared with those of 16 healthy women (Control) unrelated to these work activities.

No significant differences were detected in the main anthropometric factors analyzed, and the biochemical parameters were within normality in the study groups. However, significantly higher excretions of the four urinary biomarkers evaluated were found in the Farmers and NOE groups, although without differences between them (except in KIM-1 excretion, which was higher in NOE group). In addition, significant correlations were found between some of these urinary biomarkers and blood cholinesterases (specifically, significant correlation coefficients were identified for proteinuria, KIM-1 and NGAL in the Farmers group; and for KIM-1 and NGAL in the NOE group). These results suggest the existence of a relationship between exposure to pesticides and the development of subclinical kidney damage. Carrying out a larger study to verify these findings opens the possibility of using early kidney damage markers as a preventive measure in the progression of kidney disease in these types of workers.

P21-06

Toward a better understanding of MWCNTs phagocytosis by alveolar macrophages.

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The main role of alveolar macrophages is the phagocytosis of exogenous particles including nanomaterials, in order to eliminate them and protect the organism against their adverse effects. However, the structure of some materials do not allow macrophages to productive phagocytosis, leading to a frustrated phagocytosis. This incomplete phagocytosis was described with asbestos fibres whose length is higher than the macrophage size. The fibrous shape of carbon nanotubes arouse concern, since some of them could be several µm long. In addition, it was shown that some carbon nanotubes could induce frustrated phagocytosis. This event leads to macrophage cell death, and therefore induction of pro-inflammatory cytokine release, which participate to the establishment of a persistent inflammation that can conduct to pulmonary disease development, such as cancer or fibrosis.

We treated rat alveolar macrophages, NR8383, with carbon nanotubes of different length and diameter. We analysed proliferation and cytotoxicity through real-time cell imaging and cell morphology by May-Grünwald Giemsa staining. We evaluated the expression at the

P21-05

Pesticide exposure and risk of early kidney damage

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mRNA and protein level of transmembrane receptors and pro-inflammatory cytokines. Cytologic approach shows morphology modifications, the presence of carbon nanotube potentially inside the cells, and the cooperation of cells to remove particles. Using molecular approach, we showed a modulation of transmembrane receptor and pro-inflammatory cytokine expression, with different intensities according to the length of carbon nanotubes.

P21-07

Cytotoxicity of carbon nanofibers with different physicochemical properties

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Carbon nanofibers (CNFs) are a type of fibrous carbon material that can be used in various applications. To accelerate their practical application, a hazard assessment of CNFs must be undertaken. Three bulk CNFs, with different physicochemical properties, were dissolved in 10 mg/mL bovine serum albumin solution using an ultrasonic mixer. The resulting supernatant was filtered through a cell strainer to remove large agglomerates. The filtrates were adjusted to a concentration of 1.0 mg/mL and used as CNF stock suspensions: CNF1, CNF2, and CNF3. CNF2 was shorter than CNF1 and CNF3 with respect to the geometric mean of the length distribution obtained from the transmission electron microscopy (TEM) images. While in terms of the geometric mean of diameters, CNF3 tended to be longer and thinner than CNF1 and CNF2. Stock suspensions were adjusted to a 10- to 1000-fold dilution series in the cell culture medium for use in *in vitro* cell-based assays for 24 h using rat macrophage NR8383. Cell viability, determined by the WST-1-based assay, decreased after 24 h exposure to 10 and 100 µg/mL CNF1 and CNF3. Cell viability also decreased after 24 h exposure to 100 µg/mL CNF2. There were no significant changes in intracellular reactive oxygen species (ROS) generation after exposure to 100 µg/mL CNF1, CNF2, or CNF3. These results show that CNF reduces cell survival without generating intracellular ROS. The levels of IL-1 α , MIP-1 α , IL-1 β , IL-18, and TNF- α were higher in NR8383 treated with CNF1, CNF2, and CNF3, compared to control cells. The levels of cytokines in NR8383 treated with CNF3 were significantly higher than other treatments and the control. In NR8383 treated with 100 µg/mL CNF-1, CNF-2, and CNF-3, cell damage was extremely high, and cell fragments that did not retain their morphology were observed by TEM. In cells treated with 10 µg/mL CNF1, CNF2, and CNF3, numerous CNFs were localized throughout the cells. Damage to cells treated with 10 µg/mL CNF3 was significantly higher than those of CNF1 and CNF2. Gene expression profiling of NR8383 exposed to 10 µg/mL CNF for 24 h was performed using DNA microarrays. A significantly higher number of upregulated and downregulated genes were identified in CNF3 compared to CNF1 and CNF2 ($P < 0.05$). Some representative genes involved in inflammatory responses, response to oxidative stress and apoptosis, and extracellular matrix degradation were significantly upregulated in CNF1 and CNF3. These results showed that CNF3, with long length and narrow diameter, had high cytotoxicity, and CNF2, with short length, had low cytotoxicity. The length and diameter of CNFs significantly contribute to their cytotoxicity. This study was supported by the Teijin Limited (Tokyo, Japan).

P21-08

Toxicity of stainless and mild steel particles generated from gas-metal arc welding in primary human epithelial cells from the small airways

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Purpose: Exposure to all types of welding fumes has been classified as carcinogenic to humans. There are indications that welding acts as a cancer promoter *in vivo*, but the molecular mechanisms have yet to be clarified. The aim of this study was to generate and collect stainless and mild steel particles generated via gas-metal arc welding and to evaluate their acute toxicity in primary human small airway epithelial cells. We also used the ToxTracker reporter stem cells to screen for potential activation of biological pathways of relevance for cancer development.

Methods: Metal content and size distribution of the fresh welding aerosol were thoroughly characterized by scanning mobility particle sizer, aerosol mass spectrometry, and particle induced X-ray emission. As cell models we used primary human small airway epithelial cells as well as mouse embryonic reporter stem cells (ToxTracker). Release of metal in cell media and cellular metal content was evaluated by inductively coupled plasma mass spectrometry; cellular localization of the particles was evaluated by electron microscopy. Agglomeration of particles in cell medium was evaluated by dynamic light scattering. Cell viability was measured by the Alamar Blue assay and generation of reactive species was quantified using the DCFH-DA assay.

Results: Metal content was relatively homogenous across particle size; however, there was an enrichment in Ni and Mo together with a lower percentage of Mn for the lower size fractions for stainless steel. Neither stainless or mild steel particles were cytotoxic in reporter stem cells after 24 hours but stainless steel particles activated the Nrf2-dependent oxidative stress pathway. Both particles induced time- and dose-dependent cytotoxicity after 24–72 hours exposure in primary human small airway epithelial cells, and stainless steel particles also increased generation of reactive oxygen species. The metal cellular content was more than 10 times higher for the human small airway epithelial cells compared to the reporter cells exposed to the same nominal dose. This was, at least in part, related to differences in particle agglomeration/sedimentation in the different cell media; higher agglomeration in serum free cell medium of the small airway epithelial cells.

Conclusion: Overall, our study strengthens *in vitro* evidence regarding the differences between stainless and mild steel welding particles, the former being more cytotoxic and inducing oxidative stress in primary human small airway epithelial cells. In addition, our data indicate that careful evaluation of cellular dose is critical for comparison between studies using different *in vitro* models and particles.

P21-09**Toxicity of two different biodiesel exhausts in a murine exposure model**

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Introduction/Aim: Biodiesel is promoted as a sustainable replacement for commercial mineral diesel. As global production increases and usage becomes more widespread, concerns have been raised over the health effects of exhaust exposure. As biodiesel fuel properties and thus exhaust components, change depending on the source oil, the aim of this study was to compare the effects of exposure to exhaust generated by the combustion of diesel and biodiesel made from two different oils, canola and tallow. Both of these biodiesel types are currently in use worldwide.

Methods: Adult male BALB/c mice were separated into acute (2hr exposure) or sub-chronic (2hr exposure daily for 8 days) groups and exposed to diluted exhaust from an engine running on diesel or biodiesel created from canola or tallow (or air as a control). Exhaust was characterised for toxic gases and particulate matter physico-chemical properties. Twenty-four hours after the last exposure health outcomes including lung function, responsiveness to methacholine and local and systemic inflammation were assessed.

Results: Exhaust gas composition varied significantly between fuels with diesel and tallow containing the highest levels of respiratory irritants including nitrogen oxides and carbon dioxide and canola the lowest. Particle size spectra also differed between exhaust types with both biodiesels displaying more intense peaks in particle concentration around the ultrafine particle size (<100 nm) but also less overall particle mass than diesel.

In the acute exposure groups, exposure to tallow and diesel exhaust resulted in increased responsiveness to methacholine ($p < 0.05$) compared with air control, suggesting a shift towards a more asthmatic phenotype. In contrast, after chronic exposure only tallow exhaust exposed mice displayed hyperresponsiveness whereas canola were less responsive than the air controls. This is indicative of tallow and canola biodiesel exhaust impacting lung function via different mechanisms.

Bronchoalveolar lavage cellular inflammation was significantly higher in mice exposed acutely to tallow or chronically to diesel or tallow ($p < 0.05$) compared with air controls. Higher protein content was also observed in the lavage of mice chronically exposed to tallow exhaust ($p < 0.05$), suggesting exposure induced epithelial damage. In contrast, local and systemic mediator release was decreased in comparison to air for all treatments, suggesting dysregulation and an inability for the mice to respond appropriately to exhaust exposure.

Conclusion: Tallow biodiesel exhaust exposure resulted in significant health impacts post-acute and chronic exposure. Chronic exposure to canola biodiesel suppressed typical exhaust exposure respons-

es, including reducing inflammation and responsiveness to methacholine. This suggests that oil type greatly impacts biodiesel exhaust toxicity and changes whether it is more or less toxic than diesel.

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P21-10**Metal enriched quasi-ultrafine particles from gas metal arc welding-stainless steel induced genetic and epigenetic alterations in BEAS-2B cells**

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Purpose: Worldwide, an estimated 11 million workers have a job title of welder and around 110 million additional workers probably incur welding-related exposures. Recent evidence has identified welding fumes (WF)-derived ultrafine particles (UFPs) as the driving force of their adverse health effects. However, UFPs have not yet been extensively studied and are currently not included in present air quality standards/guidelines. Here, attention was focused on the underlying genetic and epigenetic mechanisms by which the quasi-UFPs (q-UFPs, i.e., $\leq 0.25 \mu\text{m}$) of the WF emitted by gas metal arc welding-stainless steel (GMAW-SS) exert their toxicity in human bronchial epithelial cells (BEAS-2B).

Methods: Q-UFPs were produced by GMAW-SS by an automatic welding bench and collected by a cascade impactor. Their size distribution and zeta potential were studied by Zetasizer Nano ZSP™, and their metal composition by inductively coupled plasma-mass spectrometry. BEAS-2B cells ($n=5$) were exposed acutely (24 h) or repeatedly (3 x 24 h) to q-UFPs at concentrations ranging from 0.1 to 50 $\mu\text{g}/\text{cm}^2$ to study their cytotoxicity (ATP, luminescence). Thereafter, BEAS-2B cells were exposed to q-UFPs at 1.5 and 9 $\mu\text{g}/\text{cm}^2$ for acute exposure, and 0.25 and 1.5 $\mu\text{g}/\text{cm}^2$ for repeated exposure to study the other toxicological endpoints. Oxidative stress and inflammation were studied by nuclear factor erythroid 2-related factor 2 (NRF2) and nuclear factor-kappa B (NFkB) signaling pathway activation, glutathione status, oxidative damage (i.e., 8-hydroxy-2'-deoxyguanosine, carbonylated proteins, and 4-hydroxynonenal), and cytokine secretion (i.e., tumor necrosis factor- α , interleukin-1 β , and interleukin-6) by TransAM, RT-qPCR, and ELISA/MSD/Luminex. Primary DNA damages were studied by *in vitro* comet assay. Histone H3 acetylation (i.e., H3K9ac and H3K27ac), histone acetyltransferase (HAT) and deacetylase (HDAC) activities, and miRNA profiles were studied by ELISA and TaqMan® OpenArray® Human MicroRNA Panel. Other cell signaling pathways were studied by TaqMan® OpenArray® Human Signal Transduction Panel. Student t-test was used to look at statistical differences versus controls ($p < 0.05$).

Results: Q-UFPs showed a monomodal size distribution in number centered on $104.4 \pm 52.3 \text{ nm}$ and a zeta potential of $-13.8 \pm 0.3 \text{ mV}$. They were enriched in $\text{Fe} > \text{Cr} > \text{Mn} > \text{Si}$. Dose-dependent activation of NRF2 and NFkB signaling pathways, glutathione alteration, and DNA, protein and lipid oxidative damages were reported in BEAS-2B cells acutely, and to a lesser extent, repeatedly exposed ($p < 0.05$). However, no cytokine secretion nor primary DNA strand break were seen. Histone H3 acetylation and HDAC/HAT activity decreases were reported for any exposure ($p < 0.05$). Differentially regulated miRNA and mRNA

(FC<0.66 or >1.5, $p<0.05$) closely indicated the activation of some critical cell signaling pathways related to oxidative stress, inflammation, and cell cycle deregulation towards apoptosis.

P21-11

The importance of genotyping and phenotyping of glutathione S-transferases on risk assessment of exposure to volatile organic compounds

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Some volatile organic compounds (VOCs) used in shoe manufacturing processes are n-hexane, toluene, and xylenes. In addition to the cytochrome P450 isoform called CYP2E1, other enzymes involved in the biotransformation processes of VOCs are those belonging to the Glutathione S-transferases (GST) family, considered as part of the cellular defense against numerous chemical compounds. Within this family, three main polymorphic genes have been found (GSTP1, GSTM1, and GSTT1) leading to alterations in the catalytic activity of the enzymes for which they encode. The objective of this work has been to study the usefulness of GSTM1, GSTT1 and GSTP1 genotyping and phenotyping as a tool for the evaluation of the risks of occupational exposure to solvents in footwear workers and to determine the possible genotoxic damage induced by solvents exposure through the micronucleus test (MN). Urine, blood and oral mucosa samples were collected from 272 workers: 167 exposed and 105 non-exposed. The frequencies of GSTM1 and GSTT1 genotypes, in exposed and non-exposed, were similar, presenting a variability of around 13% for the GSTP1 (Ile/Ile) and (Ile/Val) genotypes. The results show an expression level of GSTP1 almost three times higher in the exposed group; for GSTM1 and GSTT1 the differences were not statistically significant. There is a statistically significant decrease in the excretion of 2,5-hexanedione, main metabolite of n-hexane, in workers with GSTM1 null genotype, since there is no enzymatic activity for that genotype. When we study the relationship between the genotype and expression levels for GST group, results are neither significant but they showed a lower expression level in GSTM1 and GSTT1 genes in null group and in homozygote Val/Val GSTP1. For women, we found significant differences between null and non-null individuals in the expression levels for GSTM1 ($t=2.26$ $p<0.05$) and GSTT1 ($t=4.48$ $p<0.001$), as well as for GSTT1 in the exposed group ($t=5.04$ $p<0.001$). We also found a positive relationship between alcohol and tobacco consumption with the expression level of GSTM1. In relation to the results obtained when we analyzed the MN rate, we found higher frequencies in GSTP1 Ile/Val and Val/Val and in GSTM1 and GSTT1 null individuals, compared to non-null and wild genotypes, although these differences are not statistically significant. We also found higher levels of MN in the null genotypes of GSTM1 and GSTT1 and the Ile/Val and Val/Val polymorphisms of GSTP1. These results suggest that these polymorphisms may contribute to chromosomal damage if they participate in innate metabolism or if the substrate is a genotoxin, which would potentiate various types of cancer.

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P21-12

This abstract has been withdrawn.

P21-13

Dye-based cell proliferation assay to assess proliferative responses of human lymphocytes to silica nanoparticles

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Introduction: Radioactive tritiated (^3H) thymidine lymphocyte proliferation test (LPT) is considered the gold standard test to measure the ability of lymphocytes to undergo proliferation when stimulated *in vitro* by a foreign antigen. However, dye-based cell proliferation assays using carboxyfluorescein diacetate succinimidyl ester (CFSE) dye, have several advantages over LPT, such as enabling the assessment of antigen-specific responses of lymphocyte subpopulations.

In this study we aimed to use the CFSE assay to assess lymphocyte proliferation to silica nanoparticles.

Methods: Peripheral blood mononuclear cells (PBMCs) from 4 patients with silicosis and 3 healthy subjects, labelled with CFSE, were stimulated with five dilutions (1/10) of silica particle suspensions, starting from 100 ug/ml. Two types of nano-silica were used: Ludox colloidal silica (L10) and synthetic amorphous silica, JRCNM02000a (NM-200). After 7 days of incubation, proliferation was assessed by stimulation index (SI). SI is defined as the ratio of proliferating cells (% divided) of stimulated to an unstimulated condition. The response was considered positive with SI > 2.5.

Results: We firstly noted that 100 ug/ml of both types of nano-silica induced cytotoxicity, and therefore were removed from further consideration.

Mean SI $\pm$ SD	Silicosis patients (n=4)			Healthy controls (n=3)		
	CD3+ & CD19+	CD3+	CD19+	CD3+ & CD19+	CD3+	CD19+
Ludox (ug/ml)						
10	7.36 $\pm$ 6.01**	2.73 $\pm$ 2.66*	12.9 $\pm$ 14.9**	0.78 $\pm$ 0.55	0.78 $\pm$ 0.79	1.13 $\pm$ 0.97
1	2.59 $\pm$ 1.05**	1.39 $\pm$ 0.43**	4.39 $\pm$ 2.28**	0.78 $\pm$ 0.38	0.74 $\pm$ 0.38	0.84 $\pm$ 0.52
0.1	1.85 $\pm$ 0.39**	1.40 $\pm$ 0.28**	3.32 $\pm$ 3.48*	0.72 $\pm$ 0.32	0.70 $\pm$ 0.36	0.78 $\pm$ 0.37
0.01	1.75 $\pm$ 0.69**	1.49 $\pm$ 0.93*	3.34 $\pm$ 1.53**	0.76 $\pm$ 0.33	0.70 $\pm$ 0.36	0.85 $\pm$ 0.58
NM-200 (ug/ml)						
10	5.23 $\pm$ 2.93*	1.84 $\pm$ 0.43	6.87 $\pm$ 5.15	1.25 $\pm$ 0.92	1.09 $\pm$ 1.03	1.37 $\pm$ 0.85
1	2.27 $\pm$ 0.55*	1.4 $\pm$ 0.24	3.59 $\pm$ 2.28	0.98 $\pm$ 0.57	0.86 $\pm$ 0.54	1.14 $\pm$ 0.71
0.1	2.19 $\pm$ 0.19*	1.64 $\pm$ 0.38	2.70 $\pm$ 2.16	0.45 [#]	0.53 [#]	0.48 [#]
0.01	1.84 $\pm$ 0.23*	1.32 $\pm$ 0.56	3.03 $\pm$ 1.81	0.66 [#]	0.3 [#]	0.34 [#]

* $p<0.05$

** $p<0.01$ as determined by two-tailed t-test between cohorts; SI > 2.5 in italics;

[#] Only one experiment performed

At lower concentrations of 0.01 µg/ml and 0.1 µg/ml, mean SI values for T (CD3+) and B (CD19+) cells combined were <2.5, indicating lack of proliferation. By contrast, positive responses (SI > 2.5) were observed among silicotic patients at higher silica concentrations (SI value for NM-200 at 1 µg/ml was only 2.27). Considering only B (CD19+) cells, positive SI values were found in silicotic patients across all concentrations.

Discussion: CFSE assay shows to be a useful technique as it allows assessment of cell subpopulation-specific SI and enables detection of reduced cell viability, thereby avoiding misinterpretation. We showed that exposure to nano-silica induced lymphocyte proliferation in silicosis patients unlike healthy controls.

P21-14

Occupational exposure to metals and PAHs: combining literature-based exposure and *in vitro* hazard data towards a mixture risk assessment

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The environment within industrial settings is commonly characterized by the existence of a complex mixture of chemicals from different raw materials and transformation processes. Occupational co-exposure to chromium (Cr), Nickel (Ni), and Polycyclic Aromatic Hydrocarbons (PAHs) may occur in diverse workplaces, such as the aeronautic and waste management, (e.g. incineration) sectors. Such co-exposure raises concern in terms of occupational health, as these substances are recognized lung carcinogens and mainly act by genotoxic mechanisms, increasing the likelihood of interactive toxic effects. The fact that current regulatory practices are usually focused on single chemical substances, without integrating the possibility of combined or aggregated exposures and effects, may lead to a risk underestimation.

In this work, developed under the scope of HBM4EU Initiative (<https://www.hbm4eu.eu>), a literature-based mixture risk assessment (MRA) exercise for occupational exposure to metals and PAHs was performed. In addition, *in vitro* toxicity data was obtained for the same mixtures to provide support to its hazard assessment.

Human biomonitoring (HBM) data on Cr(VI), Ni and/or PAHs was extracted from occupational studies conducted in the European Union and searched in literature databases. Selected reference values were used to calculate risk quotients (RQ) for each substance based on the retrieved exposure data; the combined risk was given by the sum of the RQ, i.e., the Background Exposure Exceedance Score (BEES).

In parallel, we evaluated the combined cyto- and genotoxicity of the same chemicals (assessed by the MTT and micronucleus assays) in the human alveolar A549 cell line.

In most of the analysed studies, we observed that BEES levels, estimated from the exposure to metals mixture or to metals and PAHs, exceeded RQ levels considered acceptable for the individual substances. Only two studies, conducted in hazard waste incinerator settings, presented urinary exposure levels for the three substances. They showed a value of BEES of concern (>1) for all exposure scenarios, even for workers performing activities considered of low exposure or no-exposure, such as laboratory and administrative workers. *In vitro* assays supported that A549 cells exposure to these substances resulted in interactive cytotoxic and genotoxic effects that may underlie health effects different from those predicted from single exposures.

Our findings show the limitations of applying occupational exposure reference values defined on a single substance basis to workplaces where exposure to chemical mixtures occur, highlighting the relevance of performing MRA as a more realistic approach to guide suitable risk management measures in occupational settings.

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P22 – Regulatory toxicology (REACH)

P22-01

Reproductive toxicity of Zenolide using analogues and comply with RAAF

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Zenolide is a macrocyclic musk, which needed REACH-Annex IX registration being marketed at 100–1000 tons. Reproductive toxicity was not anticipated based on its features. It consists of a C12-dicarboxylic acid (DDDA) connected with Ethylene glycol (EG) with two ester bonds. To fulfil the REACH requirements for reproductive toxicity, reproductive organ information from a 90-day study can be used in combination with developmental toxicity, when these are sufficiently similar to OECD TG testing. To prevent animal testing, analogues can be used when the requirements of Annex XI are fulfilled. The purpose of this presentation is to show that analogues can predict the reproductive toxicity of Zenolide. To begin with, a 28-day study of Zenolide is available, in which reproductive organs are assessed, showing no effect acids and alcohols. Therefore Zenolide is unlikely present in the systemic circulation. For the acid, DDDA, a Reproscreen study shows no effects >= 1000 mg/kg bw. EG has a 90-repeated dose study showing a NOAEL of 150 mg/kg bw and no effects on reproductive organs and for developmental toxicity the NOAEL is 1000 mg/kg bw via dietary testing. Literature data on metabolism show that esters breakdown into the respective acid and alcohol. Moreover, a similar macrocyclic musk (MCM) shows no developmental toxicity and other repeated dose and reproductive effects: all NOAELs >= 1000 mg/kg bw. Based on these data the conclusion was that Zenolide is not toxic for fertility and developmental effects, meeting the requirements and saving 1200 animals. However, according to ECHA RAAF guidance, Zenolide metabolism data is needed to support the read across to its metabolites. Also a bridging study is needed to support the read across with its analogue MCM. To meet these requirements experimental work on the breakdown of Zenolide was performed as well as a Reproscreen study. The metabolism study showed that the

first ester bond cleaved within 1 minute and the second one had a DT50 of 20 minutes, supporting that Zenolide does not exist in the systemic circulation. This outcome was also derived from the Repro-screen study; oxalic acid crystals in the kidneys were found which can only be formed by complete metabolism of Zenolide to form EG. Further literature study revealed that EG developmental toxicity rat human conversion result in a developmental toxicity value $\gg 1000$ mg/kg bw. The fertility and developmental NOAELs of Zenolide remained ≥ 1000 mg/kg bw, the repeated dose toxicity NOAEL was decreased from 620 to 300 mg/kg bw because oxalic crystals were seen in Wistar but not in Sprague Dawley rats in the former 28-day study. The Annex IX registration requirements were updated accordingly using ca half of the animals as when not using read across at all.

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P22-02

Toxicological assessment of highly mentholated reduced-risk tobacco products in Sprague Dawley rats following sub-chronic inhalation exposure

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Menthol is a flavor that is commonly used additive in many products, including conventional cigarettes and other inhaled products (e.g. e-cigarettes and heated tobacco products). It is therefore important to assess potential toxicological effects of inhaled menthol in the context of heated tobacco products. This study characterized the toxicity related to highly mentholated (ranging from 1.3 to 4.2 mg menthol/stick) Tobacco Heating System (THS) compared with fresh air control, non-mentholated THS as well as two conventional reference cigarettes (3R4F and 1R6F) in a sub-chronic inhalation exposure study using Sprague Dawley rats following the OECD Test Guideline 413. Three concentrations of 'THS high menthol' aerosols (15, 23 and 50 µg nicotine/L) were delivered by nose-only exposure (6 hours/day, 5 days/week for 13 weeks) to the rats, while additional rats of selected groups were included to observe reversibility of any effects after 42 days post-exposure period. All test atmospheres were well tolerated by the rats. Respiratory minute volume and breathing frequency (both sexes) increased in a concentration-dependent manner in response to menthol levels up to a certain level (THS high menthol), after which the values for these parameters started to decrease. Pulmonary inflammation, determined by quantification of free-lung cells, and of inflammatory cytokines in cell-free bronchoalveolar lavage fluid, were lower in the THS (mentholated or non-mentholated) exposure groups compared to the conventional reference cigarettes smoke, and without any effects of menthol, which was confirmed following histopathological evaluation of the lungs, showing minimal inflammation in the THS-exposed rats. Histopathology of respiratory organs paralleled these observations and further confirmed minimal sub-chronic effects for THS groups, which is consistent with lower content of irritants in the aerosols. Microscopic findings in respiratory tract organs including epithelial cell hyperplasia and squamous metaplasia were reduced in THS-exposed groups as compared with conventional reference cigarettes groups at equivalent nicotine concentration. Findings in the non-respiratory tract organs were considered generally low incidence and severity, while exposure-related effects were not observed in the THS-exposed groups. Clinical pathological changes following exposure to aerosols of THS, such as high-

er blood neutrophil counts, elevated liver enzymes and lower energy metabolism parameters were observed, all of which reversible following recovery period, and likely represent a nicotine-effect. In general, only minimal biological effects were seen following exposure to the mentholated THS aerosols. Despite the high levels of menthol in the aerosols (equivalent up to 116 mg nicotine and 360 mg menthol per day based on human body surface area), there were no additional toxicity effects compared with conventional reference cigarettes or non-mentholated THS.

P22-03

This abstract has been withdrawn.

P22-04

Toxicological assessment of total particulate matter generated from a tobacco heating product relative to cigarette smoke using *in vitro* mutagenic and genotoxic assays

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Tobacco heating products (THPs) represent a subset of potentially modified risk products, in which tobacco is heated at temperatures up to 350°C, significantly reducing toxicant emissions relative to cigarette smoke. Although Total Particle Matter (TPM) from THPs have previously been tested *in vitro*, these evaluations have in some cases not met the OECD Test Guidelines requirements in terms of the top concentration for *in vitro* toxicological assays, thereby opening opportunities to explore significantly higher concentrations, relative to cigarette smoke TPM.

The objectives of the study were to: (a) investigate the effect of increasing the concentrations of THP TPM > 3 fold greater than cigarette TPM; and (b) assess the biological impact of potential hazards of a novel THP prototype at these enhanced concentrations and comparing results to a reference 1R6F cigarette and a baseline THP.

TPM from THPs and 1R6F, were generated under Health Canada Intense smoking conditions (55ml puff volume over 2 sec and 30 second intervals), with the exception that the THP consumables did not have their ventilation holes blocked. The TPM was captured on Cambridge filter pads and extracted with dimethyl sulfoxide. 1R6F-TPM was extracted at 24 mg/mL and THP-TPM extracted at 150 mg/mL. A suite of *in vitro* toxicological assays were used to assess the mutagenic potential of the TPMs, including the Ames test (5 tester strains, $\pm S9$; OECD 471), the mouse lymphoma (MLA) assay (L5178Y cells, 3h $\pm S9$ and 24h-S9; OECD 490) and the *in vitro* micronucleus (IVMN) assay (V79 cells, 3h $\pm S9$ and 24h-S9; OECD 487). The neutral red uptake assay (NRU) provided an acute measure of cytotoxicity (Balb/c 3T3 cells, $\pm S9$ OECD 432). All testing was conducted according to Good Laboratory Practice.

In the Ames test, 1R6F-TPM induced reproducible statistically significant ($p \leq 0.01$) concentration-dependant increases in revertant colonies in strains TA98, TA100 and TA1537 +S9 in three independent experiments. In the MLA, in each treatment condition, 1R6F-TPM induced increased mutation frequency, exceeding the global evaluation factor (GEF), and a reproducible statistically significant linear trend in two independent experiments. Similarly, dose-related increases in the proportion of MNB (micronucleated binucleate) cells were observed in the IVMN assay for 1R6F-TPM and it was cytotoxic in the NRU assay. The TPM from the THPs did not induce any concentration related increase in the number of revertants in the Ames test when tested up to 5,000 µg/plate and were not cytotoxic in the NRU assay. Concentration related increases in mutation frequency in the MLA were observed, but they did not reproducibly exceed the GEF. THP-TPM showed a reduced response in IVMN assay. The results dem-

onstrate that the TPM from these THPs, are considerably less mutagenic, genotoxic and cytotoxic than cigarette smoke and suggests THPs are potentially reduced risk products relative to cigarettes.

P22-05

In vitro toxicity assessment of paints with antimicrobial properties – a comparative study

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In this work, the *in vitro* cytotoxicity of self-disinfecting wall paints containing antimicrobial substances was assessed, using skin and lung cell lines.

Self-disinfecting surfaces have appeared as an alternative to common cleaning and disinfection protocols applied in different scenarios. These surfaces are even more trend nowadays due to the Covid-19 pandemic. We developed a wall paint formula with antimicrobial properties to be applied in areas with high propensity for infection spreading. To do so, substances with known activity against microorganisms were incorporated on a commercial wall paint. Both paints containing Bacitracin (0.6 g/L) or Colophony (3.2 g/L) showed good antimicrobial activity against several bacteria, namely *Staphylococcus aureus* and *Escherichia coli*. However, an important step of our work is to assure these surfaces' safety, both for people contacting with it and for workers handling the products.

Following ISO 10993¹, direct contact and extracts tests were performed. The surfaces were placed in direct contact with *in vitro* cell cultures of HaCaT skin cells for 24h at 37°C, 5% CO₂. In parallel, the surfaces were leached in culture media and their extracts at several concentrations were exposed to HaCaT cells and A549 human alveolar epithelial cells, for 24h at 37°C, 5% CO₂. Then, neutral red uptake (NRU), cell proliferation reagent WST-1 and lactate dehydrogenase activity (LDH) assays were performed, both on direct contact and extracts tests, for quantitative evaluation of cytotoxicity.

For direct contact tests on HaCaT cells, both surfaces containing Bacitracin or Colophony showed cell viabilities of around 90%, with NRU and WST-1 showing similar results. LDH release was around 25% for both surfaces.

Regarding the tests with the extracts on HaCaT cells, cell viability fluctuated between 85–70% for Bacitracin and between 80–90% for Colophony, according to the extract concentration. A proportional response was detected when decreasing the concentration of extract. LDH release was around 5–15% for Bacitracin and around 5–20% for Colophony. On A549 cells, the test on extracts demonstrated a cell viability of 100% for both surfaces and a LDH release of 15–25% for Bacitracin and 10% for Colophony.

These results suggest that the extract forms of the Bacitracin and Colophony are more toxic to HaCaT cells comparing to the direct contacting surfaces containing the same compounds, however, with lower LDH release levels.

Comet assay, measuring DNA damage is being performed to further evaluate the formulas' toxicity.

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P22-06

Toxicology brought to justice: Risk assessments and liability assessments related to high levels of PFAS

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In December 2013 high levels of PFAS was found in the drinking water in Ronneby, Sweden. The levels of PFAS (sum of 11) reached 10380 ng/L (for comparison the EU drinking water directive limit value is 100 ng/L). It was soon confirmed that the nearby military airport localised within the aquifer area had leached PFASs to the environment. Extensive biomonitoring in the municipality population started in June 2014 and revealed high levels in serum [1]. Median concentrations were PFHxS, 277 ng/mL (range 12–1660); PFOS, 345 ng/mL (range 24–1500); and PFOA, 18 ng/mL (range 2.4–92).

Since then, the legal rounds have been many, before the case was finally taken up in Blekinge District Court in February 2021 in a civil court trial. The question was whether Ronneby municipality has any liability towards the victims 165 exposed residents. The municipality that are responsible for drinking water argue that the right to damages should be examined individually and that the persons should be able to show a clear connection between the intake of PFAS and their personal illness.

Regulatory tools that the protection of drinking water could be broadly divided into two approaches: limit/guidance values determining allowable exposures (preventive risk assessment), and in cases where an exposure has occurred, of the right to compensation (reactive risk assessment). The basis for both regulatory systems is toxicological and epidemiological data, but there is a lack of knowledge of how the concepts of risk and uncertainty are operationalized before and after exposure.

The verdict given in April 2021 stated that Ronneby municipality should compensate the plaintiffs for personal injury in the form of elevated levels of PFAS in the blood, which means increased health risks and physical changes and deteriorations of the body. In the verdict from the district court, the WHO definition [3] of adverse effects is used for the first time in a Swedish court, i.e. a change “that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences.” The judgment has been appealed to the Court of Appeal.

We have reviewed the role of different types of risk assessment methodology and concepts in this court case, with a focus on toxicological and legal issues regarding risk, causality and harm. The commonly used approach of comparing the estimated exposures (in this case serum levels) to a guidance value estimated to be without appreciable health effects played a major role in the court case. An alternative to this deterministic assessment is probabilistic risk assessment [2]. We argue that this would be particularly useful in situations like the Ronneby case, when a deterministic (point estimates) risk assessment indicates that guidance values are exceeded or when significant equity or environmental justice issues are raised by inter-individual variability.

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P22-07**Collection and initial analysis of evidence to assess endocrine disrupting properties of Bisphenol F**L. Wiklund, ^{*}A. Beronius*Karolinska Institutet, Institute of Environmental Medicine, Stockholm, Sweden*

Identification and assessment of endocrine disruptors (ED) to minimize risks to human health and the environment is an important regulatory issue that is for example highlighted in the European Commission's recent Chemicals Strategy for Sustainability Towards a Toxic-Free Environment^[1]. The Commission is working on different actions aiming for a horizontal approach to the identification of endocrine disruptors across legislations, building on the scientific criteria implemented for biocidal products (BPs) and plant protection products (PPPs) in 2018^[2]. The aim of this study was to evaluate the available evidence on endocrine disrupting properties of the non-pesticide bisphenol F (BPF) following the ECHA/EFSA Guidance for identifying EDs under BP and PPP regulations^[3]. BPF is produced or imported at 0–<1 ton/year in the EU and is therefore not registered under REACH. Still, it is detected in urine, blood, and breast milk samples in the European population. BPF is a bisphenol A analogue, which raises concerns regarding its potential ED properties. A structured literature review and weight of evidence assessment was applied, incorporating systematic review methodology to retrieve all relevant evidence for BPF in the open literature and databases. Studies were selected based on predefined inclusion/exclusion criteria and all relevant data were extracted, using the ECHA/EFSA template. The Science in Risk Assessment and Policy (SciRAP) tools were used for data reliability evaluation. An initial analysis of the evidence was conducted to conclude on the endocrine-related adversity and endocrine activity of BPF. Our study found that the available mechanistic evidence of BPF was sufficient to evaluate an endocrine mechanism. Numerous *in vitro* studies, together with some *in vivo* and zebrafish mechanistic data, consistently showed that BPF can bind to estrogen and androgen receptors, as well as interfere with steroidogenesis. However, due to a lack of systemic toxicity investigations and low reliability of the available *in vivo* data, endocrine-related adversity of BPF was not considered sufficiently investigated. Nevertheless, some indications of effects on sperm parameters and uterus weight in rats were identified. The study demonstrates the application of systematic methods to retrieve, evaluate and integrate data from the open literature and databases for ED assessment according to the EU scientific criteria and ECHA/EFSA guidance. Our results highlight challenges of assessing chemicals that are suspected EDs based on structural similarities to other substance but for which there are no regulatory testing requirements. This study also underscores the importance of sufficient reporting of studies published in the open literature and the continued development of new approach methodologies that provide relevant mechanistic data to support the assessment of ED properties of chemicals.

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P23 – Risk prediction and assessment/risk assessment using new approach methodologies**P23-01****Development of research algorithm and safety assessment approaches for novel food sources obtained with the use of insects**^{*}S.I. Shestakova, N.V. Tyshko, E.O. Sadykova, N.S. Nikitin, M.D. Trebukh, M.S. Loginova*Federal State Budgetary Scientific Institution "Federal Research Centre of Nutrition, Biotechnology and Food Safety", The laboratory of biotechnologies safety assessment and novel food sources, Moscow, Russia*

In accordance with the Food Security Doctrine of the Russian Federation, approved by Presidential Decree N120 of 30 Jan. 2010, the country's food independence is one of the priority tasks of the state, in which sustainable development of agricultural production takes first place. The main element in the modern agro-industrial complex formation is the use of innovative technologies which allow to increase a productivity significantly and to use of the most environmentally and economically profitable ways of farming in the conditions of reducing of natural resources dependence.

In recent years, the worldwide interest in the use of alternative sources of protein, in particular insect protein, has been increased. Since insects are a new type of food that has not been used for human consumption before, the safety ensuring of such products is regulated at the state level and is the most important condition for their use. In accordance with Technical Regulation of the Customs Union 021/2011 "On food safety", food safety is defined as "...the state of food, indicating the absence of unacceptable risk associated with the harmful effects on humans and future generations ...", so the search for ways to improve the methods of novel food safety assessment should be focused on approaches that allow to study the impact on future generations.

In consideration of the increased interest of Russian producers in insects as a source of food and feed ingredients (several manufacturers grow insects for feed purposes), it is necessary to initiate the development of the state registration rules for such products based on comprehensive medical and biological research, which guarantees the safety of novel food for consumers.

The aim of the project is to develop an original algorithm for safety assessment of novel food obtained from insects, for which a comprehensive study of nutritional value and safety in *in vitro* and *in vivo* experiments will be carried out. The research material will be dry biomass of Black soldier fly larvae (*Hermetia illucens*).

The implementation of the project involves two successive steps, each of which includes a detailed study of the product. An extended list of safety indicators characterizing the biomass will also be developed. At the second stage, it is planned to conduct complex toxicological, reprotoxicological and allergological studies of dry biomass *in vivo*, as well as to study the biological value and digestibility of protein on 3 generations of Wistar rats, which will receive the product under study during the entire period of ontogenetic development. The whole duration of the second stage will be about 2 years.

This project is directed on approaches formation for food insects safety assessment that will result the draft of the methodical guidelines with the list of obligatory research for the state registration of novel food.

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P23-02

Is it time to update the EU Drinking Water Directive based on scientific data on pesticides?

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Introduction: In the European Union (EU) Directive 80/778/CEE set a concentration limit for pesticide active substances (PAS) of 0.1 µg/liter, or 0.5 µg/liter for multiple substances. There is no background or official document that explains the rationale for such limit. The limit has remained unchanged in all revisions of the Drinking Water Directive, including the last proposal. This despite the fact that toxicological and ecotoxicological information of PAS has greatly increased that would allow for an individualized risk based definition of concentration limits.

Methods: This limits apply to both surface and ground-water that may be intended for human use. We derived health-based water limits for PAS and compared them with the legal limit. First we identified the acceptable daily intake (ADI) for over 400 PAS, allocated 10% of the ADI to intake from drinking water and assumed the standard water intake of 2 liters/day for a 60 kg adult. ADIs were taken from the European Food Safety Authority (EFSA) for those PAS that are/have been authorized in EU. Regarding ecotoxicological effects, a comparison was made with the Regulatory Acceptable Concentrations (RAC) established by EFSA in the last 10 years, in order to have values derived by the current methodology.

Results: Regarding the toxicological assessment, the health-based limits for PAS authorized in EU (N=312) would be lower than 0.1 µg/liter for one compound (bromadiolone), whereas it was up to 10, 100, 200, 500, 1 000, 10 000, and >10 000 times higher for 1, 27, 20, 63, 56, 45, 74, and 25 PAS, respectively. In addition, based on monitoring data in Italy we estimated a health-based mixture limit derived assuming, as a worst case, additive effects for the 8 molecules most frequently found: this limit would be 178 times higher than the 0.5 µg/liter legal limit. Regarding ecotoxicological risk (N=170) the RAC was found to be >100, up to 100 and up to 10 times lower than 0.1 µg/liter for 5, 3 and 23 PAS, respectively. It was up to 10, 100, 1 000, 10 000, 100 000 and >100 000 times higher than 0.1 µg/liter for 40, 50, 27, 15, 6 and 1 PAS, respectively. **CONCLUSIONS.** PASs stand out when compared to all other chemicals in the EU legislation because their limits in drinking water are not health based but are set to the same level for all irrespective of their toxicological characteristics. This level is very low, much lower than that for other chemicals such as arsenic (carcinogen, with a limit of 50 µg/liter), lead (50), chromium (50), cadmium (5) or mercury (1). As a consequence, water supplies may be shut down for exceedances of the limit for PAS that would be without any health consequences for the consumer, or PAS may not be authorized or their use strongly reduced because of modeled, or more rarely measured, exceedance of the limit. In both cases, costly actions with no, at best, benefit to human health. It cannot also be excluded that this limit might be too high for certain PAS.

P23-03

Safety assessment features of novel insect-derived food sources: experimental diets

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The search of alternative food sources and ensuring the possibility of usage for the population feeding is critical to humanity's recovery from the impending food crisis.

In recent years the worldwide interest to the alternative protein sources use, in particular insect proteins, has been increased. Since insects are novel food that have not previously been eaten by humans, the safety of such products in the Russian Federation is regulated at the state level. The creation of the safety assessment system for insects-derived food, proof the effectiveness and use the system in the framework of the state registration procedure for such food is an important scientific task.

Since the solution of the problem implies the research of the alimentary factors influence in the *in vivo* experimental studies, the formation of diets which provides an adequate macro- and micronutrients supply, and allows to include the maximum possible amount of the studied product, is an extremely important aspect.

The semi-synthetic casein diet (SCD) for laboratory animals, developed by the Federal Research Centre of Nutrition, Biotechnology and Food Safety^[1] and widely used in toxicology studies since 2009, has all required characteristics. This diet was formed on the basis of comparative assessment of the diet AIN93 [2] and the previously used by the FRC of nutrition SCD. The main components of the new diet are casein (23.9 g/100 g of the diet), responsible for the protein component, maize starch (59 g/100 g) – carbohydrate component, sunflower oil (5.0 g/100 g) and lard (2.0 g/100 g) – fat component. The diet also includes L-cysteine (0.2 g/100 g), choline (0.25 g/100 g), tret-Butylgidoquinone (0.001 g/100 g), microcrystalline cellulose (5.0 g/100 g), salt mixture (3.5 g/100 g), a mixture of water-soluble and fat-soluble vitamins (1.0 and 0.1 g/100 g, respectively). The salt mixture and the mixture of vitamins are similar to AIN93. The total caloric value of the diet is 358.4 kcal/100 g.

In the formation of experimental diet the primarily issue is a determination of the limiting factor, which is used to calculate the maximum amount of the test product that can be included in the diet without changing the chemical composition and caloric value. Thus, in case of inclusion in SCD the Black soldier fly (*Hermetia illucens*) dry biomass, that contains 37.9 g of protein and 37.3 g of fat per 100 g of biomass, the limiting factor will be fat, so the maximum possible amount of biomass per 100 g of diet will be 20 g, which corresponds to 20% of the diet. This amount of biomass contains 7.6 g of protein, so the equivalent amount of casein should be excluded from SCD. Therefore, 100 g of the experimental diet containing dry biomass of Black soldier fly will include 12.7 g of casein protein, 0.5 g of vegetable and 1.7 g of animal fat, 20 g of biomass (7.6 g of protein and 5 g of fat).

This work was supported by Russian Science Foundation grant 20-16-00083.

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P23-04**Rethinking carcinogenicity assessment for agrochemicals**

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For the past 40 years, questions have been raised about the relevance and regulatory utility of rodent cancer bioassays in human health risk assessment. As a result, a working group of experts from different sectors have formed the Rethinking Carcinogenicity Assessment for Agrochemicals Project (ReCAAP) to determine the appropriateness of and criteria for waiving rodent cancer bioassays for the registration of food-use pesticides. A weight of evidence (WoE) reporting framework, which outlines a suggested assessment of publicly available information, was used to draft carcinogenicity study waivers to determine if sufficient information was available to perform a health protective chronic risk assessment without conducting rodent cancer bioassays. Information used in the WoE include exposure, mode-of-action, physiochemical properties, metabolism, and sub-chronic toxicological data from standard risk assessment endpoints. Using this framework, ReCAAP evaluated 15 pesticides registered over the past ten years with the chemical distribution spanning 10 tumor types, 15 chemical classes, and six cancer classifications (including subclasses). The reporting framework criteria and example carcinogenicity waivers will be presented. This effort has established criteria for when the mouse and/or rat cancer bioassay can be waived while ensuring that pesticide human health risk assessments are protective.

P23-05**Virus Response Measures in Case of Infection with a Previously Unknown Virus or COVID-19 in Employees at Research Facility**

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, the virus responsible for coronavirus disease 2019 commonly known as COVID-19) was first identified in November 2019, and caused outbreaks in China, South Korea, Japan, and Thailand in January 2020. Shortly afterwards in March 2020, the World Health Organization (WHO) recognized the global COVID-19 outbreak as a pandemic (Reuters, Geneva, March 11, 2020). The new coronavirus is of the same type as the ones that caused SARS and the Middle East respiratory syndrome (MERS). However, it is more infectious than the other coronaviruses that have been identified thus far, as is evident from the rate at which the number of cases multiplied within a short period of time.

Given such a situation, both public and private institutions are making efforts for protection against the infection. Infection prevention measures are necessary, and research facilities that are workplaces of scientists are no exception. Research facilities in general already have adequate infection prevention measures in place in the operating areas. Advanced prevention is followed especially to pre-

vent the transmission of infections from laboratory animals to humans, or vice versa. In addition, there are strict international rules for the handling of biological samples, such as blood and organs. Nevertheless, there is currently no defined guideline for the prevention of human-to-human transmission within research facilities, or for an emergency response in case of confirmed infection with an unknown virus, among the employees. This report aims to present suggestions for possible measures to be implemented in the event of an infection with a highly infectious virus such as SARS-CoV-2, or an unknown bacterium or virus, within the research facilities.

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P23-06**Adsorption of horseradish peroxidase on metallic nanoparticles – effects on reactive oxygen species detection using 2'-7'-dichlorofluorescein diacetate**

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The fluorescent probe 2',7'-Dichlorofluorescein Diacetate (DCFH-DA) together with the enzyme Horseradish peroxidase (HRP) is widely used in e.g. nanotoxicology to study reactive oxygen species (ROS) production by nanoparticles (NPs). ROS has been shown to correlate to toxicological effects such as membrane damage, DNA damage and reduced cell viability.

This study examined interactions between HRP and metallic NPs of Mn, Ni and Cu in saline, PBS and DMEM (Dulbecco's Modified Eagle Medium). Previous studies show the possible inactivation of HRP and the self-initiation of DCFH-DA at excess H₂O₂ concentrations. Interactions not studied include adsorption of HRP onto metallic NP surfaces and influences on released metals. These are aspects of importance since the activity of HRP can be altered if adsorbed to the NPs, interacts with released metals, and/or influence the release rate.

HRP adsorption onto NPs of Cu, Mn and Ni was studied using Attenuated Total Reflection Fourier Infrared Spectroscopy (ATR-FTIR) and UV-VIS Spectroscopy. The extent of released metals after 1 h exposure at 37°C in the different fluids was determined using Atomic Absorption Spectroscopy (AAS) with a graphite furnace accessory. Metal speciation predictions in solution were performed in parallel using chemical equilibrium speciation calculation software (JESS and Visual MINTEQ). Nanoparticle Tracking Analysis (NTA) was employed to study possible agglomeration of HRP due to metal ion release, and Fluorescence Spectroscopy to detect ROS using the DCFH-DA assay with and without the addition of HRP.

The metal NPs interacted differently with HRP in terms of adsorption and release of metals. From these variations in HRP-particle interactions follow an evident difficulty in the applicability of HRP to assess ROS production. Adsorption of HRP is reported to reduce the HRP activity. No adsorption of HRP took place on the Mn NPs in PBS while HRP adsorbed to both Ni NPs and Cu NPs in all fluids. The adsorption of HRP on the Cu NPs was higher in saline compared with PBS. Due to the high pH sensitivity of HRP, changes in solution pH as

a result of extensive metal release will result in an additional uncertainty of detected ROS. Small effects on pH were observed in this study. Since the buffer capacity of PBS is not sufficient to handle the addition of the alkaline DCFH solution, any pH adjustment before particle addition and ROS measurement clearly influence the results, both with and without HRP in the system. Increased concentrations of released metals did not induce any evident HRP agglomeration.

In all, the ROS measurements using the DCFH-DA assay combined with HRP on reactive metal NPs (Cu, Mn, Ni) in different biologically relevant synthetic fluids clearly elucidate that artefacts are introduced as a result of HRP-surface and metal release interactions. This may result in erroneous interpretations of ROS production from metallic NPs.

P23-07

Photosafety evaluation during drug development – how to use safety margins in support of human risk assessment

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Phototoxic properties of systemically applied pharmaceuticals may be the cause of serious adverse drug reactions. Despite being clinically manageable in principle, they can limit the use of a drug depending on the indication. Protective measures against sunlight can be applied very reasonably during a few days but may not be practicable for chronic treatments. Thus, both patients and health authorities are unlikely to accept a relevant photosensitization risk in such situations.

Typically, a reliable preclinical photosafety assessment strategy combining *in vitro* and *in vivo* approaches is usually applied early on. For most drug candidates, photosafety evaluation can be based purely on spectroscopic measurements and *in vitro* results (Bauer, Regul Toxicol Pharmacol, 2014). However, a few compounds will need confirmation *in vivo*. Such studies involve multiple dose levels covering the pharmacologically efficacious dose and the maximal tolerated dose with the aim to define the no-observed adverse events level (NOAEL) and to derive critical PK parameters (i.e. T_{max} , C_{max}).

The results from 41 acute, oral murine photo-local lymph node assays (photo-LLNA) demonstrated the utility of this approach (Schumann, Toxicol Sci, 2014). Phototoxicity *in vivo* is clearly a dose-dependent effect. The applied level of simulated sunlight (normalized to 10 J/cm² UVA) is sufficient to elicit phototoxic responses using reference compounds and corresponds well to typical sunlight exposure of patients. Therefore, NOAEL-derived safety margins versus therapeutically relevant drug levels based on C_{max} are an appropriate method to support human risk assessment and are regulatory accepted (ICH S10, 2013).

Historically, in particular antibiotics such as fluoroquinolones or tetracyclines were extensively evaluated regarding clinically relevant photosensitivity reactions. However, during the last 20 years the majority of pharmaceutical companies have introduced preclinical photosafety testing strategies in order to avoid late stage surprises. Therefore, clinical photosensitivity testing of investigational drugs is not performed on a routine basis and only close to clinical phase 3 development trials due to the required exposure levels. The outcome of such studies is not published systematically. However, two recent cases demonstrated the relevance and utility of safety margins based on preclinical data. Vemurafenib was found clinically phototoxic at therapeutic exposure levels. *In vitro* and *in vivo* results were suggestive of this outcome (Boudon, Toxicol Sci, 2014). In contrast, Pradigastat did not demonstrate any phototoxicity in a dedicated clinical photosensitivity study (Bauer, Photochem Photobiol Sci, 2016) which confirmed the preclinical assessment indicating a margin of at least 15-fold based on the NOAEL *in vivo*.

P23-08

A transcriptomics-based new approach methodology (NAM) identifies points of departure (PoDs) of adaptive stress in HepG2 cells

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In a next-generation risk assessment (NGRA), safety decisions are based in large part on the margin of safety, which is the ratio between the maximum plasma concentration for a given chemical exposure scenario and the point of departure (PoD), the concentration at which the chemical induces bioactivity in relevant *in vitro* assays^[1]. When using high throughput transcriptomics data, current approaches for PoD estimation rely on single key gene targets. However, since genes do not work alone but within complex molecular networks, they lack the diagnostic capability to provide a comprehensive view of biological activity. In this context, we developed a computational pipeline to investigate pathway dose-response relationships and estimate PoDs. Here we first employed the decomposition matrix-based Pathway-Level Information Extractor (PLIER)^[2] in combination with MSigDB prior knowledge, to identify latent variables (LVs) describing relevant biological activities. We then investigated LVs dose-response relationships and computed PoD using the ToxCast pipeline (tcpl)^[3]. We applied this methodology to a relevant public dataset of MCF7 cells exposed for 6hr to 44 compounds selected from 3 classes including those with effects on specific molecular targets expressed in MCF7 cells, chemicals causing broad cytotoxicity, and a set of herbicides that are active against targets that are not expressed in MCF7 cells. We showed that our workflow is able to capture known biological activity for compounds with specific molecular targets (i.e., estradiol agonists and antagonists) and confirmed absence or low activity for herbicides. We then transferred its application to a Unilever in-house dataset obtained by exposing HepG2 cells for 24hr to a relevant panel of chemical compounds across six concentrations with the goal of estimating adaptive stress PoDs, since adaptive responses may not be of concern in risk assessment. Our results confirmed a lack of activity for low-risk compounds (caffeine, coumarin, niacinamide and phenoxyethanol) and the presence of oxidative and ER stress in compounds known to elicit these adaptive responses (andrographolide and triclosan and flutamide, respectively). In addition, we estimated LVs PoDs and compared them with global PoDs obtained using BM-Dexpress, a widely accepted method, and BIFROST, a recently developed Bayesian approach. These results prove the effectiveness of using PLIER in combination with tcpl to estimate pathway-based PoDs and support the development of a framework with the ability to estimate more reliable PoDs with the long-term goal of improving current risk assessment strategies for chemicals using NAMs.

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P23-09**Inhalation toxicity assessment of an aerosolized sunscreen product with an *in vitro* pulmonary model**

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Purpose: Evaluation of the toxicity of cosmetic products generating a cloud of particles and its ingredients must take into account the different exposure routes. Although the main exposure route is dermal, inhalation is a potential one for this type of cosmetics. Previously, the systemic toxicity assessment after exposure by inhalation to a cosmetic product was carried out. Thus, it was interesting to complete this study by assessing local toxicity in the upper respiratory tract using an *in vitro* pulmonary model.

The aim was to assess the repeated dose toxicity of an aerosolized sunscreen product on a three-dimensional model of fully differentiated human upper airway epithelium: MucilAir™.

Methods: The potential toxic effects after two weeks of daily exposure were evaluated on an epithelium reconstructed from a pool of nasal donors. The cells were exposed to the tested product at the apical level, at 3 concentrations: 10%, 50% and 100%. Several endpoints were analyzed: cytotoxicity, epithelial functions (cilia beating frequency, mucociliary clearance), airway inflammation/remodeling (IL-8/MMP9) and histology.

Results: Exposure at 50% and 100% of sunscreen for two weeks has induced dose- and time-dependent cytotoxicity. The IL-8 and MMP9 release was also altered.

On the contrary, exposure at 10% didn't affect the airway epithelial functions. The cell viability didn't decrease. The IL-8 and MMP9 release and the mucus production didn't increase. However, epithelium reshaping and vacuole formation were observed. Comparing this exposure with the respirable particles released by the sunscreen (0.15%), this corresponds to an exposure 66 times higher. Exposure at 10% is a conservative situation. Better results are therefore expected at a lower concentration.

Conclusion: Overall, the study showed that exposure to the sunscreen diluted at 10% (a conservative situation) didn't induce local and systemic toxicity, in the upper respiratory tract. The epithelium was therefore functional, able to exercise his clearance and defense activity.

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P23-10**A pharma stepwise approach to the evaluation of Nitrosamines in drug products: regulatory scenario and the role of *in silico* approaches***S. Zucchi¹, M. Pavan², E. Mastrocola¹, A. Bassan², L. Durando¹, L. Ragni¹¹ Angelini Pharma, Global RD PLCM, Ancona, Italy;² Innovatune, Padova, Italy

N-nitrosamines are a class of high potency mutagenic carcinogens referred to as a “cohort of concern” according to the ICH M7 guideline^[1].

Following the important and restraining regulatory changes that have been prompted by the discovery of unacceptable levels of NAs

impurities in marketed pharmaceuticals, Angelini Pharma is implementing a stepwise approach for the evaluation of NAs in drug products.

Currently, interim limits have been applied to each product based upon maximum daily intake, as suggested by relevant major regulators. However, two main factors could influence promptness and adequacy in the identification of NAs, the analytical methods and lack of sufficient substance specific data (e.g.: Acceptable intake, TD50).

The proposed workflow is based on considerations contained in the “Assessment report on Nitrosamine impurities in human medicinal products” as per European Medicinal Agency (EMA) guideline^[2]. A compound specific limit, consistent with the ICH M7 guideline, can be derived from available carcinogenicity potency data that is expressed in terms of TD50 (i.e., the daily dose causing tumours in 50% of animals in a life-time bioassay). For N-nitrosamines without TD50 values, a default class-specific TTC (i.e. Threshold of Toxicological Concern) of 18 ng/day can be used, and this is considered a conservative value being sufficiently protective; however, deviation from this default approach can be justified on a case-by-case basis based on considerations from a structure-activity-relationship and read-across approach.^[2] This means that a prediction of a carcinogenic potency can be obtained by comparing compounds with similar structures and by using scientifically based assumptions and available data from other analog substances. This read-across approach follows the principles developed within the Read-Across Assessment Framework (RAAF) framework by ECHA^[3]. Structural similarity is the main prerequisite for using read-across, and this needs to be combined with an evaluation of similarity in terms of mechanistic, physicochemical, pharmacokinetic and metabolic properties. It is observed that the choice of the most suitable analog(s) is crucial and the most important step since the range of carcinogenic potency of N-nitrosamines spans four orders of magnitude. The choice accounts for the knowledge the structural features affects their carcinogenic potency. Knowledge on mechanism underlying carcinogenicity of N-nitrosamines and availability of reliable carcinogenicity potency data are key elements to apply a robust read-across. In this respect, Angelini Pharma welcomes international activities by scientists that are working to advance understanding of SAR of N-nitrosamines for the derivation of protective category limits when experimental data are not available.

The use of database and alternative approaches for the identification of NAs, represent a crucial asset in the safety procedures, in a rapidly changing regulatory scenario.

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P23-11**Quantitative sensitizing potency assessment using GARDskin dose-response**

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Proactive identification and characterization of sensitization hazards are central aspects of risk assessment of chemicals. Current legislations and trends in predictive toxicology advocate a transition from *in vivo* methods to non-animal alternatives, with a number of methods for hazard assessment of skin sensitizers currently available.

However, non-animal methods capable of providing quantitative assessment of sensitizing potency are currently lacking.

The GARDskin assay is a next-generation *in vitro* assay for hazard assessment of skin sensitizers, currently progressing towards regulatory acceptance. Recently, the GARDskin Dose-Response (DR) testing strategy was introduced, in which test chemicals are evaluated by the GARDskin assay in a titrated range of concentrations, in order to investigate the dose-response relationship between GARDskin classifications and test chemical concentration. As such, it provides a quantitative estimation of sensitizing potency, referred to as cDV₀, which corresponds to the least required dose able to generate a positive response in the GARDskin assay. The cDV₀ value obtained for a test chemical may be viewed as an analogue to the LLNA EC3 value, based on which further hazard characterization and risk assessment may be performed. Statistically significant correlation between the GARDskin DR cDV₀ and the LLNA EC3, as well as with human No Expected Sensitization Induction level (NESIL) estimations has been confirmed, thus enabling direct extrapolation between the different metrics. Here, we further introduce the GARDskin DR protocols, as proposed in a standardized testing strategy. By studying a concentration range of 6 concentration points titrated from the experimentally derived GARD input concentration in biological duplicates, a test chemical-specific cDV₀ is established by linear interpolation. We illustrate how these results can be used on their own to facilitate direct potency-associated ranking of test chemicals. Furthermore, we demonstrate how obtained cDV₀ values can be extrapolated to LLNA EC3 values with a 95% confidence interval, thereby also facilitating potency-associated subcategorization of test chemicals according to UN GHS classification criteria. Lastly, we illustrate how results generated with GARDskin DR can be directly incorporated into existing strategies for Quantitative Risk Assessment using an entirely *in vitro* setup.

P23-12

Nitrosamines: EMA versus FDA

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Since 2018 EMA and FDA have been investigating the presence of nitrosamine impurities in certain drug products. Starting from the nitrosamines affair in sartans, EMA and FDA addressed the issue in a succession of independent documents. However, although both Agencies have applied the approach suggested in ICH M7 (R1), the latest published documents express significantly different positions. First and foremost EMA has derived a class specific Acceptable Intake (AI) for the nitrosamines cohort of concern. This value equal to 18 ng/person/day applied to all nitrosamines for which no TD50 value is available to derive an AI, or no read-across approach can be successfully applied. Of note, the class specific AI for nitrosamines was determined deriving a class specific TD50 by using the TD50 data of all nitrosamines listed in Lhasa carcinogenicity potency database (LCDB) and use of the lower 5th percentile. This TD50 is then used to calculate the excess risk which would in theory not exceeded with 95% probability by any nitrosamine. The second difference lies in the approach for medicinal products with multiple nitrosamines. EMA suggests two approaches: i. to limit the sum of N-nitrosamines to the limit of the most potent one found; ii. limits for individual nitrosamines could in principle be acceptable as long as the total risk does not exceed the 10⁻⁵ tumour risk level, so the quantity of each nitrosamine represents a fraction of the acceptable 1:100,000 tumour risk level. According to FDA if more than one of the nitrosamine impurities with defined AI is detected and the total quantity of nitrosamine impurities should not exceed 26.5 ng/person/day (the AI for the most potent nitrosamines) based on the maximum daily dose (MDD). On the one hand it seems that the FDA approach is more re-

strictive, on the other hand the FDA position seem to open up to a greater dialogue with the interested parties. Finally, the FDA and EMA positions differ in the sensitivity of the required analytical methods. Overall, the different positions of the EMA and the FDA affect the pharmaceutical and chemical-pharmaceutical companies.

P23-13

How do the existing read-across frameworks work for nanomaterials?

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Risk assessment of chemical substances is always a challenging process. It can be supported by using the potential of the *in silico* methods such as the read-across approach. Several frameworks and methodologies can be found, e.g. the Read-across Assessment Framework (RAAF) developed by ECHA, which describes how the analysis is carried out using the read-across approach. However, they are focused on classical chemical substances, not nanomaterials. Thus, our goal was to evaluate publicly available read-across frameworks in the context of ENM. Especially, in view of the recent update of the REACH regulations (Annex VI), which introduced the concept of "nanoform" of the substance.

We examined the possibilities as well as the challenges for nanomaterials when applying available frameworks by carrying out read-across case studies for selected nanoforms of nano-SiO₂. Structural properties of five ENMs and data related to their ecotoxicity were extracted from the JRC Repository characterization dossier on nano-SiO₂ amorphous materials and the corresponding NanoReg2 H2020 project deliverable. From all endpoints available, toxicity results towards the Carp leucocyte cell line were considered as the most appropriate. For the purposes of the case study, we decided to treat one of the nano-SiO₂ as a target (NM200) and the four others (NM 201-204) as source analogues. The analysis consisted of several steps: i) identification and characterization of all nanoforms; ii) development of grouping hypothesis; iii) assignment to groups; iv) data gathering; v) applicability assessment; vi) filling data gaps. After passing through all the stages we were able to estimate the toxicity of target ENM.

The formulated hypothesis of the read-across approach for the assessment of ecotoxicity was as follows: SiO₂ nanoforms can be separated into two distinct groups based on how the following properties influenced cytotoxicity in fish cells: i) surface area, ii) coating mass, iii) size distribution in stock and media solutions, iv) polydispersity in stock and media solutions. This leads to the follow-up hypothesis of novel SiO₂ ENMs with similar physicochemical/structural parameters inducing similar toxicological activities in fish cells. Subsequently, we employed similarity analysis in the space of the mentioned properties. Based on the calculated Euclidean distances, the target nanoform (NM200), has been placed within the group of toxic source analogues (NM201 and 204). Therefore, according to the worst-case approach, one can assume that the target nanoform will be highly toxic to fish cells.

The results and lessons learned from this exercise will be discussed further in the context of the work carried out in the PATROLS project.

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P23-14

The role of PFAS in non-alcoholic fatty liver disease: a structure-activity relationship on hepatotoxicity

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Non-alcoholic fatty liver disease (NAFLD) is one of the most prevalent liver diseases, characterizing various liver disorders ranging from steatosis and steatohepatitis to hepatocellular carcinoma^[1]. Environmental toxicants play a role in the heterogeneous pathophysiology of NAFLD^[2]. Amongst these chemicals are per- and polyfluoroalkyl substances (PFAS), which can be summarized into two groups depending on their carbon chain-length: long-chained PFAS (with eight or more carbon atoms) and short-chained PFAS (with less than eight carbon atoms)^[3]. Whereas the effects of a few selected long-chained PFAS on human health and the environment are well documented, those of short-chained PFAS are, however, not yet fully understood^[4,5,6,7]. The present study therefore aimed to develop a predictive model linking PFAS chain-length and headgroup to hepatotoxicity.

Several well-established toxicological assays were used to assess hepatotoxicity (MTT), oxidative stress (DCFH) and mitochondrial membrane potential (TMRE) in HepG2 cells induced by 13 different PFAS. Initial data demonstrated a clear link between both headgroup and chain-length with toxicity. Longer chain-length were observed to be more hepatotoxic and induce more oxidative stress. Similarly, PFAS with an acidic or sulfonic headgroup were significantly more toxic than PFAS with an alcoholic headgroup. Similar behavior regarding chain-length was observed in the TMRE assay. However, it was observed that PFAS increased mitochondrial membrane potential, which might explain oxidative stress induction by PFAS.

In order to determine the mechanisms leading to oxidative stress, we subsequently studied cytochrome P (CYP) inhibition by PFAS^[8,9,10]. The following CYP's were studied: 3A4, 2C19, 2D6, and 2E1. Initial inhibition data, expressed as IC50 values, demonstrated a link between PFAS structure and CYP inhibition. For CYP 3A4, 2C19 and 2D6, PFAS with a sulfonic headgroup had lower IC50 than PFAS with an acidic headgroup. PFAS with an alcoholic headgroup on the other hand only inhibited CYP2E1.

A similar structure-activity relationship was observed both with the *in vitro* and CYP assays. Long-chain PFAS were hepatotoxic, potent CYP inhibitors, induced oxidative stress, and increased mitochondrial membrane potential. PFAS with an alcoholic headgroup only inhibited CYP2E1 and appeared to be less hepatotoxic, induced less oxidative stress and did not increase mitochondrial membrane potential. Data collected so far not only reinforce a potential link between CYP inhibition by PFAS and oxidative stress, but also confirm a strong structure-activity relationship for PFAS hepatotoxicity.

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P23-15

Food use of microbial synthesis products: application experience, prospects

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The experience in the use of microbial synthesis products in feed for farm animals goes back several decades. The most important tasks in the USSR were solved in the period since 1964 till 1970, which determined the possibility of developing a new branch of microbiological industry: at first, the biological value and safety of microbial synthesis products, in particular dry microbial biomass (further – protein-vitamin concentrate, PVC), and secondly, the conditions guaranteeing full safety of their use in animal husbandry were determined.

Safety requirements for PVC due to the principal novelty of these products and the use of new energy sources, such as natural gas and petroleum hydrocarbons in their production, implied the need for large-scale medical and biological studies aimed at identifying possible adverse biological effects in various types of experimental animals. At the same time, not only conventional methods of identification of acute, chronic toxicity, carcinogenic, mutagenic and teratogenic effects were used, but also highly detailed studies of PVC impact on the state of main physiological systems of the body, metabolic processes and enzyme systems. PVC studies had been carried out on tens of thousands of experimental animals, including several populations of white rats, dogs, rabbits, guinea pigs and monkeys. The results of these studies were safety, nutritional and biological value confirmation, justification of PVC introduction norms into the ration of farm animals and birds.

Thus, since 1970 till 1990, microbial synthesis products were used in farm animal feed in an industrial scale. During this period no ad-

verse effects on animal or human health were detected at the population level, which unequivocally confirms the validity of the results of biomedical research and demonstrates the safety of these products.

In recent years, worldwide interest has increased in the use of alternative sources of protein, in particular, the protein of microorganisms. Due to the fact that the food obtained on the basis of microbial synthesis are classified as food of a novel type, ensuring the safety of such products is regulated at the state level.

The most important step in the registration procedure is comprehensive safety examination, including toxicological and allergological studies, assessment of mutagenic and carcinogenic potential and study of the effects on reproductive function and offspring development. Integration of the existing scientific background and the latest approaches in toxicology, biology, medicine, genomic and proteomic technologies will make it possible to create a comprehensive system that guarantees the safety of the novel product, taking into consideration all modern requirements for food safety.

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P23-16

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P23-19

The UK Committee on Toxicity (COT) and on Carcinogenicity (COC) of Chemicals in Food, Consumer Products and the Environment: Guidance for synthesising and integration of epidemiological and toxicological evidence

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The UK Committees on Toxicity (COT) and on Carcinogenicity (COC) regularly review epidemiological and toxicological evidence in their risk assessments. However, there is a need for guidance on the approaches used by the Committees to integrate these evidence streams, in part to ensure public transparency. To that end, the Committees established the Synthesising and Integration of Epidemiological and Toxicological Evidence Subgroup (SETE) to review and document current practice and provide applicable guidance on how different evidence streams should be integrated in a transparent manner, giving appropriate weight to both.

SETE recognised that issues on which advice from the Committees is sought varies considerably and hence the guidance proposed should be sufficiently flexible to address this.

Scoping and problem formulation were identified as the crucial first step in the process. This ensures the right questions are asked, helps make the most efficient use of resources and identifies the most appropriate approaches to use in the assessment. An established system or guidance should be followed where feasible.

For both, epidemiological and toxicological evidence a prescriptive checklist or scoring approach is not recommended. However, identifying the strengths and weaknesses of studies is an important

part of the process. The decision-making process should be robust, transparent, evidence-based, defensible and documented but equally importantly, it should be easy to use. Collaboration and ongoing dialogue between epidemiologists, exposure experts and toxicologists are strongly advised. Information on mode of action (MOA) can be invaluable for evidence integration as it underpins weight of evidence considerations by providing the mechanistic link between empirical observation and biological plausibility.

All lines of evidence should be considered, with no specific hierarchy *a priori*. One way to clearly depict the influence of the different lines of evidence on the null hypothesis of causality is via visual representation. Decisions on whether there is sufficient information to reach a conclusion or whether a causal relationship in humans is more likely or less likely can be reached based on where the causal interference appears on a graph. The impact of the different lines of evidence is influenced by several factors, including the impact of the strength or weakness in the data, the relative weighing of epidemiological and toxicological studies and the uncertainties associated with the data. When more information is added/becomes available the placement of the evidence can be easily adjusted and a determination made whether the conclusion remains valid.

The conclusion should be stated, with an estimate of the overall uncertainty and, where appropriate, guidance on how data gaps could be filled.

SETE prepared a report and guidance, which have been accepted by the COT and COC. The guidance will be trialled by the COT for 2 years and then reviewed.

P23-20

This abstract has been withdrawn.

P23-21

How to increase confidence in the defined approach for skin sensitisation with propyl paraben?

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Skin sensitisation is a key adverse health effect to be addressed in the safety assessment of cosmetic ingredients. Regulatory demands have urged the development of Next Generation Risk Assessment (NGRA) using New Approach Methodologies (NAM) and Defined Approaches (DA) instead of animal models. An illustrative NGRA case study shall demonstrate whether the use of propyl paraben at 0.2% in a face cream is considered safe for consumers. A sequential stacking tier testing DA based on NAM data predicted propyl paraben to be a non-sensitiser (No Cat.) with a probability to be a skin sensitiser of 16% (well below the cut-off of $\leq 30\%$), while some NAM input data showed positive test results (e.g. KeratinoSens™ and U-SENS™ assays). To increase confidence, structurally related parabens (methyl-, ethyl-, butyl- and isobutyl paraben) were considered. They revealed NAM and DA hazard predictions similar to those of propyl paraben, whereas animal and human data indicated only some rare cases of skin allergy. As the NAM results for propyl paraben did not differ from those of other parabens, it was decided to consider propyl paraben a non-sensitiser supporting the DA outcome.

On the other hand, an *ab initio* NGRA not using the *in vivo* data from analogues but only considering additional NAM assays and metabolism data, led us to conclude propyl paraben to be a weak sensitiser in a conservative approach. This was particularly due to the absence of the animal and human evidence for the parabens, introducing uncertainty with the positive NAM results. So, taken togeth-

er the DA outcome and the *ab initio* NGRA, propyl paraben was conservatively considered at most a weak skin sensitiser and the use concentration of 0.2% in a face cream was regarded as safe for consumers in terms of the risk of skin sensitisation.

P23-22

In vitro and *in vivo* thyroid evaluations after exposure to mixtures of triazole fungicides

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Background: Mixture effects of chemical substances have been discussed also in the context of endocrine disruption. A key endocrine target organ of chemical substances in rodents is the thyroid. Thyroid effects may be caused by a variety of mechanisms, of which increased hepatic metabolism of T3 and T4 and a compensatory up-regulation of TSH is the most prominent one following exposure to environmental chemicals (ECs) in animal experiments.

Methods: A 28 day feeding study in rats examining the effects of treatment with the hepatotoxic fungicides cyproconazole, epoxiconazole and prochloraz as well as combinations thereof was performed as already described by Heise et al 2015. Serum hormone concentrations were analysed by MagPix multiplex assays *post hoc*. Induction of hepatic metabolism was measured by qRT PCR and hepatic T3/T4 clearance was measured by a microsomal enzyme activity assay. With cyproconazole, additional *in vitro* assays with human cells or enzymes were conducted covering thyroidal I-uptake (NIS), TH uptake via MCT8, thyroperoxidase (TPO), iodothyronine deiodinases (D1–D3) and iodothyronine deiodinase (IYD) inhibition. Activities of Dio1 and Iyd were measured in kidney and liver together with hepatic TH-relevant transcripts (e.g. Thrsp).

Results: A decrease in serum T4 levels and an increase in TSH levels were observed in rats treated with 1000 ppm cyproconazole and with the azole mixtures that contained a corresponding amount of cyproconazole. At the same doses, liver hypertrophy and an increase in UGT gene expression and induced T3 and T4 metabolism were observed, indicating that the effect on thyroid hormones may be secondary to hepatic enzyme induction. No such effects were seen with lower doses of cyproconazole nor epoxiconazole and prochloraz (tested up to 1000 and 900 ppm, resp.) and also at TH-specific key functions assays conducted with cyproconazole only.

Conclusion: Cyproconazol induced thyroid hormone changes in rats but not in *in vitro* systems. Furthermore, liver enzyme induction appears to be a key mechanism in thyroid hormone disturbance by cyproconazole and its mixtures. The results also lend mechanistic support to the definition of the cumulative assessment group for effects on the thyroid by EFSA (2019) which included only cyproconazole but not epoxiconazole and prochloraz. Additionally, the present study also shows how to combine different mechanistic assays using one single sample collection for establishing the mode of action on individual or mixtures of compounds.

P23-23

A BMDC assay for studying the respiratory sensitisation mechanism: first results with platinum salts.

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Many chemicals used in industry can cause occupational allergies. The European Classification, Labelling and Packaging (CLP) regulation classifies these chemical agents in two hazard categories: skin and respiratory sensitisers. Appropriate preventive strategies can only be developed if the sensitising potential of industrial substances is accurately predicted. In this context, our research team at INRS (French National Research and Safety Institute) has developed an *in vitro* Bone Marrow-derived Dendritic Cells (BMDC) model to predict the sensitising power of chemicals. This assay is based on the modification of cell surface markers in response to sensitisers whatever the category they belong to.

Although the mechanisms induced by skin sensitizers are well known, those triggered by respiratory ones are not well described in the literature. It has been shown that intracellular pathways such as Nrf2/Keap1, or those involving MAPK, are involved in dendritic cells responses to skin sensitisers. An analysis of these signalling pathways might allow us to better understand the mechanisms of respiratory sensitisation.

The aim of the current study was to investigate MAPK and Nrf2/Keap1 pathways in BMDC exposed to different platinum salts classified as respiratory sensitisers or not sensitisers.

Methods: The tested platinum salts were : two respiratory sensitisers, the ammonium hexachloroplatinate (HCP) and the ammonium tetrachloroplatinate (ATCP), along with two non-sensitizers, the hydrogen hexahydroxyplatinate (HHP) and the tetraamineplatinum (II) chloride hydrate (TPCH). Following chemical exposure, cells were fixed, permeabilised and stained with antibodies against phosphorylated forms of MAPK (p-p38, p-ERK) and against total Nrf2. Staining patterns were analysed by cytometry. To complete flow cytometry, RT-qPCR experiments were carried out to study Nrf2 activation.

Results: No MAPK nor Nrf2 pathways activation was found in BMDC exposed to the non-sensitizers HHP and TPCH. On the contrary, exposure to both platinum respiratory sensitizers HCP and ATCP induced p38 phosphorylation and intracellular Nrf2 accumulation. The activation of the Nrf2/Keap1 pathway induced by these respiratory sensitizers has been confirmed by RT-qPCR.

Conclusion: The p38 phosphorylation and intracellular Nrf2 accumulation were analysed with a cytometry-based method to study the response of BMDC exposed to different platinum species. Both pathways activation were successfully used to identify compounds with sensitisation potential as well as ones with no sensitisation activity. These mechanisms, p38 and Nrf2/Keap1, are both described in the cellular response to skin sensitizers. The current study shows that these pathways could be activated by respiratory sensitizers in a BMDC model. These results confirm that respiratory sensitizers share common pathways with skin ones in the intracellular signalling cascade.

P23-24**Food proteins: safety assessment and *in vitro* digestion***A. Fernandez Dumont¹, M.M. Epstein², J.-L. Bresson⁴, F.J. Moreno³¹ European Food Safety Authority, Parma, Italy;² Medical University of Vienna, Division of Immunology, Allergy and Infectious Diseases Department of Dermatology, Vienna, Austria;³ CIAL (CSIC-UAM), Madrid, Spain;⁴ Descartes University, Paris, France

Purpose: There is a generalised public demand to increase alternative food protein sources. Food proteins derived from biotechnology or other means can meet this demand, but they must undergo a comprehensive safety assessment before they enter the market. Typically, a 'weight-of-evidence approach' integrates information from multiple sources and types of data to reduce uncertainty and improve reliability and predictability. Currently, *in vitro* digestion data are primary elements in the safety assessment. However, improvements are needed to frame the overall usefulness of these data in the risk assessment (RA).

Methods: The classical pepsin resistance test defined by Codex Alimentarius^[1] provides information on protein digestion for the RA. However, this test has several inherent limitations as a predictor of digestion and allergenicity. EFSA published a guidance document^[2] proposing more refined digestion conditions to provide more reliable knowledge about a novel protein's fate in the gastrointestinal (GI) tract. Additionally, EFSA commissioned experimental work^[3] that provided a comprehensive dataset on *in vitro* digestion methods. EFSA has recently considered these methods and published a statement on *in vitro* protein digestibility tests in the allergenicity and protein safety assessment of genetically modified plants.^[4]

Results/discussion: There is still great controversy on the usefulness of the current methodologies used to understand protein stability and its fate in the GI tract. To frame and build a more robust strategy in the safety assessment, we need a better understanding of protein-GI tract interactions. In response, EFSA asked specific questions, including^[4]: what is the usefulness of *in vitro* digestion in the overall protein safety assessment?; what are the most suitable *in vitro* digestion models? What is an adequate item to test? What criteria should be used to identify digestion fragments as relevant for RA (abundance, persistence, size; others)?^[5] How can this information be integrated into a weight-of-evidence approach? What follow-up actions would be required to assess the relevant proteins/fragments identified in previous steps? Answering these questions would be essential to better integrate *in vitro* digestion data into the overall safety assessment of proteins. EFSA is committed to dedicating continued efforts to this vital topic by promoting discussions at the international level to develop a scientifically robust consensus approach.

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P23-25

This abstract has been withdrawn.

P23-26**Human Relevance Assessment of Adverse Outcome Pathways and associated New Approach Methodologies: A Pragmatic Workflow**C. Veltman¹, L.T. van der Ven¹, *H.J. Heusinkveld¹, E. Menegola², M. Luijten¹¹ National Institute for Public Health and the Environment (RIVM), Centre for Health Protection, Bilthoven, Netherlands;² Università degli Studi di Milano, Department of Environmental Science and Policy, Milan, Italy

According to current approaches, safety assessments of chemical substances predominantly rely on *in vivo* testing. Multiple ethical, scientific, financial and legal considerations and limitations call for the use of alternative testing strategies that are based on new approach methodologies (NAMs). The human relevance of such alternative strategies is however often uncertain. Therefore, there is a need for a harmonized and accepted approach for the assessment of human relevance. We developed a workflow for human relevance assessment of Adverse Outcome Pathways (AOPs) and associated NAMs. The workflow starts with an established AOP (or similar structured description of a toxicological pathway), of which the adverse outcome is relevant to humans and that has sufficient weight of evidence. Human relevance is assessed through structured questions related to the different components of the pathway, to the pathology of human syndromes that have a similar adverse outcome, and to quantitative aspects. The latter comprise both interspecies differences and *in vitro* – *in vivo* differences. The structured questions lead to a combined evidence score for human relevance. The workflow developed was tested in a case study, through application to an AOP describing craniofacial malformations after *in utero* exposure to triazoles. This case study provided moderate to strong support for human relevance of both the various components of the AOP and its associated NAMs, with further support from human genetic syndromes, and without conflicting quantitative information. Furthermore, it demonstrated that the workflow is a promising approach that allows for a more transparent, scientific evaluation of human relevance of AOPs and associated NAMs. Therefore, we consider the workflow an important step forward for application of AOPs and related NAMs in human health risk assessment.

P23-27**An interlaboratory comparison study upon particle deposition and effects using an air-liquid interface lung model***H. Braakhuis¹, A. Bannuscher², B. Drasler², K. Meldrum³, S. Doak³, B. Birk⁴, M. Clift³, B. Rothen-Rutishauser², R. Vandebriel¹¹ RIVM, Bilthoven, Netherlands;² AMI, Fribourg, Switzerland;³ SU, Swansea, UK;⁴ BASF, Ludwigshafen, Germany

For hazard testing of airborne engineered nanoparticles (NPs), air-liquid interface (ALI) exposure systems have been developed for *in vitro* tests with lung cell cultures in order to mimic realistic inhalation exposures. Within the EU-project PATROLS, we have further advanced an epithelial lung-cell model with the aim to produce standard operation procedures (SOPs) allowing to reproduce the experiments in different laboratories. The lung model consists of a co-culture of the human bronchial epithelial cell line Calu-3 and macrophages, either human blood monocyte derived macrophages

(MDM) or macrophages differentiated from human THP-1 monocytes (dTHP-1). For NP exposure, we have chosen a commercially available exposure device, i.e. the VITROCELL Cloud system.

As a first step to advance these models towards regulatory use we initiated interlaboratory comparison studies. The first stage compares deposition upon aerosolization of quartz silica particles (DQ12), titanium dioxide (TiO₂ NM-105) NPs and the chemical fluorescein. First results show similar deposited doses for the different particles between three participating laboratories, i.e. 300–500 ng/cm² for a single nebulization of 500 µg/mL.

The second stage compares effects of exposure to DQ12, TiO₂ NM-105 and LPS (positive control) in the Calu-3 ALI system with and without MDM or dTHP-1. Two nebulizations of 500 µg/mL were used to achieve 600–1000 ng/cm² which is similar to doses that induce inflammation *in vivo*. Trans-epithelial electrical resistance (measure of barrier function), cell viability (using WST-1 and LDH), and secretion of pro-inflammatory mediators (IL-6, IL-8 and TNF-α) were measured. First results show no effect of the exposures on TEER and cell viability. LPS increased the release of pro-inflammatory mediators in both Calu-3 alone and in co-culture with macrophages. DQ12 and TiO₂ slightly induced the release of pro-inflammatory mediators in the co-culture model.

Initial results show high intra-laboratory reproducibility in both studies, investigations remain ongoing and interlaboratory reproducibility continues to be evaluated as results from participating laboratories are being collected. The studies are further generating detailed SOPs that will be made available in the course of 2021.

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P23-28

Selenium Health Benefit Values, a biomarker for tunna mercury risk assessments

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Mercury (Hg) is a well-known toxic element, diffused in the environment, especially in the Mediterranean Sea. The bioaccumulation of Hg in fish is of great concern, especially for high-level aquatic predators above all for species of large human consumption and high nutritional value such as tuna. Hg levels are high enough in some fish to cause adverse health effects in people who eat large amounts. However, fish, particularly oceanic ones, are relatively rich in selenium (Se), which offers protection against Hg toxicity. The modified Se health benefit value (HBVSe) is a risk assessment criterion based on the molar concentrations of methylmercury (MeHg) and Se present in a fish or seafood. Considering only Hg content is inappropriate. For this reason, we determined the levels of Hg, Se and MeHg in farmed tuna (*Thunnus thynnus*), both red and dark muscle. The determinations were made on both raw and cooked tuna. A total of 43 tuna samples delivered by Balfegó & Balfegó from l'Ametlla de Mar (Spain North-east Mediterranean area), were analyzed. For all samples, the Hg content was below the legal limit (1 mg/kg, wet weight). In raw tuna, the results showed in red muscle were 0.628 mg Hg/kg, 0.647 mg Se/kg, 0.455 mg MeHg/kg, 4.1 molar ratio Se/MeHg and 7.5 HBVSe. In dark muscle, the results were 0.712 mg Hg/kg, 15.38 mg Se/kg, 0.528 mg MeHg/kg, 79.43 molar ratio Se/MeHg and 193.6 HBVSe. The results showed that with cooking the levels were higher. In red muscle, the results were 0.760 mg Hg/kg, 0.862 mg Se/kg, 0.600 mg MeHg/kg, 4.2 molar ratio Se/MeHg and 10.12 HBVSe. However, in dark muscle the levels were 0.927 mg Hg/kg, 17.04 mg Se/kg, 0.706 mg MeHg/kg, 67.8 molar ratio Se/MeHg and 214.1 HBVSe.

The fish that have the least amount of Se tend to bioaccumulate the most MeHg. In our study increased amounts of Se have been shown to increase MeHg efflux. Tuna Se concentrations optimal levels comes with the added benefit of diminishing their MeHg contents (Molar ratio Se/Hg >1 and HBVSe >0). Since the HBVSe is based on the biochemical mechanism of MeHg toxicity, it provides an objective index for assessing the relative effects of MeHg exposures. Their HBVSe indicates they are all a net source of surplus Se and are thus predicted to protect against risks associated with MeHg exposure.

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P23-29

Nanofibre inhalation toxicology: providing the foundations for safe-by-design *in-silico* models

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Introduction: Model nanofibres such as carbon nanotubes (CNTs) present unique challenges to human hazard assessment due to their unique physico-chemical characteristics such as geometry, length and biopersistence. Paradoxically, they have tremendous applications in the consumer and industrial markets. Therefore, understanding their toxicity and moreover predicting their toxicity could be extremely beneficial where bulk manufacture of new nanofibres is concerned. The aim of this work was to gather and analyse literature-sourced information pertaining to nanofibre physico-chemical features with pre-existing data to produce structure-activity-relationships (SARs) which could then be implemented in the development of *in-silico* models to predict nanofibre (human) pathogenicity.

Methods: Focussing upon CNTs, due to the broad nature of their surface chemistry, geometry, and wide-ranging applications, original peer-reviewed research articles were sourced from the online databases PubMed and Scopus. Specifically, papers were sought from 2009 onwards, building off the holistic review by Johnston and colleagues for all papers prior to this time (Johnston *et al.*, 2010). Keywords were chosen to gather as much relevant literature as possible following this, focussed search-strings were implemented, *in vitro* AND carbon nanotubes OR CNT AND toxicity & *in vivo* AND carbon nanotubes OR CNT AND toxicity. Assessment of the papers collected from this literature search was conducted using the GUIDEnano approach (Fernández-Cruz *et al.*, 2018) to obtain a comprehensive list of quality-screened publications. Once the nanofibre physico-chem-

ical features have been related to the observed toxicity, the pathogenicity of novel nanofibres can be predicted based off existing data using *in-silico* models.

Results and Discussion: A total of 33 publications were assessed for their usefulness using the GUIDEnano approach. The GUIDEnano analysis revealed 87% of publications were approved with high quality content (a Q-Score of 0.8). The physico-chemical features of the nanofibres as well as the biological responses of each paper has been extracted and catalogued in a spreadsheet for further analysis on deducing structure-activity-relationships (SARs). This has been achieved by breaking down the data by assay, exposure time, model used and the key physico-chemical characteristics of the nanofibres.

Summary: Data has been extracted on CNTs as model nanofibres to deduce the SARs within *in vitro* and *in vivo* hazard characterisation tests. The key physico-chemical characteristics and biological response data will now need to be tested using *in silico* modelling to test the predictivity of the model.

P23-30

High throughput chronic cardiotoxicity evaluation of human iPSC-derived cardiomyocytes in a pro-maturation environment

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In pre-clinical drug development, cardiac contraction analysis of potential drug candidates is one of the crucial steps to ensure a successful and reliable transition to clinical stages. The use of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) continues to increase in the assessment of toxicological side effects of newly developed compounds, due to their reproducibility and low ethical concern.

However, their premature phenotype causes issues concerning non-physiological responses and acute testing within limited time-scales (min to h) after compound application remains the primary application so far, partly due to the inability of common cell-based assays to analyze cellular behavior reliably over prolonged periods of time.

Here we describe the bio-compliant 96-well FLEXcyte technology with its pro-maturation effect on hiPSC-CM maturation as well as its applicability for chronic cardiotoxicological studies.

We selected 15 kinase inhibitors and 3 anthracyclines with well-known cardiotoxic profiles to evaluate the reproducibility of clinical data.

Cells from commercial sources were cultured on freely-swinging, ultra-thin and hyperelastic silicone membranes. Rhythmic contraction of the hiPSC-CMs resulted in dynamic deflection changes quantified by means of capacitive distance sensing. The resulting beat patterns were analyzed for essential inotropic parameters including amplitude, frequency, slopes of contraction and relaxation, area under curve and arrhythmic events. For the assessment of chronic compound effects, inotropic properties of the cells were recorded daily for five days.

Compounds considered as clinically cardio-safe showed negative inotropic effects only at micromolar doses, while compounds with demonstrated cardiotoxic profiles showed both time and dose dependent inotropic effects as well as arrhythmic events at nanomolar concentrations. Our results indicate that the FLEXcyte technology enables the assessment of physiologically relevant inotropic effects on hiPSC-CMs at a chronic level beyond the current perspective of preclinical cardiac risk assessment.

P23-31

Identification of non-intentionally added substances in reusable plastic containers in contact with food

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European regulations regarding the safety of materials intended to come into contact with food allow the presence of not-intentionally added substances (NIAS) in such materials. In order to determine whether these NIAS pose a risk to the consumer, it is necessary first to identify such substances and afterwards assess their hazardlessness and to establish appropriate exposure scenarios. In the case of NIAS, since the substances have not been intentionally added to the formulation of the plastic material, their determination and quantification is complicated, which makes exposure assessment difficult.

Our final goal is to assess the risk to the human health of the NIAS migrating from reusable plastic materials used in kitchen stuff and food containers to meals, but in this work, we will be focused on the identification of such NIAS. Different market samples of two of the most common plastic materials in the sector, polypropylene and low-density polyethylene were selected and migration assays with 10% ethanol and 3% acetic acid were performed. The conditions of the migration assays were: i) lunch boxes 4 h at 100°C; ii) cutting board 2 h at 70°C; iii) dosing bottle 10 days at 40°C; and iv) big spoon 1 h at 100°C. Afterwards, the extracts were analyzed using HPLC with mass spectrometry QTOF detector.

Up to 44 different NIAS were found in the sampled plastic materials. These NIAS included several CMR (carcinogenic, mutagenic and reprotoxic) chemicals as (among others) octamethylcyclotetrasiloxane, tetrahydrofurfuryl methacrylate and anthraquinone and also several bisphenols as bisphenol F and bisphenol E. In most cases, the concentrations of NIAS in the migration extracts were below 10 ppb, except for the dimer of caprolactam cyclic (around 1 ppm in both ethanol and acetic acid extracts) and dipropylene glycol (20–60 ppb in ethanol extracts).

The presence of CMRs and bisphenols flags the necessity of the urgent development of realistic exposure scenarios for proper risk characterization.

P23-32

Rare earth element as the emerging contaminants in black tea – risk assessment resulting from the dietary exposure

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In the recent years, a great increase of the interest in the rare earth elements (REEs) has been observed. Due to their specific properties, they are used in several areas including medicine, agriculture and some branches of modern technologies, e.g. electronics, mobile communication, LED light bulbs, catalysts, electric cars or fuel additives. Consequently, more and more of the elements are getting into the environment, accumulating in soil, water, organisms, and thus entering into the food chain. This may lead to an increase in the exposure to REEs. Inhalation and consumption of contaminated foods and beverages have been indicated as the main pathways of the exposure to REEs.

Until now, no incidents of acute human poisoning caused by ingestion of REEs have been reported. However, concerns regarding the potential adverse effects of chronic exposure to low levels of REEs on human health are currently being emphasised. It is known that REEs can accumulate in liver, blood, brain, lungs or bone. Chronic exposure to low dose REEs modify the bone structure and generate DNA aberration of bone marrow cells, also cause oxidative stress – related effects. The REE ions impair the stability of DNA and even cause DNA damage. Long-term exposure to REEs may also decrease the cognitive abilities and lower IQ scores in children.

Because REEs are perceived as emerging contaminants, it is crucial to assess the dietary exposure and the risk posed to the consumers' health. To assess the potential risk it is therefore necessary to monitor the presence of REEs in foods and beverages.

After water, tea is the most frequently consumed beverage in the world. Moreover, teas have been already reported to contain elevated concentration of REEs comparing to other foods. Thus, the main focus of the study was put on determination of REEs in representative samples of black teas available on the Danish market.

Levels of REEs in black teas were determined by inductively coupled plasma mass spectrometry (ICP-MS) after acid pressure digestion based on the principles of the European standard methods EN 13808:2014 and EN 15763:2009. Risk assessment of the exposure to REEs resulting from tea consumption was performed using the data from the Danish food survey on ingestion of tea and determined levels of REEs. As the REEs has only started to be perceived as the emerging contaminants, there are no reference values such as NOAEL, ML, or ADI established in the European Union. For the purpose of the risk assessment, a provisional TDI levels for some of the REEs, based on available toxicological data, have been suggested.

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P23-33

Honeybee risk assessment for Neonicotinoids in Korea

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Concerns about the risk of pollinators caused by pesticides are becoming an international issue. In Korea, the risk of pesticides to honeybees is evaluated according to the pesticide registration system. For the tiered risk assessment system in Korea, honeybee adult acute (contact and oral) toxicity (Tier 1), foliar residual toxicity (Tier 2) and semi-/field test are evaluated. However, the current risk assessment method only evaluates toxicity to adult bees and assumes only contact exposure, so there is a problem that it is insufficient to assess the effect of the hive community. Therefore, research is being conducted to improve the method of risk assessment for bees at the pesticide registration stage in Korea. In this study, in order to study the applicability of the honeybee risk assessment system in EU and USA to Korea, we evaluated the risk for honeybee using two models (BeeRex and Bee-Tool) for pesticide products containing Neonicotinoids (Imidacloprid, Thiamethoxam, Clothianidin, Dinotefuran) that registered in Korea. As a result, according to the criteria in EU and USA, all 98 products were unacceptable risk to bees, so it was necessary to conduct a higher tier assessment. And according to the results of Dinotefuran, it was confirmed that among larval toxicity, chronic toxicity determines the risk rather than acute toxicity. In addition, since the

EU model (Bee-Tool) can additionally evaluate the risk from exposure to drinking water, it can be used for risk assessment due to ingestion of water of rice paddy fields in Korea, where the ratio of rice paddies in agricultural area exceeds 55%.

P23-34

Assessment of the skin sensitizing potential of pandemic-associated medical devices using the GARDskin Medical Device assay

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The current SARS-CoV-2 pandemic have led to an increased use of medical devices such as face masks and nitrile gloves, within occupational groups of medical care as well as within the general population. Consequently, the incidence reports of adverse effects associated with use of such medical devices have increased manifold, including reports of allergic skin reactions.

The cause of allergic skin reactions, referred to as Allergic Contact Dermatitis (ACD) is the immunological process known as skin sensitization, which is induced by so-called chemical sensitizers. Assessment of skin sensitizing potential of leachables from medical devices is a part of biocompatibility testing of medical devices and is typically performed by use of the Guinea Pig Maximization Test (GPMT), in accordance with the ISO 10993 series of standards. However, recent developments of *in vitro* assays for assessment of chemical sensitizers motivates the exploration of such methods in the context of rapid pandemic-associated testing.

The GARDskin assay^[1] is a next-generation *in vitro* assay for hazard assessment of skin sensitizers, currently progressing towards regulatory acceptance. The method evaluates the transcriptional patterns of a genomic biomarker signature in a human dendritic-like cell line following exposure, in order to provide hazard assessments of tested substances. The method has been adapted to testing of solid materials from e.g. medical device products, by application of extraction protocols using polar- and non-polar extraction vehicles, in accordance with ISO 10993-12.

Here, we present results from testing of a commonly used face mask and a nitrile glove in the GARDskin Medical Device assay. Results indicate that the face mask does not leach any compounds with skin sensitizing potential (among four different batches tested), while the nitrile glove was classified as a skin sensitizer. These results harmonize with preexisting experience of similar models of nitrile gloves, which are known to be associated with adverse skin reactions, potentially induced by skin sensitization. Furthermore, these results may have implications on the continued use of similar medical devices throughout the pandemic and beyond, as the appropriate application and removal of face masks may indeed benefit from the discontinued simultaneous use of nitrile gloves.

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P23-35**Metribuzin-induced non-adverse liver changes result in rodent-specific non-adverse thyroid effects via Uridine 5'-diphospho-glucuronosyltransferase (UDPGT, UGT) modulation***W.H. Bomann¹, H. Tinwell², F. Kluxen³¹ Toxconsult, LLC, Toxicology, Overland Park, USA;² Bayer SAS, Regulatory Toxicology, Lyon, France;³ Adama GmbH, Regulatory Toxicology, Koeln, Germany

Abstract: Metribuzin is a pre- and post-emergence herbicide that inhibits photosynthesis and has been used for over 40 years. Extensive toxicity data indicate that metribuzin has an overall favorable toxicological profile. It has no genotoxic, carcinogenic, reproduction or developmental toxic potential. Metribuzin's main target organs are the liver and the thyroid in rats. Metribuzin shows a specific thyroxine (T4) profile in rat studies with T4 increases at low doses and T4 decreases at higher doses. To understand the mode of action (MOA) as well as the human relevance of the rat observations, several mechanistic studies were performed. These experiments demonstrated an UGT-mediated thyroid MOA in rats. *In vitro* studies demonstrated this UGT effect in rat hepatocytes, but not in human hepatocytes. Furthermore, direct thyroid effects via thyroid peroxidase (TPO), deiodinases 1, 2, 3 (DIO) or the sodium importer system (NIS) could be excluded. Therefore, an UGT-mediated thyroid MOA of metribuzin is proposed which is not relevant to humans.

P23-36**Hazard assessment of disinfection by-products, bromo chloroacetic acid and bromo dichloroacetic acid, in drinking water**

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Under the current Japanese Water Supply Act, 8 items of chlorine disinfection by-products: chloroform, dibromochloromethane, bromodichloromethane, bromoform, total trihalomethane, chloroacetic acid, dichloroacetic acid, and trichloroacetic acid are regulated with the "Drinking Water Quality Standards (DWQS)". On the other hand, six other haloacetic acids (HAAs) of chlorine disinfection by-products are currently listed in "Items for Further Study" under the Act. Because the health-based guidance values (HBGV) for the 6 HAAs have not been established, the risk assessment of these items is provisional, and they need to be continuously assessed and revised. To investigate possibility for establishing the HBVG for these HAAs, we made a literature search for them. There was reliable toxicological information for bromo chloroacetic acid (BCAA) and bromo dichloroacetic acid (BDCAA) while no toxicological information was available for other 4 HAAs. Based on the recent reliable information, we conducted the hazard assessment for health-based effects of BCAA and BDCAA. Toxicological profile of BCAA and BDCAA was similar, and the both showed a similar carcinogenic potency in rat and mouse 2-year drinking water studies. To find a proper point of departure for HBVG, the benchmark dose analysis was applied for incidences of all neoplastic endpoints in rats and mice by using EPA Benchmark Dose Software (Ver 3.2). The most appropriate BMDL₁₀ of BCAA was considered to be 3.27 mg/kg/day for female mouse hepatocellular carcinoma while that of BDCAA was considered to be 2.83 mg/kg/day for male rat malignant mesothelioma in all organs. As genotoxic carcinogens, Virtually Safe Doses at 10⁻⁵ risk of BCAA and BDCAA were calculated to be 3.27×10⁻⁴ mg/kg/day and 2.83×10⁻⁴ mg/kg/day, respectively. These values will be useful to establish the target values of BCAA and BDCAA to be monitored in drinking water for future risk assessment.

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P23-37**Cisterna Magna Administration: A Robust Alternative Non-Systemic Route of Delivery for New Drug Entities in Non-Human Primates***A. Dütting¹, T. Voß¹, F. Runge¹, J. Luft¹, B. Pajazit¹, S. Korte¹,
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The field of gene therapy has matured in recent years to bring forward potential treatment or cure for life-threatening monogenetic neurological diseases. Delivery of new drug entities involving adeno-associated virus (AAV) vectors requires a robust delivery technique enabling blood-brain barrier crossing. While injection into the lumbar intrathecal space is well established, direct injection into the *Cisterna cerebellomedullaris* (also known as *Cisterna magna*) may be needed, e.g. if high exposure of the posterior fossa is required. This poster describes *Cisterna magna* delivery of an AAV vector to cynomolgus monkeys, a procedure that is not well described in the existing literature.

The study was conducted in accordance with EU Directive 2001/20/EC and German Drug Law to support clinical trials, and was approved by the local authority and an Institutional Animal Care and Use Committee. Nine healthy Asian cynomolgus monkeys (*Macaca fascicularis*; > 3 years, > 3 kg), serologically negative for antibodies against AAV, were anaesthetized with ketamine and medetomidine. Animals were placed in abdominal recumbency on a flat surface with the head bended forwards. The neck area was shaved, disinfected and a micro incision of the skin was conducted. A 20G Pencan Paed™ pencil-point needle was inserted through the skin incision and advanced into the suboccipital space until cerebrospinal fluid (CSF) was obtained, followed by slow injection of the AAV-based test article over approximately 1 minute. After dosing, artificial CSF (aCSF) was given to flush the dead space volume. Immediately following dosing, the animal was held for 10 minutes in an up-right position (reverse Trendelenburg position) with its head bent backwards to (a) reduce post dose leakage and (b) maximize target distribution. Thereafter, sedation was antagonized with atipamezole and the animal was returned to its home cage. In this study, a statistically sufficient number of animals per group received a fixed concentration with varying volumes up to 2.0 mL of test article. At 4 and 24 hours after dosing, a physical and neurological examination was conducted by the attending veterinarian, comprising evaluation of body temperature, heart and lung auscultation, abdominal palpation, general sensory reactions, cerebral and spinal reflexes, and foot grip reflex. Test article administration into the *Cisterna magna* was successful in all of the animals, and well tolerated up to the highest dose volume. This route of administration has been associated with side effects such as bleeding or general compromise of the central nervous system, but none of these was observed in this study when conducted according to the described procedure. Post-dose neurological examinations did not reveal any abnormalities. Based on a study in nine animals, direct injection into the *Cisterna magna* is a safe and efficient method to deliver test articles into the brain without requiring guidance by computed tomography.

P23-38

Risk assessment of elemental impurities in medicinal clay using an *in vitro* gastrointestinal digestion model for analysis of the bioaccessible amount of heavy metals

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Since 2014, elemental impurities (EIs) in existing and new drug products have to be assessed and controlled as described by the ICH Q3D guideline^[1] using the principles of risk management. For risk assessment, Permitted Daily Exposure (PDE) values have been established for oral, parenteral and inhalative routes and the observed or predicted level of each EI in the drug shall be related to its specific PDE.

In general, based on product knowledge, its starting materials and manufacturing methods combined with a specific analytical assessment, the total content of each metal is determined in the final product as basis for the risk assessment, thus representing a worst-case approach.

However, in special matrices, not the total amounts of EIs but rather the amounts potentially available for absorption in the gastrointestinal tract reflect the actual toxicological burden for humans from oral ingestion of the drug product.

Using the example of medicinal clay, which is traditionally used e.g. in medicinal products against digestive disorders^[2], an *in vitro* model was developed to determine the bioaccessible fraction (BF) of heavy metals in clay. Medicinal clay from geogenic origin, naturally contains heavy metals such as lead (Pb), arsenic (As), cadmium (Cd), nickel (Ni), cobalt (Co) or vanadium (V)^[2], all of which fall under the scope of the Q3D regulation. Being a fine, porous powder, medicinal clay possesses a high binding capacity and tends to retain compounds such as metals in the gastrointestinal tract^[2]. Therefore, total metal content significantly overestimates the actual risk. Only the fraction of heavy metals released from the clay into the digestive juices is potentially available for internal absorption and thus represents the basis for risk assessment^[3,4].

The BF of the elements was determined using an *in vitro* digestion model mimicking the biochemical conditions in the human gastrointestinal tract. DIN 19738^[5] outlines such a model for environmental soil samples, which was adapted and validated for the clay matrix. The method implies a two-step extraction simulating gastric and subsequent intestinal digestion using synthetic digestive juices. The BF of the heavy metals was then compared with the total contents of the clay samples. Results from six representative batches of medicinal clay revealed the BF of the investigated metals in medicinal clay to be far lower than their total contents, thus going along with a reduced heavy metal exposure. The digestive bioaccessibility of Pb was found to be 17% at maximum. The further BFs were determined to be 75% (Cd), 6% (As), 15% (Ni), 41% (Co) and 10% (V).

The results of this study clearly demonstrate that the heavy metal exposure by ingestion of medicinal clay is much lower than hitherto suspected. Therefore, we recommend element specific correction factors when relating analytical results to the given PDE in order to estimate the risk from heavy metal uptake via medicinal clay.

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P23-39

Can We Get the Full Picture? Comparison of a Series of Volatile Chemicals' Action on Gene Expression in A549 Cells as Part of a Read-Across Approach*W. Zobl¹, C. Drake¹, M. Wehr¹, T. Hansen¹, J. Knebel¹, J. Koschmann², M. Niehof¹, D. Ritter¹, S.E. Escher¹¹ Fraunhofer Institute for Toxicology and Experimental Medicine, Chemical Safety and Toxicology, Hannover, Germany;² geneXplain GmbH, Wolfenbüttel, Germany

Aim: This case study examines the extent to which whole-genome transcriptomics are capable of generating a comprehensive picture of the induced biological processes within read-across groups of volatile compounds, thereby increasing confidence in the assessment that no relevant differences in the mechanism of action of structurally similar compounds have been overlooked. We apply the concept to distinct groups of volatile compounds with both similar structure and shared lead effects observed in *in vivo* toxicity studies with repeated dose inhalation exposure.

Background: Under several regulations, Read-Across can be used for hazard assessment as an alternative to animal testing. In essence, the idea is to infer toxicological properties of data poor target compounds from analogue data on structurally similar source compounds. However, it is often a challenge to show that two or more compounds cause the same type of toxicity through a common mechanism of action without overlooking other effects. *In vitro* studies can generate additional lines of evidence, which can strengthen a judgement on biological similarity.

Materials and methods: A549 cells cultured in an air-liquid interface setup were exposed to three groups of structurally similar compounds with known inhalatory repeated dose toxicity *in vivo*. For each compound, gene expression was analysed after three days with 1h daily air-borne exposure at three concentrations each using TempO-Seq[®] probe-based whole genome sequencing. Differentially expressed genes (DEGs) were evaluated for their consistencies and divergences within and across compound groups. Resulting compound profiles were characterised in terms of pathways enriched in Reactome^[1] and TRANSPATH[®]^[2]. Further, highly interconnected gene products, which were likely to regulate part of the observed differential expression through transcription factors were traced using TRANSFAC[®]^[1] and TRANSPATH[®].

Results: Differential gene expression analysis revealed evidence for a high mechanistic similarity of tested volatile vinyl esters and secondary amines in a relatively realistic scenario of inhalatory exposure. These findings were detailed and put into context by enriched pathways and enriched regulators of the measured gene expression. Altogether, conclusiveness and differences of these different layers were evaluated as well as uncertainties involved in judgements on biological similarity of the tested compounds.

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P23-40**An *in vitro* approach to determine the human relevance of anti-spermatogenic effects of an industrial solvent in rat reproductive toxicity studies.**

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Reduced sperm counts were observed in male rats in an OECD 422 guideline study following repeated administration of 50 – 500 mg/kg/day to an industrial solvent. However, the observed effects on fertility were not dose-dependent and the mode of action (MOA) for anti-spermatogenic effects is unknown, hindering interpretation of these data for human risk assessments. In a subsequent OECD 443 study with 10 weeks pre-mating exposure, spermatogenic effects were more pronounced. We used *in vitro* approaches to evaluate the MOA and directly test interspecies susceptibility using organotypic *in vitro* models of rat, non-human primate, and human spermatogenesis. Human and rat *in vitro* metabolism studies were performed to support *in vitro* – *in vivo* (IVIVE) modeling and species comparison based on external dose (oral equivalent doses; OEDs) to account for pharmacokinetic and pharmacodynamic differences. Chemicals may affect sperm maturation directly, or indirectly through the somatic environment. Thus, dose-response data were collected in both monoculture germ cells and an 3D seminiferous tubule co-culture model that recapitulates the interaction between somatic environment and germ cells. The rat co-culture demonstrated dose-dependent decreases in secondary spermatocytes (OEDs >= 89 mg/kg/day), and reduced expression of mRNA markers of spermatogonia, pachytene spermatocytes, and round spermatids (OED = 267 mg/kg/day), consistent with *in vivo* observations. In contrast, the monkey co-culture did not show dose-dependent decreases in these mRNAs, but rather demonstrated increased trends. The human germ cell monoculture model showed no toxicity at doses <= 300 uM (OED = 86 mg/kg/day). Effects observed in the rat *in vitro* co-culture were not observed in the monkey at OEDs <= 1376 mg/kg/day, which is higher than the maximum tolerated dose *in vivo*. These quantitative and qualitative differences in the rat and primate response to the compound indicate that *in vivo* rat anti-spermatogenic effects are not likely to be relevant to humans.

P23-41**WGCNA *in vivo* rat liver co-expressed modules to derive human *in vitro* reporters associated with pathology**

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Animal testing *in vivo* is the standard for safety assessment of drugs and chemical, but translation of risk assessments to human is uncertain. Testing in human cells *in vitro* can solve the species translation problem but *in vitro* models might not replicate *in vivo* complexity. Herein, we propose a pipeline that addressed both these translational problems and derive biomarkers relevant for *in vivo* liver injury, but applicable to *in vitro* human earlier screening safety phases, impacting both efficiency and efficacy of drug safety testing.

We applied weighted gene co-expression network analysis (WGCNA) on the publicly available TG-GATEs dataset of rat *in vivo* liver

including more than 150 compounds^{1,2}. We obtained co-regulated clusters of genes (modules), to bridge individual gene variations to emergent global properties as key events in a toxicity pathway. Modules are functionally annotated and allow dynamic visualization of the transcriptome under experimental conditions (compound, dose, time) in a dedicated R Shiny App, called TXG-MAPr. Modules activation or repression is used to model the presence of absence of drug induced liver pathologies, defining modules, and the mechanisms represented by the networks, statistically associated with toxicity phenotypes³. We identified the module including DDIT3, a major player of endoplasmic reticulum (ER) stress response, and a module including ATF4 regulated genes, hub downstream the ER stress and the integrated stress response (ISR), as associated with the occurrence of hepatocellular single cell necrosis (apoptosis) in rat *in vivo*. To determine if this response is preserved in human liver models, we assessed preservation of the rat module using large transcriptomic datasets obtained from Primary Human Hepatocytes and HepG2⁴. The ATF4 and DDIT3 modules were preserved in both human pre-clinical systems, and the module member genes are induced similarly by the same compounds (e.g. Tunicamycin, Diethyl Maleate). We identified TRIB3 as a novel candidate ISR and ER stress biomarker related to an adverse phenotype representative of the module response, and developed a BAC-GFP HepG2 to monitor TRIB3 induction in early drug screening. Compounds positive for TRIB3 and single cell necrosis induction *in vivo* activate the HepG2 TRIB3-GFP response.

In conclusion, we demonstrate a pipeline to derive translationally conserved biomarkers that bridge the species and *in vivo*-to-*in vitro* gaps, and suggest that the ATF4-dependent ISR/ER stress can be monitored by TRIB3 induction in a relevant human model as an indicator of a stress response associated with *in vivo* adversity.

The work received funding from EU-ToxRisk project (grant agreement No 681002) and the Innovative Medicines Initiative 2 (IMI2) Joint Undertaking for the TransQST (agreement 116030) and eTRANSAFE (agreement 777365) projects. This Joint Undertaking receives support from the European Union's Horizon 2020 research and innovation program and EFPIA.

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P23-42**A review of hazards and risks associated with Indonesian jamu aiming to promote its safety in use**

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Although most people and even some medical practitioners assume that jamu, representing a traditional Indonesian medicine system, has fewer side effects than conventional drugs, the potential health

risks associated with the use of jamu cannot be neglected. The toxicity data and knowledge on the occurrence of adverse reactions as a result of jamu consumption are still limited. As a result, the increasing use of jamu has raised concerns among scientific and regulatory communities especially given the case studies reporting adverse health effects resulting from misuse, misidentification of the botanical species or (deliberate) contamination with extraneous plants or bioactive constituents. The present review presents an overview of the current state-of-the art related to the hazards and risks of active pharmaceutical ingredients (APIs) as well as of constituents that are genotoxic and carcinogenic reported to be present in jamu. We evaluated the potential risks for consumers upon use of the adulterated jamu by comparing the intake estimates to existing health-based guidance values (HBVGs). The risk assessment of compounds that are both genotoxic and carcinogenic was performed using the Margin of Exposure (MOE) approach. Based on the overview thus obtained it appears that drug-adulteration presents important hazards responsible for potential adverse effects, due to overdosing. The potential hazards of exposure to APIs mainly relate to the presence of botanical constituents that may cause liver damage, renal impairment, kidney failure, or steroid dependence. Genotoxic carcinogens may raise concern when the MOE resulting from prolonged daily intake from use of jamu is below 10000. This may be the case for jamu containing alkenylbenzenes, pyrrolizidine alkaloids, and/or aristolochic acids. It is concluded that risk management actions including prioritizing regulatory actions to reduce potential risks connected to the exposure to genotoxic carcinogens via consumption of jamu, is urgently required to support the safety in use of jamu. A collaboration between farmers, manufacturers/producers, academia, risk managers, government, health professionals and consumers may be essential to prevent the risk of potential adverse health effects of these botanical preparations on the Indonesian market.

P23-43

The re-evaluation of sweeteners by the European Food Safety Authority (EFSA)

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Purpose: This presentation aims at providing an overview on the current and future EFSA's work on the re-evaluation of sweeteners as well as at elucidating the main steps of the protocol on the assessment of the hazard identification and characterisation of the sweeteners. According to Regulation (EC) No 1333/2008^[1], all food additives permitted before 20 January 2009 should be subject to a new risk assessment by EFSA and a programme for the re-evaluation of approved food additives has been set up by Commission Regulation (EU) No 257/2010^[2]. The sweeteners to be re-evaluated are fifteen substances: sorbitols (E 420); mannitols (E 421); acesulfame K (E 950); cyclamates (E 952); isomalt (E 953); saccharins (E 954); sucralose (E 955); thaumatin (E 957); neohesperidine DC (E 959); neotame (E 961); salt of aspartame-acesulfame (E 962); maltitols (E 965); lactitol (E 966); xylitol (E 967) and erythritol (E 968).

Methods: In order to gather all information available, EFSA launched calls for data to invite the interested business operators to submit all data available (covering both technical and biological/toxicological^[3,4] data as well as occurrence data^[5]). These data will be complemented with any relevant literature published since the latest opinions of the Scientific Committee on Food (SCF) or EFSA. To ensure impartiality and methodological rigour along the process, two protocols have been developed: one on the assessment of the hazard

identification and characterisation of sweeteners^[6], and the other one focussing on the exposure assessment^[7]. Both protocols underwent a public consultation period and the comments received were considered in the finalisation of the two protocols.

Results: In this presentation, the main features of the protocol on hazard identification and characterisation, summarising the different steps to be applied during the risk assessment, will be presented. This protocol defines the strategy to be applied: addressing the questions to be answered (problem formulation), collecting (Extensive literature search) and selecting data (screening the studies for relevance), appraising the relevant evidence (evaluation of the risk of bias and data extraction), and analysing and integrating the evidence (weighing and synthesis of the body of evidence) in order to draw conclusions that will form the basis for the scientific opinions. Some developments on the implementation phase of the protocol on hazard identification and characterisation will be also presented.

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P23-44

A Risk Assessment Workshop inspired by a EUROTOX Specialized Course

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In 2018, the Slovenian Society of Toxicology hosted a EUROTOX Specialized Course – Regulatory Toxicology (SCRT). A five-day course, with 19 one hourly lectures, five working group sessions, five reports of outcome and a written exam aimed to provide knowledge and understanding of methods for toxicological risk assessment (RA) in regulatory processes for different categories of chemicals. The lectures were followed by practical work in five study groups. The course was attended by 31 participants from six different countries (<https://www.dropbox.com/s/a2s4hvlxspao0ix/Eurotox%20Course%20REg%20Tox%202018%20.pdf?dl=0>). At the end of the course, the participants filled in an evaluation questionnaire. Overall, the participants were very satisfied with the course organization and its contents; in particular the quality of lectures, practical exercises and work in study groups. A vast majority (96%) of the participants would recommend the course to their colleagues.

The positive experience with the EUROTOX SCRT inspired us to design a one-day risk assessment workshop in 2019. The registered participants were advised to study the handouts of the EUROTOX SCRT in advance, and to indicate their preference for the foreseen practical exercises. Following an introductory lecture on principles of RA the 35 participants were allocated into six groups for the practical exercises, namely: 1) classification of a chemical X* based on the available genotoxicity studies, 2) exposure assessment to a plant protection product Y* for given scenarios, 3) RA of cadmium as a food contaminant, 4) RA of nano-form titanium dioxide in a cosmetic product, 5) RA of lead using predictive model for internal dose based on soil monitoring data, 6) RA of bisphenol A based on human biomonitoring data. Each group was supplied with the relevant literature and exercise data. The groups had four hours to solve their exercises and to prepare presentations under the supervision of respective tutors. After the presentations and the discussions, the participants were asked to evaluate the workshop. Only 24 returned the evaluation questionnaires. The workshop contents, the tutors and the organization were marked as excellent in 79%, 92% and 71%, respectively. Methods of tuition were given excellent marks in 63%, very good in 29%, good in 8%; usefulness excellent in 62%, very good in 17%, good in 17%, poor in 4%. The overall assessment of practical exercises was marked as excellent in 59%, very good in 25%, good in 8%, no answer in 8%. The majority of participants (92%) reported that their expectations were met, and that they would like to attend further workshops of the kind. The results of evaluation feedback prompt us to recommend the inclusion of toxicological RA workshops into a curriculum of a newly established School of Public Health at the National Institute of Public Health.*The names of chemicals were not disclosed as the exercises 1 and 2 were created from confidential data.

P23-45

This abstract has been withdrawn.

P23-46

Derivation of the no expected sensitization induction level for dermal quantitative risk assessment by the Research Institute for Fragrance Materials

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Some fragrance ingredients may have the potential to induce skin sensitization in humans but can still be safely formulated into consumer products. Quantitative risk assessment (QRA) for dermal sensitization is required to determine safe levels at which potential skin sensitizers can be incorporated into consumer products. The no expected sensitization induction level or NESIL is the point of departure for the dermal sensitization QRA. Sensitization assessment factors (SAFs) are applied to the NESIL to determine acceptable exposure levels at which no skin sensitization induction would be expected in the general population. The purpose of this work is to elucidate the key steps involved in the derivation of the NESIL for use in the QRA of fragrance ingredients. Generally, this process involves, collating and analyzing all available historical data on a given fragrance ingredient, determining the chemical's potency, and finally deriving a weight of evidence (WoE) NESIL. When a material is determined to present a skin sensitization hazard, the WoE NESIL is set based on a NOEL (no observable effect level) generated from a confirmation of no induction in humans (CNIH) study. When limited or no information is available on a chemical, a read-across may be used to fill the data gap or the dermal sensitization threshold (DST) approach may be applied where appropriate before further testing is considered. We provide a few case studies illustrating how the WoE approach is

applied to generate a NESIL for fragrance ingredients. These case studies demonstrate how target data, read-across, or the DST are considered when setting a NESIL and also highlight the importance of using a WoE approach to set a NESIL. We outline the process as it currently stands at the Research Institute for Fragrance Materials (RIFM), but it is dynamic and is bound to change with evolving science as new approach methodologies (NAMs) are actively incorporated.

P24 – Gut microbiota and toxicity

P24-01

Microbiota release of bound mycotoxins contributes to human exposure: *in vitro* and *in vivo* evidence

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Mycotoxins are toxic fungal metabolites in cereal foods and pose a potential health risk for consumers. In addition to fungal mycotoxins, plant-derived mycotoxin metabolites are frequently found in cereals. These mycotoxin sugar-conjugates may contribute to overall exposure and toxicity in humans. Our previous work used a range of *in vitro* model systems to predict the intestinal fate of masked mycotoxin-glucosides in the human gut. We have demonstrated that DON-3-glucoside, the most commonly detected mycotoxin-conjugate in cereals, is stable under small intestinal conditions and is not absorbed through the intestinal epithelium intact^[1]. Following transport to the large intestine and contact with the gut microbiota, DON-3-glucoside could be rapidly hydrolysed and free DON released, as demonstrated in human faecal batch cultures. However, the *in vivo* degradation of DON-3-glucoside and absorption from contaminated food has not been demonstrated in humans. Hence, this study assessed the exposure of human volunteers to DON from barley porridge naturally contaminated with DON-3-glucoside at a low, safe level.

Following 3 days of cereal-restricted diet, 9 healthy fasted volunteers (3 male, 6 female) consumed one portion of barley porridge containing 664 ng DON-3-glucoside. All urine excreted was collected at defined time points (1, 3, 5 and 24 hours post-dosing) and a pooled urine sample was collected overnight. Urinary excretion of free DON was monitored using an established extraction and LC-MS/MS method⁽²⁾. Results for average urinary DON in early time points (1–5 hours) were compared to overnight samples and 24 hour time points by ANOVA and post-hoc t-test.

From *in vitro* results we found that DON-3-glucoside is not absorbed in the small intestine while free DON is rapidly transported through the gut epithelium. This finding is confirmed *in vivo* where only small proportions (average 9–20%) of the DON-3-glucoside dose were excreted as DON in the early urine samples 1–5 hours post-dosing. The majority of urinary DON was detected in the overnight sample (average 81% of dose, $p < 0.05$ compared to 1–5 hour time points), suggesting the release of DON from DON-3-glucoside in the lower GI tract. Marked inter-individual differences were observed in the kinetics of DON excretion in human urine in the present study, which also confirms previous *in vitro* results highlighting different DON-3-glucoside hydrolysis rates between microbiota samples from different donors.

In summary, we demonstrate for the first time that exposure to DON-3-glucoside through contaminated food results in urinary excretion of free DON through microbial release and colonic absorption. This finding confirms that sugar-bound mycotoxin conjugates can contribute to mycotoxin exposure and toxicity in humans and need to be included in future risk assessments.

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P24-02

The safety of artificial sweeteners examined by an integrated metabolomics and gut bacterial profiling approach of Acesulfame K and Saccharin treated Wistar rats

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Gut bacteria are known to play key role in the host metabolism and physiology. Hence, it makes it vital to understand the metabolic functions of the gut microbiota to elucidate better, its role in host metabolism. Omics technologies promote better extrapolation of the gut microbial functionality. Here, we examine the effects two doses of artificial sweeteners, Acesulfame potassium (Ace-K) and Saccharin on the metabolomes (plasma and feces) and fecal 16S bacterial composition to elucidate their influence on the gut microbiome.

Ace-K was gavaged in two doses 40 & 120 mg/kg body weight/day and Saccharin, 20 & 100 mg/kg body weight/day respectively in a 28-day oral toxicity study with Wistar rats following OECD 407 guidelines. Blood plasma samples were collected on days 7, 14 and 28, while feces at day 28. Targeted MS-based metabolome profiling of plasma and feces and a fecal 16S marker gene sequencing was conducted. For both sweeteners treatments, specific plasma metabolic patterns could be identified.

The results indicated that the sweeteners induce very minor changes in the gut microbiota according to the diversity- and relative abundance analyses. In line with this, fecal metabolomes of sweeteners-treated animals also showed very low alterations compared to control animals. In contrast, the plasma metabolomes of the sweeteners treated animals show the more profound alterations. Saccharin induced about three fold higher statistically significant changes than Ace-K in both sexes. Saccharin treatment significantly altered metabolites belonging to amino acids, lipids, energy metabolism, bile acids and related classes. Differences between males and females could be observed in the regulation of conjugated primary and secondary bile acids, an upregulation in these metabolites was observed in females, whereas downregulation in males. Interestingly, plasma metabolome profiles of Ace-K treated rats also had a sex-based effect. In males, larger alterations in glycine conjugated primary and secondary bile acids could be observed compared to females. Previously established plasma biomarkers known to be associated with altered gut composition (indole derivatives, hippuric acid) did not show any change, this too is in line with the conclusion that Ace-K and Saccharin have very low/no influence on the gut microbiota. Although both the sweeteners showed no/low effects on the fecal microbiota and metabolite profiles but had a clear sex-based impact on the conjugated bile acid levels in plasma. MetaMapTox, an in-house database, which consists of metabolic profiles from 1000+ compounds including agro-chemicals, pesticides, antibiotics, was used

to predict the modes of action of the two sweeteners and we found lack of association to any kind of toxicity, for the doses administered in the study. Overall, there were no indications that the plasma metabolome changes should be considered as adverse, but rather as an adaptation to the administration of the sweeteners.

P24-03

Uptake, fate, and gut toxicity of perfluorooctanoic acid (PFOA) *in vitro*

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In human, dietary intake is the main route of exposure to perfluorooctanoic acid (PFOA). Gut is the first barrier against PFOA, as well as its first target. Surprisingly, little is known regarding its effects on the intestinal barrier. Our objective was to evaluate *in vitro* the uptake, fate, and toxicity of PFOA after an acute or a repeated exposure on human intestinal cells.

Caco-2 cells and a co-culture of Caco-2 with mucus-secreting HT29-MTX cells (90/10) were considered. Cells were grown on 96-well plates or Transwells until full differentiation (day 21), and exposed apically for 24 h to PFOA (0.1–100 µM). Dose-response viability was determined. [¹⁴C]-PFOA distribution was investigated in apical and baso-lateral (BL) compartments, inserts and cells, by liquid scintillation counting; [¹⁴C]-PFOA metabolism was evaluated by radio-HPLC profiling. Regarding repeated exposure, cells were grown on Transwells and exposed apically to PFOA every two days from day 10 to day 21. Transepithelial electrical resistance (TEER) was continuously monitored. At the end of the exposure period, cell viability was measured; expression of tight junction (TJ) and proliferation genes was determined by qPCR. After exposure to PFOA 100 µM, Caco-2 cells were prepared for high-resolution chemical imaging, based on Electron Microscopy (EM) and Secondary Ion Mass Spectrometry (SIMS).

Acute exposure to PFOA had no effect on cell viability even at PFOA 100 µM. Regarding PFOA distribution, no concentration effect was observed whatever the cell model. Total radioactivity recovery was equal to 97.5 ± 3.6%; radioactivity in the BL compartment was higher for the Caco-2 cell monoculture (57.9 ± 5.5%) than for the co-culture (36.8 ± 1.2%). No [¹⁴C]-PFOA metabolite was detected on radio-HPLC profiles in both compartments, showing that intestinal cells do not metabolize PFOA. During repeated exposure of Caco-2 cells to PFOA, a dose-dependent TEER stabilization over time was found, which was correlated to a significant decrease in *Ki67* gene expression; no cell viability decrease nor alterations in TJ gene expression were observed. SIMS high-resolution imaging enabled to localize PFOA at the subcellular level with a preferential localization in the cell cytoplasm. PFOA was also localized in the insert membrane, suggesting its capacity to pass through the cell monolayer consistently with radio-HPLC profiling results.

In conclusion, [¹⁴C]-PFOA distribution within compartments was different according to the cell model. Viability of Caco-2 cells after acute or repeated exposure remained unchanged. Expression of TJ genes was not affected. However, a dose-dependent TEER stabilization over time was observed, related to a decreased *Ki67* gene expression. This altered cell proliferation should be further investigated. The applicability of high-resolution chemical imaging to detect and localize PFOA was successfully tested and provided insights into its uptake mechanisms and intracellular fate.

P24-04**Interindividual differences and similarities in *in vitro* human gut microbial degradation of fructoselysine and carboxymethyllysine**

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The human gut microbiome plays an important role in host health, and microbial metabolic activity complements host physiology. Also, certain foodborne xenobiotics can be (de)toxified or (in)activated by the gut microbiome, which in turn can have consequences for host health. Therefore, interactions of the human gut microbiome with foodborne xenobiotics are relevant to consider, particularly when assessing toxicokinetics in novel assessment methods and alternative test strategies. The Amadori product fructoselysine is one of the dietary relevant precursors in the formation of the abundant advanced glycation end product (AGE) carboxymethyllysine. AGEs are formed upon heating and processing of food products. They are widely present in the Western diet and have been associated with several (chronic) diseases and inflammation. Therefore, AGEs are considered potentially hazardous to human health.

The human gut microbiome can degrade fructoselysine and carboxymethyllysine. To study interindividual differences in these microbial reactions, we developed and optimized an *in vitro* model based on anaerobic incubations using human fecal slurries. For fructoselysine, kinetics of degradation followed Michaelis-Menten saturation and were quantified, and substantial interindividual differences in the ability to degrade fructoselysine were elucidated. Of the samples collected from 16 individuals, 11 were able to degrade fructoselysine, with $V_{\max}$, K_m , and k_{cat} values that differed up to 15-fold, 10-fold and 4-fold, respectively. Bacterial taxonomic profiling of the fecal samples by 16S rRNA gene sequencing revealed that the relative abundance of 15 genera from 9 families differed statistically significantly between the degraders and non-degraders. The genus *Ruminococcus_1* showed the strongest correlation ($\rho \geq 0.8$; $P < 0.05$) with individual $V_{\max}$ levels of fructoselysine degradation and accompanying butyrate production, implying a possible role in these reactions [1]. Carboxymethyllysine degradation as well as fructoselysine degradation was studied using individual samples from another 20 donors, also revealing large interindividual differences. Further analyses revealed no correlation between an individual's ability to degrade carboxymethyllysine and fructoselysine.

Overall, it was concluded that the presented *in vitro* model is a useful tool to study, quantify, and profile human gut microbial metabolic activities, which, depending on the substance in question, can be a relevant addition to novel assessment methods.

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P24-05***In vitro* modeling of inflammation-induced intestinal barrier damage and repair with therapeutic candidates**

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Proper functioning of gastro-intestinal tract depends on the functional physiology and intactness of mucosa lining of the gut. Disruption of the intestinal barrier integrity can cause inflammation in patients that can lead to chronic diseases such as trauma or inflammatory bowel diseases. To mimic disrupted intestinal barrier *in vitro* we exposed EpiIntestinal (*in vitro* reconstructed 3D human small intestine epithelium) to various combinations of cytokines that were previously shown to play a role in the pathogenesis of inflammatory bowel disease. Prolonged exposure of EpiIntestinal tissue to the combination of tumor necrosis factor alpha (TNF α) and interferon gamma (IFN γ) results in approximately 30% decrease in barrier integrity as established by measurement of trans-epithelial electric resistance (TEER). This decline was associated with induction of several proinflammatory cytokines including interleukins 6 and 8 (IL6, IL8). Besides the effect on intact intestinal epithelium, treatment with TNF α and IFN γ hindered reconstitution of mechanically injured/wounded tissues. In cytokine treated tissues wounds were healing much slower than controls and newly grown tissue appeared structurally different. To test if this model of inflammation-induced barrier disruption can be used to screen potential therapeutic compounds, we exposed wounded tissues to relevant concentrations of anti-inflammatory compounds. Most of the tested candidate drugs were capable to counteract the disruption of barrier integrity. In addition two of them also shown the ability to promote healing of tissues exposed to the combination of mechanical wound and cytokines. In short, the reconstructed 3D small intestine epithelium model (EpiIntestinal) can be used to mimic the intestinal barrier disruption associated with chronic inflammation, which may be useful in identification of potential therapeutic agents.

P24-06**Impact of polyethylene microplastics on human gut microbiota as assessed in an *in vitro* gut model**

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Purpose: For the last half-century, the global plastic production dramatically increased leading to a significant accumulation into the environment. Polyethylene (PE) is one the most produced plastic worldwide. Plastics can be degraded into smaller pieces under 5 mm (microplastics, MPs) leading to the contamination of various environmental media and ultimately the food chain. MPs enter the digestive tract with food and drinks but also air via mucociliary clearance. However, to date, their fate and impact on the human digestive tract clearly remain under-studied, especially interactions between MPs and gut microbiota.

Methods: The impact of chronic exposure to commercially available PE MP (1-10 μ m) on the human gut microbiota composition and metabolic activity was evaluated by using the Artificial Colon system (ARCOL). ARCOL is a one-stage fermentation system which integrates the main physicochemical (pH, temperature, retention time, ileal ef-

fluent composition and anaerobiosis) but also microbial (luminal and mucus-associated microbiota) parameters of the human colon. Bio-reactors were inoculated with fecal samples from four adults (two men, two women) ranging in age from 23 to 52 years old (four biological replicates). After one-week stabilization of the microbiota in the bioreactor, PE MPs (21 mg) were daily injected for 14 days. Samples were regularly collected from the atmospheric and luminal/mucosal phases for determination of microbiota composition (qPCR on targeted populations and metabarcoding 16S) and activity through short chain fatty acid (SCFAs) and gas measurement. Luminal and mucosal media were observed by scanning electron microscopy (SEM) before and after PE MP exposure.

Results: In the present study, the repeated exposure to PE MPs neither affected gas and SCFA total production and profiles. qPCR analysis indicated no impact of PE MP exposure on total bacteria. Sequencing analysis also revealed no effect of PE MPs on microbial diversity indexes, but an influence on human gut microbiota structure at the family and genus levels (not phylum) that was individual-dependent. SEM showed bacteria from the digestive lumen adhering to the PE MPs. These first results highlight that PE MPs, in their pristine form, don't seem to induce major perturbations in adult human gut microbiota. Reciprocally, next analyses will aim to unravel gut microbiota ability to modify physico-chemical characteristics of PE MPs (e.g. formation of biomolecular corona). The potential of the AR-COL model could be extended to the simulation of specific digestive conditions such as the infant at-risk population.

P24-07

Dual DNA and DNA repair protein damage by acrolein in colon epithelial cells

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Acrolein is a ubiquitous environmental toxicant in frying oil, automobile fumes and cigarette smoke. It has been discovered recently that acrolein is produced by human gut bacterial strains through bacterial glycerol metabolism, creating a potential source of continuous exposure of the intestinal epithelium to acrolein. Acrolein can react with biomolecules, including DNA and proteins, to form covalent adducts. The adverse effects of acrolein-DNA adducts (Acr-dG) are thought to be countered by the nucleotide excision DNA repair pathway, which relies on initiation by the repair recognition protein XPA. However, XPA has been shown to degrade after exposure to acrolein. Consequently, acrolein may have a combined toxic effect by damaging DNA and degrading the DNA repair enzymes needed to repair the damage. However, the relevance of this process in intestinal cells, which may be exposed to continuous low levels of acrolein due to microbial metabolism is not known. The goal of this project was to compare how DNA damage and repair factors are impacted by acute vs. continuous exposure of intestinal epithelial cells to acrolein. Thus, We exposed human colorectal adenocarcinoma cells (SW480 cell line) to acrolein in two ways: 1) a single time point exposure and 2) a cell-culture-compatible acrolein bioproduction system used to simulate continuous exposure. Acr-dG adducts were measured by LCMS. In the acute exposure scenario, adducts formed in a dose-dependent manner, with the lowest observable adduct formation level being at 50 mM acrolein. With the acrolein bioproduction system, Acr-dG adducts were observed to form at levels equivalent to those from acute exposure concentrations of 50–75 uM acrolein. Furthermore, a dose-dependent degradation of XPA was observed following exposure to acrolein. These results suggest colon cells are susceptible to the dual detrimental effects of DNA damage formation and compromised repair due to Acr-dG adduct formation and degra-

dation of repair enzymes. Furthermore, the data validate a novel bioproduction method for *in vitro* studies of the effects of continuous exposure to acrolein. Further investigation using the enzyme-based continuous acrolein exposure technique are expected to advance knowledge concerning the toxicological implications of microbial production of acrolein and the combined effects of DNA and DNA repair protein damage in intestinal cells.

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P24-08

DNA and protein adducts form from acrolein produced by the human gut bacteria *Limosilactobacillus reuteri*

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Humans are exposed to acrolein from the dietary intake of fried food, charred meat and alcoholic beverages, exposure to automobile exhaust, cigarette smoke and wood burning. Industries manufacturing plastics and acrylics may emit acrolein into the environment as a waste product. It is also a metabolite produced endogenously from lipid peroxidation, polyamine catabolism, threonine utilization and the catabolism of drugs. Recently, specific strains of gut bacteria have been found to encode a glycerol diol dehydratase (GDH) that transforms glycerol to 3-hydroxypropionaldehyde (3-HPA), which can spontaneously form acrolein. As an electrophilic α,β -unsaturated aldehyde, acrolein reacts with nucleophilic biomolecules as a molecular initiating event producing reactive oxygen species and DNA and protein adducts. These reactions can lead to cell death and mutations, thus potentially provoking the onset and development of neurodegenerative disorders, cancer, and cardiovascular diseases. However, to our knowledge, the reaction of bacterially released acrolein with proteins and DNA has not been reported. Therefore, we tested the formation of the guanosine-acrolein adduct γ -hydroxy-1-N2-propano-2'-deoxyguanosine (γ -OH-Acr-dG) and lysine-acrolein adduct N-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine) when calf-thymus DNA or bovine serum albumin (BSA) were allowed to react with two known acrolein-producing pro-biotic *Limosilactobacillus reuteri* bacterial strains individually in the presence of increasing concentrations of glycerol. Experiments were performed in the absence of bacteria or glycerol as negative controls. γ -OH-Acr-dG adduct levels was determined by LC-MS and FDP-lysine was monitored by immunoassay. When *L. reuteri* strains were grown in the presence of increasing concentrations of glycerol (0, 75, 150, 300 or 600 mM), 3-HPA and acrolein were produced in a dose-dependent manner after 2 h. When we exposed each strain to DNA in the same experimental conditions, we observed that the formation of γ -OH-Acr-dG adducts increased when additional glycerol was present. When each strain was incubated with BSA and increasing concentrations of glycerol, a dose-dependent increase in the levels of FDP-lysine adducts was observed for only one strain. We confirmed that no adducts were detected in the absence of bacteria or glycerol. These results indicate that acrolein produced from glycerol catabolism by gut bacteria can react with DNA and protein, forming adducts as a potential initiating step for adverse biological effects. Moreover, we developed an experimental approach that can be used to further investigate adduct formation by bacterially produced acrolein. Further work is needed to address the relevance of this process in cells to adequately account for acrolein production by glycerol-catabolizing probiotic strains in the assessment of their safety.

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P25 – Mixture toxicology

P25-01

Tentative assessment of recycled post-consumer plastic for packaging of cosmetic products

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Post-consumer recycled plastic (PCR) may contain and release thousands of different substances, which may migrate to cosmetic products if the PCR is used as packaging. Many such substances represent a hazard to human and environmental health^[1,2]. The purpose of this project was to find a method to assess the safety of PCR when used for packaging of cosmetic products.

Among thirteen samples of high-density polyethylene (HDPE) and four samples of polypropylene (PP) seven were selected for screening and/or more detailed analysis. Screening of all seven samples was performed by GC/MS after extraction with isooctane and by multi-target analysis after extraction with 95% ethanol. The latter extracts were also analyzed for degradation products of antioxidants and stabilizers (PE-phenols). Four samples were analyzed for migration of polyaromatic hydrocarbons (PAH) and primary aromatic amines (PAA). Four samples were analyzed for content of fluoride and per-fluoroalkyl- and polyfluoroalkyl substances (PFAS).

None of the four samples showed a content of PFAS or fluoride. Migration of various PE-phenols were found in amounts up to 48 mg/kg product simulant, with 2,6-di-tert-butyl-1,4-benzoquinone as the highest. 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione was found to migrate in all seven samples up to 26 mg/kg product simulant. PAA was found to migrate from all four samples with 2-methoxyaniline and o-toluidine in the highest amounts (0.54 mg/kg simulant each). PAH migrated from six out of seven samples in amounts up to 6.2 mg/kg simulant, with phenanthrene as the highest. Pyrenes were not found. No hazardous metals were found to migrate, except Al, which was found to migrate in amounts up to 0.6 mg/kg simulant.

A few of the substances found were selected for safety assessment in agreement with the methods given by SCCS^[3]. Assuming use of the PCR as small packages for baby body lotion and assigning 10% of the tolerable daily intake (TDI) to cosmetic products we found that in many cases the degradation product 2,4-di-tert-butylphenol exceeds the acceptable level. Both phthalates and tris-(2,4-di-tert-butylphenyl) phosphite in some cases exceed the acceptable level, as well.

If the PCR plastic material is to be used in baby body lotion, the following criteria can be derived from the calculations:

For substances, which are easily absorbed through skin ($P=1$), and a TDI value of 1 mg/kg bw/day, the measured migrant concentration can be maximum 300 mg/kg).

For substances, which are easily absorbed through skin ($P=1$), and a TDI value of 10 mg/kg bw/day, the measured migrant concentration can be maximum 3000 mg/kg).

For substances, which are only partially absorbed through skin

($P=0.1$), and a TDI value of 10 mg/kg bw/day, the measured migrant concentration can be max. 30000 mg/kg).

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P25-02

Stereotactic surgery with frameless neuronavigation for dosing into the cerebellum – dentate nucleus – in cynomolgus macaques

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Introduction: Gene therapy utilizing adeno-associated virus (AAV) vectors is a promising area of drug development for several neurological disorders requiring direct targeting with bilateral administration of an AAV vector into the cerebellar dentate nucleus (DN).

Methods: An IACUC-approved regulatory study with single bilateral administration of a viral vector into the DN. Magnetic resonance imaging (MRI) was used to visualize the regions of interests. 26 (F/M) cynomolgus macaques (3 dose-groups; 8 days, 4, 13, 26 and 52-week recovery).

Total intravenous anesthesia (TIVA) was conducted and monitored (HR, BP, SpO₂, EEG).

After preparing surgical area, an MRI compatible post was attached to the skull with ceramic screws. A fiducial array was mounted on the post prior to imaging for co-registering of each animal to their scan. Animals were transferred to the MRI (Siemens, Aera, 1.5T) and a 3D T1 MPRAGE sequence was used with a standard knee coil (0.7mm isotropic voxels, 5 averages, 30 min).

Following MRI, animals were transferred back to surgical suite and imaging data was loaded into neuronavigation software (Brainsight®). Targets and trajectories to targets were defined and utilized to ensure correct placement of catheters (MRI Interventions, NGS-NC-01). A position sensor was used to aid with co-registration of images to the animals. All critical structures including sinuses, skull and dentate nuclei were identified in 3D. Animals were placed in a 4-pin surgical c-clamp to keep the head rigid during surgery. A median suboccipital approach was performed to expose the occipital bone. Surgical tools were tracked in real-time with an adjustable surgical arm and locked in place when the predetermined trajectory to DN was identified in the software. A 4mm burr hole was drilled in the skull bilaterally (Aesculap, Elan 4) and an injection caliper was applied to guide the catheter to target. Catheters were connected to infusion lines containing control or test article and were inserted along the calculated trajectories and the determined depth to administer the AAV vector into the DN. Infusion rate was 0.10µL over 12 min and an additional 5 min was given following infusion before the catheter was retracted from the brain; hemispheres were dosed successively.

Wounds were closed and animals treated with analgesics/antibiotics. A post-surgery MRI scan was acquired to verify correct location of injection and to rule out any acute hemorrhage.

Results: Animals recovered fast and uneventful from a 6–8 h TIVA anesthesia. One animal encountered postsurgical hemorrhage as identified in post dose MRI. This animal showed neurological disorders at recovery from anesthesia and was euthanized. No other animal showed neurological abnormalities.

Discussion: Intracranial administrations were performed successfully into the dentate nucleus and correct targeting was verified in a post MRI. Postsurgical hemorrhage as a complication needs to be verified in post-surgery MRI scan.

P25-03

Real-life PFAS placenta mixtures and estrogen activities

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Introduction: Perfluoroalkylated substances (PFAS) are a diverse group of human-made chemicals used in a wide range of consumer products, including food packaging, cosmetics and impregnation products. Humans are exposed to PFAS mainly through food and drinking water. Both human and *in vitro* studies of individual PFAS compounds suggest that they interfere with the estrogen system. However, humans are exposed to complex PFAS mixtures where compounds have the potential to interact additively (^[1] Kjeldsen *et al.* 2013). We have previously showed that the xenoestrogenic activity of PFAS mixtures extracted from serum of 702 Danish pregnant women was associated with lower birth weight and length (^[2] Bjerregaard-Olesen *et al.* 2019).

In the present work, we have extracted real-life PFAS mixtures from human placentas and determined their estrogenic activity using two cell culture assays.

Methods: PFAS mixtures were extracted from 25 Spanish human placenta homogenates. The placenta homogenates (16 g) were dissolved in hexane, and passed through a glass column with dried aluminium oxide. The eluate was further extracted using solid phase extraction, high-performance liquid chromatography, and weak anion exchange as described in ^[3] Bjerregaard-Olesen *et al.* 2015. The estrogenic activity of the PFAS extracts was measured with the MVLN estrogen receptor (ER)-luciferase reporter gene assay and the proliferation assay (E-Screen). Correlations between the assays will be evaluated and linear regression analyses used to explore association between the PFAS induced estrogenic activity and women's characteristics and birth outcomes.

Results: In the ER-luciferase reporter gene assay, 13 of the 25 (52%) PFAS placenta extracts elicited significant ER activity when tested alone. The median fold induction was 114% (93%–128%). In the presence of 17 β -estradiol (E2), 17 of the 25 (68%) extracts significantly enhanced the E2-induced ER activity. The median fold induction was 124% (88%–144%).

In the E-screen assay, 15 of 24 (62.5%) PFAS placenta extracts elicited significant proliferative effect. The median fold induction was 188% (100%–367%).

We will present further results at the meeting including correlations between the two assays, and between placenta PFAS concentrations and estrogenic activities of PFAS placenta mixtures, as well as associations with women's characteristics and birth outcomes.

Conclusion: Our preliminary results indicate that real-life PFAS placenta mixture display estrogenic activity, and enhance the response of E2.

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P25-04

Unravelling cocktail effects of complex chemical mixtures using integrated -omics: a case study with agricultural fungicides

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New approach methods (NAMs) based on -omics technologies are useful tools to identify biomarkers and processes regulated in response to xenobiotic exposure. Using mechanism-based approaches, we can improve the understanding of adverse effects induced by chemicals and their combined effects in mixtures, called "cocktail effects", to pave the way to improved human health risk assessment of finished products. In this study, molecular and cellular mechanisms in skin sensitization and immunotoxicity were investigated using *in vitro* transcriptomics- and proteomics-based technologies focusing on agricultural fungicides to study the cocktail effects of complex chemical mixtures.

A human myeloid cell model resembling dendritic cells (DCs) was used to investigate a selection of commercial fungicide formulations available in Sweden, and their active ingredients and adjuvants tested alone or in binary or tertiary combinations thereof (e.g. active ingredient and adjuvant) in order to resemble the commercial formulations. A transcriptomic biomarker profile associated with hazard assessment of sensitization potential was evaluated following cellular exposure. In addition, the DC activation marker CD86 expression was monitored by flow cytometry. Through a proteomics approach, we characterized the response of the DCs to the fungicide materials, exposing four independent batches of cells for 24 h.

All qualified test materials were found to induce altered transcriptional levels of evaluated genomic biomarkers, indicative of potential sensitization hazard. In the proteomics approach, more than 5000 protein groups could be identified and when comparing protein regulation induced by fungicides and formulations, respectively, using the same cut-off criteria (false discovery rate 0.01), formulations induced over 50% more significantly regulated protein groups. Protein regulation patterns after mixture and formulation exposures were complex and need to be interpreted taking cytotoxicity of the respective materials into account. Changes in line with expected (e.g. antioxidant stress response for the surfactant group) and more recently suggested protein and pathway regulations were identified such as autophagy and cholesterol homeostasis.

In conclusion, this study illustrates the increased complexity of biological pathway regulation induced by chemicals mixtures such as present in fungicide formulations. We present an overview of regulated cellular processes in response to single fungicide materials compared to binary and tertiary mixtures including e.g. adjuvants, which will be followed up by more traditional biochemical and immunological approaches to validate and complement this data. Our study supports the importance of developing approaches to predict and model product toxicities considering all ingredients in mixtures.

P25-05

Mixture effects of co-formulants and two plant protection products in a liver cell line

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Background: Plant protection products are extensively used in the agricultural sector to protect desirable plants against diseases, pests and other unwanted plants. Plant protection products consists of one or more active substances and a number of co-formulants. Active substances provide the functionality of the plant protection product and are also assumed to contribute to the entirety of its toxicity. Consequently, active substances are evaluated according to standard test methods set by legal data requirements before they are approved. In contrast, the relevance of co-formulants for toxicity when in combination is not always clear. However, mixture effects have been reported between active substances within plant protection products and with co-formulants in both *in vivo* and *in vitro* studies.

Aim: This study aims to investigate the differential effects of two plant protection products, their (hepatotoxic) active substances in combination and some of their co-formulants on the HepaRG liver cell line.

Method: The cytotoxicity of the two plant protection products, their active substances in combination, and their co-formulants on HepaRG cells, were investigated using Neutral Red uptake assays and WST-1 assay. Cyproconazole and azoxystrobin were investigated in combination and epoxiconazole and fluxapyroxad were investigated in combination, while a few known co-formulants from each plant protection product tested were investigated individually. A low density gene expression array (Human Molecular Toxicology Pathway-Finder 384HT) was used to provide information at the gene expression level from said substances. The amounts of CYP450 enzymes and transporters in 50 protein extracts from the HepaRG cultures were also quantitatively investigated. In addition, HPLC-MS-MS was employed to determine whether the uptake of the active substances was increased in the presence of the co-formulants as compared to the active substances alone.

Results: Cell viability results show a dose-response relationship for cytotoxicity in HepaRG cells with increasing concentrations of the substances used. Products were more cytotoxic than their active substances alone. Gene expression profiles in cells exposed to products and their active substances were similar with minor differences. Proteomics results show differences in CYP3A4 quantity between effects caused by products and their active substances.

Conclusion: Plant protection products can show increased cytotoxicity as compared to their active substances alone.

P25-06

Toxicokinetic mixture effects of co-formulants and active substances in plant protection products *in vitro*

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Background: Plant protection and biocidal products consist of one or more active substance(s) and a varying number of co-formulants. Currently, the authorisation process for plant protection products (PPPs) relies on the testing of acute and topological toxicity only. Contrastingly, the evaluation of active substances includes a more comprehensive set of toxicity studies. Nevertheless, mixture effects of active ingredients and co-formulants may result in increased toxicity.

Methods: Therefore, we investigated effects of surface active co-formulants on the toxicity of two PPPs focussing on qualitative and quantitative toxicokinetic effects on absorption and secretion. The respective products are based on the active substances abamectin and fluroxypyr-meptyl and were tested for cytotoxicity in presence or absence of the corresponding surfactants and co-formulants using Caco-2 cells. In addition, the effect of co-formulants on increased cellular permeation was quantified using LC-MS/MS, while potential kinetic mixture effects were addressed by fluorescence anisotropy measurements and ATPase assays.

Results: The results show that the surface active co-formulants significantly increase the cytotoxicity of the investigated PPPs, leading to more than additive mixture effects. Moreover, analytical investigations show higher efflux ratios of both active substances and the metabolite fluroxypyr upon combination with certain concentrations of the surfactants. The results further point to a significant and concentration dependent inhibition of P-gp transporters by most of the surfactants as well as to increased membrane fluidity.

Conclusion: Altogether, these findings support the hypothesis that surfactants can contribute to increased cytotoxicity of PPPs and do so by increasing the bioavailability of the respective active substances. Consequently, in these cases the consideration of toxicokinetic mixture effects is required in PPP testing.

P25-07

Beauvericin and enniatin B1: an *in vitro* integrated approach for risk assessment

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Beauvericin (BEA) and Enniatin B1 (ENN1) are considered natural contaminants of emerging concern, and data on risk are urgently required.

This study aims to evaluate potential toxicological effects of ENN1 and BEA mixture, through an *in vitro* integrated approach with human and specie-specific intestinal models.

1. Human intestinal barrier with Caco-2 cells (Sigma Aldrich 86010202) differentiated on inserts in medium with 10% fetal bovine serum (FBS) were exposed from the apical (AP, oral exposure) side to ENN1 (0.5, 1.5, 3 μ M) and BEA (0.5, 3, 6 μ M) alone or combined (ENN1 0.5–1.5–3 μ M + BEA 3 μ M), for 24 h;
2. Human intestinal barrier with Caco-2 cells differentiated on inserts in serum-free medium were exposed from the AP side to

ENNB1 (0.5, 1.5, 3, 6 μ M) and BEA (3 μ M) alone or combined (ENNB1 0.5–1.5–3–6 μ M + BEA 3 μ M), for 24 h;

- Species-specific intestinal barrier with IPEC-J2 cells (DSMZ – German Collection of Microorganisms and Cell Cultures GmbH, ACC-number: 701) cultured on inserts were treated from the AP side with ENNB1 (0.5, 1.5, 3 μ M) and BEA (0.5, 3, 6 μ M) alone or combined (ENNB1 0.5–1.5–3 μ M + BEA 3 μ M), for 24 and 48 h.

A significant trans-epithelial electrical resistance (TEER) increase was observed in Caco-2 cells differentiated in FBS-medium after exposure to the highest concentration of ENNB1 (3 μ M), while in the Caco-2 serum-free model such increase was already observed at the lowest concentration (0.5 μ M ENNB1), together with a trend of concentration-dependent increase in IL-8 secretion after 24-h exposure to ENNB1.

On IPEC-J2 model, a synergic action between ENNB1 and BEA could be hypothesized, on the TEER increase after 48-h of co-exposure. Furthermore, a positive correlation between mycotoxin concentrations and TEER values was observed with the human and species-specific models, particularly evident with respect to ENNB1.

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P25-08

Estimation of agonistic and antagonistic moieties from toxicological mixture experiments

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Endocrine-disrupting compounds are usually presented in the environment in a form of complex mixtures. These mixtures may have both agonistic and antagonistic effects on the respective toxicological receptor. The resulted dose-response curves of such mixtures are partial agonistic. We present a simple method how to analyze a partial agonistic dose-response curve to obtain the values for agonistic and antagonistic moieties only. By this method, we could estimate the effect of agonists with the absence of antagonists in a mixture and vice versa. For the validation, we measured several mixtures of 17 β -estradiol and fulvestrant by the estrogen receptor binding assay (BMAEReluc/ER α). By analyzing the resulted dose-response curves we obtain concentrations of the agonist in the mixture which were in good agreement with the added concentrations of 17 β -estradiol. The presented method is useful for the characterization of an unknown mixture. It could “filter” the effect of antagonists and estimate an estradiol equivalent (EEQ) consisting of only agonists for unknown mixtures. As a result, it may enhance the risk assessment associated with environmental samples.

P25-09

Reevaluation of Concentration addition: ligands with different maximal effects cause curvature of isoboles

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Concentration addition as a classic null model for toxicology and pharmacology is based on Loewe's mathematical formulation and the linearity of the isoboles. Novel mathematical models, however, propose curved isoboles in certain conditions. This study aims to test the hypothesis of the curvature of isoboles in experimental measurements. With the assumption of linear isoboles, a partial agonist acts as an antagonist above its maximal effect level. The isoboles automatically convert to a positive slope. For curved isoboles, a partial agonist acts as an antagonist at higher effect levels than its maximal effect alone. The discrepancies between effect levels were studied with an estrogen receptor binding assay (BMAEReluc/ER α) using a mixture of 17 β -estradiol and fulvestrant as a partial agonist. A mixture of 17 β -estradiol and fulvestrant acts as a partial agonist and causes the diminishing of the effect level of 17 β -estradiol at a significantly higher level than the maximal effect of their partial-agonistic dose-response curve. Measured, elevated effect levels were well predicted by the mathematical model. Nonlinear isoboles resulted from a non-synergistic mixture change our understanding and definition of synergism or antagonism and prompt further attention in receptor theory.

P25-10

Aloe vera gel and sea-buckthorn oil protect human fibroblasts against silver nanoparticles cytotoxicity

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Background: Bionanocomposite materials are a hybrid class of new materials, with a low negative impact on the environment and with a high positive impact in biomedical field (cellular regeneration, tissue engineering) and pharmaceutical applications (controlled drug release, gene therapy, antimicrobial properties).

Aim: We aimed to create a bionanocomposite based on collagen I (col), Aloe vera (*Aloe barbadensis miller*) and silver nanoparticles (AgNP), to combine the proliferative effect of biomaterials with the antimicrobial effect of AgNP.

Material and method: Nanocomposite gels were obtained from alginate (an algae-based biopolymer) (2%) and collagen I solution, extracted from *Hypophthalmichthys molitrix* (0.01%) homogenized by magnetic stirring at RT, at 500 rpm/15 min, followed by glycerol addition, to a ratio of 3:3:1 (vol:vol:vol/Alg:col:Gly). Subsequently, aloe vera powder, sea buckthorn oil and/or colloidal NPAg solution were added to the base solution. Homogenization of the compounds and formation of the gelled structure was obtained by centrifugation at 2500 rpm/5 min. Toxicity and regenerative properties were tested on human dermal fibroblasts (HDFa- ATCC) using LDH, MTS assays and quantification of cell division in real-time, using Biostation (Nikon).

Results: 8 different aloe vera bionanocomposite gels (out of which only 4 w/ AgNp) were obtained and tested, compared with the gel-base, as well as non-treated cells. The gels dissolved differently in complete cell culture medium and some were more prone to polymerize in solution at room temperature. Although the alginate alone slightly increased cell proliferation, when compared to non-treated control, the gel-base did not significantly influence the viability index of treated cells. The best result was obtained by addition of sea-buckthorn oil (153% increase in cell viability at 100 mg/ml gel). Addition of AgNp significantly increased cytotoxicity of this gel concentration (57% viability), but the toxicity decreased with lower concentrations. At concentrations of 100 µg/mL, none of the tested gels exerted cytotoxicity.

Conclusion: Although AgNp could be toxic for human fibroblasts, the addition of biomaterials extracted from natural sources (such as collagen, aloe vera and sea-buckthorn oil) could mitigate this effect and help create a medical device with both antimicrobial and regenerative properties.

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P25-11

Evaluation of biological and epigenetic marks after exposure of MucilAir® human bronchial epithelia to mixtures of gaseous and particulate pollutants

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Context: Ambient air is a heterogeneous and complex mixture with a high biological reactivity, considering the presence of many pollutants, which might be gaseous or particulate. Over the last fifty years, some epidemiological studies showed the prevalence of respiratory and allergic pathologies, as well as the emergence of symptoms specific to environmental pollution.

The purpose of this work is to compare the biological effects on the airways of two kinds of mixtures, one characteristic of indoor air, the Volatile Organic Compounds (VOCs), and the other one representative of outdoor air, the fine particulate matter (PM_{2.5}).

Methods: Using an original *in vitro* 3D-model adapted for chronic exposures (MucilAir® epithelium), we assessed the modulation of both biological and epigenetic responses of a human reconstituted bronchial epithelium after repeated exposures to the two mixtures of pollutants. The epithelia used were reconstituted from primary cells of healthy subjects. Environmental particles were collected in two areas from the Hauts-de-France region (France), with an industrial or traffic influence. These samples are similar in size, with a diameter between 0.3 and 2.5 µm, but are chemically different. Synthetic atmospheres comprising the main VOCs at the levels encountered in indoor air were generated using a specially developed gas generation and dilution bench.

The atmospheres tested were distributed to the respiratory epithelia using an “air-liquid” exposure system ensuring direct contact between the apical side of the epithelium and the pollutants. Successive periods of exposure and non-exposure were conducted for up to

three weeks with two exposures per week with an interval of 48 hours between exposures.

After exposures, epithelia were sacrificed and then inflammatory response, oxidative stress and epigenetics marks were evaluated using RT-qPCR and ELISA.

Results: Our preliminary results showed a different modulation of the biological response according to the nature of the pollutants entering in contact with the epithelium (gaseous for VOCs, particulate for PM_{2.5}).

The inflammatory response evaluated by gene expression (RT-qPCR) and cytokine production (ELISA) indicated an increase of IL-8 only after PM_{2.5}-exposure whereas an increase of GM-CSF was noted after exposure to each of the two pollutants. No CXCL10 gene induction was observed after PM-exposure whereas a dose and time dependent induction was detected after VOC-exposure.

Epigenetic marks assessed by the gene expression of DNA methyltransferases and Histone Deacetylases showed an induction of both DNMT3b and HDAC2 after exposure to PM_{2.5} and VOCs respectively.

This project contributes to a better understanding of the cellular and molecular effects of airborne pollutants according to their nature (particular or gaseous) on the development of respiratory pathologies.

P25-12

Endocrine disruptive potentials of pollutant mixtures present in two size fractions of indoor dust from various microenvironments assessed by *in vitro* bioassays

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People living in industrialized countries spend more than 90% of their lives in indoor environments, i.e., houses, offices, schools, cinemas, restaurants etc. Such environments may serve as a sink for a number of known and unknown chemicals which can be trapped into the dust, accumulate on surfaces and suspend into the indoor air. This complex mixture of chemicals in the dust matrix may be taken up through ingestion, inhalation or dermal absorption, representing a potential concern for the health of those exposed. Works published elsewhere have shown that the composition of the indoor dust varies significantly depending on its particle size fraction. The specific surface area of particles is inversely proportional to the particle size, thus the fine particles have higher sorption capacity for chemicals that can pose a risk for health. The current work assessed the potential endocrine disruptive effects of indoor dust from seven different microenvironments in the Czech Republic, considering two size fractions, fine with particles smaller than 0.25 µm and bulk dust (particles <2 mm) extracted with two different solvents (methanol and hexane: acetone 1:1) in order to provide a polar and non-polar fraction of the samples. The battery of bioassays examined dioxin-like activity, (anti)androgenic, (anti)estrogenic, and (anti)thyroid effects, using human cell lines stably transfected with luciferase reporter gene under the control of Aryl Hydrocarbon Receptor (AhR), androgen receptor (AR), estrogen receptor (ER) and thyroid receptor (TR), respectively. The analyses have shown that the indoor dust contains chemical mixtures with potential to disrupt the above mentioned receptors' signaling with clear differences between the different size fractions and type of solvent used for extraction. Chemical analyses have been carried out and the data are used for effect modelling and for estimation of contribution of the analysed chemicals to the assessed biological potentials. The results of this study can be used to support risk assessment of chemicals present in the indoor environment as well as prioritization of the risk drivers.

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P25-13**The Grouping of Chemicals with Effects on Reproduction and Development for Cumulative Risk Assessment in Europe**

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The EU pesticides regulation 1107/2009 (2019) requires that known cumulative and synergistic effects of pesticide are taken into account when the methods to assess such effects are available. The European Food Safety Authority (EFSA) is developing approaches to identify substances triggering cumulative risk assessment by assigning chemicals to cumulative assessment groups (CAGs) based on common toxic effects on the same target system. In an evaluation conducted by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) on behalf of EFSA (2016) to identify substances with effects on the reproductive and developmental system, 9 CAGs were defined and of the 129 substances analysed, 124 were included in these CAGs. This high number of CAGs and the allocation of almost all the substances to one or more CAGs, was the consequence of the proposed approach which considered any observed specific effect occurring through different phases of the reproductive cycle, from embryo to adult as relevant, without taking into account the impact of systemic and of parental toxicity.

This work proposes a revised approach for grouping substances in CAGs for reproductive and developmental effects. It consists of a decision scheme to correlate prenatal developmental and reproduction toxicity endpoints with the various phases of the reproductive cycle together with a guidance for discriminating between direct effects on the reproductive system or on development of the offspring and effects which are secondary to other and/or parental toxicities.

As of the 124 substances allocated in the CAGs, only 42 were approved for registration according to the EU pesticides database (accessed 21 January 2021) and only 13 were classified or proposed to be classified for reproductive toxicity by the Committee for Risk Assessment of the European Chemical Agency (ECHA RAC) or EFSA, the revised grouping scheme has been aligned with the three categories defined in the classification, labelling and packaging (CLP) criteria for classification for reproductive toxicity (ECHA, 2017) i.e., adverse effects on sexual function and fertility, on development of the offspring, on or via lactation.

Following refinement, only 6 CAGs remained resolving the problem of over-recording and duplication of the same effect in more than one group. In the allocation of the substances to CAGs only primary effects should be considered together with the dose level and severity of the specific effects seen for each ingredient, so that this information can be factored into the cumulative risk.

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P25-14**Concurrent exposure to multiple chemicals in the Canadian population: Biomonitoring data from the Canadian Health Measures Survey**

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People are concurrently exposed to numerous chemicals. Here we sought to explore multi-chemical exposures in the general Canadian population. We first developed the Exposure Load metric, which represents the number of chemicals measured above a defined threshold. We then used nationally-representative biomonitoring data from the 2012–2015 Canadian Health Measures Survey (CHMS) to calculate Exposure Loads for the Canadian population. These Exposure Loads were based on five thresholds: the analytical limit of detection and the 50th, 75th, 90th, and 95th percentiles. A total of 44 biomarkers representing 26 chemicals were reported in the 2012–2015 CHMS. Complete biomarker data were available for 1858 individuals aged 12–79 years. Each individual was assigned a value of 0 or 1 for each of the 44 biomarkers depending on whether that biomarker was measured below or above the concentration threshold, respectively. Each of the 26 chemicals was then assigned either a value of 0 if all biomarkers had a value of 0 or a value of 1 if at least one of the biomarkers had a value of 1. These values were then summed for each individual to produce the Exposure Load, which had a theoretical range from 0 (all chemicals below the threshold) to 26 (all chemicals above the threshold). We report distributions of Exposure Loads for the total population, as well as by age group, sex, and smoking status. When considering the analytical limit of detection as the threshold, most Canadians had an Exposure Load between 13 and 18. When considering the 95th percentile as the threshold, most Canadians had an Exposure Load between 0 and 3, although a small number of Canadians had Exposure Loads as high as 15. Adolescents aged 12–19 years had significantly lower Exposure Loads than adults aged 20–79 years. No differences in Exposure Loads were observed between males and females. Smokers had significantly higher Exposure Loads than nonsmokers, which was understandable given that tobacco smoke is a known source of some chemicals in our study. These findings indicate that Canadians are concurrently exposed to many chemicals at low concentrations and to fewer chemicals at high concentrations. A subset of the population is exposed to many chemicals at high concentrations and the prevalent chemical combinations were explored for these individuals. This information will establish real world multi-chemical exposures in the general population and assist researchers in identifying priority chemical mixtures.

P25-15**Toxicokinetic interactions of industrial chemical mixtures as internal exposure modifiers***S. Karakitsios^{1,2}, I. Petridis¹, V. Kokaraki³, D. Sarigiannis^{1,2,3}

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Single chemical exposure is an exception rather than the rule in the general and occupational environments. The risk assessment of chemical mixtures is particularly challenging due to the number of chemicals combined, limited knowledge on composition, the toxicokinetics and toxicodynamics of components. For this study three relevant mixtures of chemicals were investigated. The first one is the bisphenols' mixtures consisting of bisphenol A, S and F, the second one is the phthalates' mixture consisting of DEHP, BBzP, DnBP and DiNP and the third one of BTEX (benzene, ethylbenzene, toluene and xylene). In order to evaluate the inhibitory effect on the metabolism as a result of co-exposure, a broad range of mean daily intake levels (bodyweight normalised) have been tested for bisphenols and phthalates, starting from 0.01 µg/kg bw/day up to 100,000 µg/kg bw/day., while for the BTEX, the TLV's for the four chemicals considered here are (a) benzene 0.5 ppm (b) toluene 33.0 ppm (c) xylenes 50.0 ppm (d) ethylbenzene 50.0 ppm have been used. From the results of the co-exposure interaction, it was clearly shown that under environmentally relevant exposure levels and even at the level of EFSA's temporary tolerable daily intake (tTDI) of 4 µg/kg bw/d for bisphenol A and 50 µg/kg bw/d for DEHP, the effect of interaction on the internal dose (expressed as increase of the Area Under Curve, AUC) is negligible (below 1%). However, as expected, the interaction effect is higher when the daily intake level increases. The interaction is significant for intake levels above 10,000 µg/kg bw/d, which might be relevant only for occupational settings. Similarly, for BTEX, the changes in internal dose upon inhalation exposure are dose dependent, and they become more evident as we move closer to the TLV.

From all the case studies, it is evident that co-exposure to chemicals that are subjected to interaction at the level of metabolism, is crucial at high exposure levels, that are mostly met in occupational settings. Thus, although in environmentally relevant exposure levels, interactions at the level of metabolism regarding cumulative exposure might not be a major concern, they have to be taken into account when estimating or apply the exposure-response relationships. Hence, these results indicate, that a broader framework of interactions that affect bioavailability upon cumulative exposure might need to be investigated, as well as integration of toxicokinetic and toxicodynamic models (and more specifically biology based dose response models); in our view this has to be the next step in cumulative exposure risk assessment, and qAOPs (quantitative adverse outcome pathways) seem to be the ideal substrate towards this direction.

P26 – Toxicology in life cycle analysis**P26-01****Broadening the chemical coverage to derive human toxicity dose-response factors for non-cancer endpoints***N. Aurisano¹, L. Huang², S. Jang³, W.A. Chiu³, R. Judson⁴, O. Jolliet², P. Fantke¹

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When assessing impacts of chemical life cycle emissions and of chemicals in products on human health, chemical-specific points of departure (PODs) are crucial to derive toxicity-related effect factors. Recent updates of the globally recommended approach for deriving human dose-response factors for non-cancer endpoints in life cycle impact assessment (LCIA) proposes prioritizing the usage of regulatory assessment-based PODs. Such data are available only for several hundreds of chemicals and are missing for the wider range of currently marketed chemicals. As an alternative, experimental animal data can be used to estimate PODs that most closely mimic one that would be selected in a regulatory assessment context. The aim of this study is thus to use available *in vivo* data to estimate suitable PODs for substances lacking regulatory assessments in order to broaden the coverage of chemicals for application in LCIA. As a first step, we extracted experimental animal data available from the US EPA ToxValDB, focusing on oral repeated-dose studies and three non-cancer effect-level types: lowest observed adverse effect level (LOAEL), no-observed adverse effect level (NOAEL) and benchmark dose lower bound (BMDL). The extracted dataset needed further curation before use in LCIA applications, the main steps of which included harmonization of the reported test species names, conversion of units to mg/kg-d, as well as different extrapolations from subchronic or subacute studies to chronic, from the other effect-level types to benchmark dose (BMD) and from tested animal species to humans. After curation, for each chemical with at least 10 studies, we fit the resulting data to a lognormal distribution. For chemicals with less than 10 studies, we fit the distribution while applying a fixed standard deviation of $\log_{10}=0.55$, based on the average standard deviation across data-rich chemicals in our curated dataset. In total, we derived PODs for 8241 chemicals based on the 5th percentile of the fitted lognormal distribution. The resulting $\log_{10}$ POD values ranged by orders of magnitude from -5.57 mg/kg-d to 4.04 mg/kg-d across the considered substances, with a median $\log_{10}$ POD = 0.92 mg/kg-d. For the 749 chemicals having available regulatory PODs, we observed a good correlation with our derived 5th percentile PODs ($R^2=0.66$ and RMSE = 0.65 of the $\log_{10}$ -transformed values). These results suggest that the proposed method is able to derive PODs consistent with regulatory values in a high-throughput manner, thus substantially increasing the coverage of chemical substances for application in LCIA.

This abstract does not necessarily reflect US EPA policy.

P26-02

Global Human Toxicity Potential assessed with the ProScale method for use in Normalisation in LCA

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Life Cycle Assessment (LCA) is widely used to quantify the overall environmental impact of products, and methods are increasingly harmonised through e.g. the Product environmental Footprint (PEF) initiative of the European Commission^[1].

So far, methods to assess human toxicity impacts cover the indirect impact of chemicals emitted into the environment. ProScale was developed as a method to fill this gap for a hazard and exposure-based quantitative scoring system for comparing direct chemical risks (corresponding to near-field human toxicity) to workers, professionals and consumers associated with products in a life cycle perspective^[2].

Examples of applying ProScale are emerging^[3,4], showing that the method works and can be integrated into LCA. But in order to align with other impact assessment approaches for the benefit of LCA and PEF practitioners, there is also a need for a global normalisation score^[5] for ProScale. The study described here presents the results of the first calculation of two such Normalisation scores for ProScale, inhalation and dermal.

Method: ProScale scores are calculated (as detailed in^[1]) as $PSS = HF \times ECF \times PHF \times MF$, where: PSS denotes the ProScale Score (separate for inhalative, oral and dermal exposure routes); HF is the Hazard Factor for a substance, derived based on H-phrases hazard bands and OEL/DNEL; ECF is the Exposure Concentration Factor, and is based on rescaled ECETOC TRA^[6] exposure estimates; PHF is a Person-Hour Factor describing the person-hours of work needed per unit output or input of a process (product or service); and MF is the Mass Flow, describing the amount of a substance needed to produce a product (kg per functional unit).

For the global Normalisation score, we focused on petrochemistry and plastics. For about 60 individual processes, ProScale scores for inhalation and dermal exposure were calculated. Then, the global

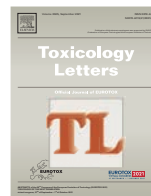
annual production, or in some cases production capacity, was identified through a range of literature sources, normally covering a year in the period 2015–2018.

Result: The resulting Normalisation scores are: ProScale(inhalation): $1.2E+14$ ProScale points, and ProScale(dermal): $1.2E+13$ ProScale points. Among the assessed and included processes, the three dominating processes are crude oil extraction (43% and 69% for inhalation and dermal, respectively), crude oil refining (27/7) and steam cracking (20/18).

Discussion: As a first version of normalisation scores, the numbers calculated here could be used, but cover a limited part of the global economy. Possibly important other processes are for example metal production processes. The domain of residential exposure from building material is also not included at this point. This may mean that when using these scores in regular LCA projects, direct human toxicity potential as calculated with ProScale will potentially be slightly overestimated. Further work is planned to elaborate the issue further.

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LP | Late Breaking Abstracts

LP-01

Modelling Toxicokinetics of BPA Analogues in Zebrafish

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Endocrine disrupting compounds (EDCs) in the environment have become a matter of increasing concern due to their ability to interfere with hormones thus resulting in effects such as impaired reproduction and development. Bisphenol A (BPA) is an EDC which has been extensively studied and is being phased out of some industrial applications due to its estrogenic properties causing adverse effects to humans and wildlife.^[1]

Despite BPA being restricted, many BPA analogues are still being used for various industrial purposes, although limited research is available on their hazard profiles. Recent studies suggest that some of these analogues have similar estrogenic properties and many have been detected in consumer products and the environment including surface water where they may affect fish.^[2] In fish, estrogenic compounds activate estrogen receptors in e.g. the liver, which could cause reproductive effects, making the liver an important target for toxicity. Quantification of the dose at the target organ for various BPA analogues is therefore crucial for assessing the hazard they can pose to fish reproduction.

We aimed to develop a physiologically based toxicokinetic (PBTK) model for BPA analogues in zebrafish, a widely used model species in toxicology. PBTK models are a means to predict dose at target organs such as the liver. To this end, we used and extended a previously published PBTK model for BPA in zebrafish^[3] to simulate and accurately verify measured liver concentrations of BPA and a sub-set of environmentally-relevant BPA analogues. We incorporated maternal transfer and spawning to the previous published PBTK model in order to improve predictions. Additionally, we measured *in vitro* metabolic rates of these bisphenols to more accurately parameterize and understand toxicokinetic differences between these compounds.

Our PBTK model showed good agreement with literature experimental data for BPA and Bisphenol AF (BPAF) capturing the liver accumulation of bisphenols observed in zebrafish. Our novel data on metabolic rates of bisphenols in fish liver homogenate also shows large differences to those measured in human systems, suggesting the use of human parameters in fish modelling may underestimate the bioaccumulation.

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LP-02

Improving *in vivo* predictions from *in vitro* hepatocyte models: model selection, protein effects, and transporter regulation

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The unbound intracellular concentration (ICC) is the driving force for processes that occur inside the hepatocyte, including metabolism, induction and inhibition (metabolic and transporter), efflux-based drug interactions, and hepatotoxicity. The absence (or reduction) of any of these processes can result in sub-optimal predictions of *in vivo* effects. Data will be presented from studies with obeticholic acid (Ocaliva®) demonstrating that fully polarized hepatocyte models (sandwich-cultured human hepatocytes) with optimized uptake and efflux functions dramatically improve the prediction of *in vivo* effects (metabolic and transporter inhibition and hepatotoxicity) compared to non-polarized models (suspended and plated hepatocytes). Recent data has also demonstrated that the effect of protein binding on hepatic uptake, intracellular concentration, and estimation of intrinsic clearance for some drugs cannot be correctly predicted based on the free (unbound) drug concentration. One notable example is telmisartan where the hepatic uptake and intracellular concentration are underpredicted by approximately 90-fold. Improved methods for integrating the effects of plasma protein binding in hepatocyte models will be presented and discussed. Although P450 regulation has been well studied for many years, and is routinely evaluated in drug development, regulation of hepatic transporters is only recently being evaluated. Cholestatic drug-induced liver injury (DILI) in humans has been associated with bile salt export pump (BSEP) inhibition; however, *in vitro* BSEP IC₅₀ concentrations do not correlate with *in vivo* cholestatic DILI severity. Regulation of bile acids through FXR

mediated pathways and induction of both canalicular (BSEP, MDR3) and basolateral (OST α/β) efflux transport is an important adaptive mechanism in the prevention of cholestatic hepatotoxicity. In addition to BSEP inhibition, we will present data showing that integration of inhibition of basolateral efflux and/or interference with the adaptive response (FXR antagonism) allows for more accurate prediction of cholestatic DILI.

LP-03

Quercetin protects SH-SY5Y cells against sterigmatocystin-induced toxicity: prevention of NF- κ B nuclear translocation and down regulation of HO-1 expression

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Sterigmatocystin (STE) is a polyketide mycotoxin produced by fungi commonly found in food and feed commodities. In the last few years a global growing attention is grabbed by STE as it causes genotoxicity, hepatotoxicity and nephrotoxicity in animals and high incidence of liver and stomach cancer has been reported in areas with high levels of STE contamination. A network of interacting mechanisms has been associated to STE toxicity. Among these, oxidative stress appears to be of particular interest. However, attempts to alleviate STE-induced toxicity through the use of natural substances are poorly reported in literature. In the present study a more detailed understanding of the molecular mechanism underlying STE-induced toxicity was carried out in human neuroblastoma SH-SY5Y cells, with specific reference to oxidative stress and inflammatory response. Furthermore, the role of the dietary antioxidant quercetin (QUE) in preventing STE-induced toxicity was investigated. As determined by qRT-PCR, the exposure to STE (0.78, 1.56 and 3.12 μ M) was found to induce a concentration-dependent up-regulation of the gene expression levels of the stress-inducible enzymes HO-1 and NOS-2, two known indicators of oxidative stress. Increased HO-1 expression levels has been shown to represent an adaptive response to increased oxidative stress, as well as it has been demonstrated that alteration in NO signalling through increased NOS-2 expression leads to mitochondrial enzymes, nucleic acids and proteins damages and cell death. In agreement with the existing literature showing QUE as a powerful antioxidant, pre-treatment of cells with QUE 10 μ M completely abolished the STE-induced up-regulation of NOS-2. However it did not completely prevent HO-1 expression. As regards the effect of STE on inflammatory response, our results showed the down-regulation of the pro-inflammatory cytokines TNF- α and IL-6 following STE exposure, suggesting a negative immunomodulatory effect of the mycotoxin. In QUE pre-treated cells, TNF- α and IL-6 were significantly further reduced, which indicates an anti-inflammatory role of QUE. Taken together, the results of the present study provide further insights into the molecular mechanisms involved in STE toxicity and reveal the protective role of QUE in STE-induced toxicity through its antioxidant and immunomodulatory properties that make this dietary flavonol attractive for protection against toxic effects associated with STE exposure.

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LP-04

Using New Approach Methods to predict local acute irritation properties of the pesticidal actives Captan and Folpet

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The current toxicological registration packages for Captan and Folpet rely exclusively on animal tests for acute endpoints. It was thus investigated whether new approach methods can correctly predict Captan and Folpet hazards in order to replace future animal tests for their products. A toxicological evaluation of the compounds may be challenging due to their reactivity at the site of first contact.

The test battery comprised tests according to OECD TGs 431, 439 (Reconstructed human Epidermis), 437 (Bovine Cornea Opacity/Permeability test), 492 (Reconstructed human Cornea-like Epithelium), 442C (DPRA), 442D (KeratinSens), 442E (U-Sens), GARD skin and EpiAirway assays.

While the tests predicted known local acute irritation properties of the actives, eye irritation potential was underpredicted. No skin irritation potential was indicated, which is similar to results of available acute rabbit studies but different to skin reactions seen in rats. These differences may be associated with species-dependent tissue morphology, e.g. stratum corneum thickness between rat and human skin, and the mode of action of the substances.

Skin sensitization potential was correctly predicted but might be biased by the mode of action. EpiAirway assay correctly predicted acute inhalation toxicity potential based on local irritation properties.

LP-05

Development of an alternative predictive model of aerosol deposition in macaque monkeys and associated means of administration

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The respiratory system is a preferred entry point for pathogens and non-pathogens agents (pollens, dusts etc). Furthermore, the inhaled route is also used for the administration of therapeutics for the treatment of respiratory pathologies.

Preclinical studies using animal models are essential and sometimes undisputable (development of inhaled drugs, FDA rule) in order to study, understand and predict toxic and therapeutics aerosols effect in the upper and lower respiratory tract. In this context, the quantity of deposited aerosol and its distribution in the respiratory tract is a key factor influencing the results.

Moreover, with the 21st century challenges regarding the SARS-COV-2 pandemic, potential air transmitted pathogens and increase of respiratory diseases, having robust tools to study aerosols deposition is necessary. The macaque has been identified as a relevant animal model in terms of anatomy, physiopathology for inhalation studies but the complexity and availability of this animal, make it, among other things, a valuable but difficult and expensive model to access. It therefore appears necessary and fundamental to use alternative

methods for preliminary studies without animal in order to reduce the number of animal studies and increase the quality of studies. In our project, we have developed an *in vitro* 3D model anatomical model of the macaque airway. We use a high precision CT-scans of Non-Human Primates and the 3D printing technique with stereolithography method.

We have developed a refined *in vitro* 3D Cast model, which accurately mimics the upper airway of 3 macaques and the lower airway down to the second bronchial division.

However, the 3D model needs to be validated. To validate this model, a comparative study between the aerosol deposition obtained with the 3D model and the deposition obtained with the three macaques was carried out by scintigraphy imaging. Aerosols will be generated using three nebulizers generating three different particle sizes in order to target and study three different deposition areas in the airways: the first nebulizer with a 10µm in terms of Volume Median Diameter (VMD) predicting a major deposition in the upper airways (90%), the second nebulizer with a 1, 35µm in terms of Mass Median Aerodynamic Diameter (MMAD) predicting a deposition in upper and lower airways (50%) and the third with a 0, 36µm of MMAD predicting a major deposition in lower airways (90%).

If the 3D model is validated by the comparison between *in vitro* and *in vivo* results in terms of scintigraphy deposition measurement, it will allow to reduce the number of experiments on macaques and will participate to the improvement of the quality of studies.

LP-06

Genotoxicity and inflammatory potential of stainless steel welding fume particles – an *in vitro* study on standard vs Cr(VI)-reduced flux-cored wires and the role of released metals

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Welders are a large occupational group exposed to metal-containing particles in the fumes generated during the welding process. Exposure to welding fumes has been linked to several respiratory health effects which serves as a driving force to develop new methods that generate less toxic fumes. The individual contribution of the various metals found in welding fumes is not fully understood, but the carcinogen hexavalent chromium (Cr(VI)) has been proposed to be largely involved in the toxicity of stainless steel welding fumes. To explore the role of released metals for welding particle-induced toxicity and test the hypothesis that a reduction of the Cr(VI) content in

the fumes of FCWs results in welding fume particles less hazardous to welders, we compared the toxicity of stainless steel welding fume particles generated with standard flux cored-wire (FCWs) electrodes to those generated with Cr(VI)-reduced FCWs *in vitro*. The endpoints of cell viability, DNA damage and inflammation were examined using human bronchial epithelial cells (HBEC-3kt) and human monocyte derived macrophages (THP-1) as models. To elucidate the role of particles versus released metals, the cells were exposed to either the welding fume particles or solely its released metal fraction. The welding fume particles generated with Cr(VI)-reduced FCWs released small levels of Cr(VI), indicating a successful development and they were further found to be considerably less cyto- and genotoxic compared to the those of standard FCWs. The released fraction of the fumes generated with standard FCWs, being predominantly Cr(VI), was concluded to be toxic and may therefore explain a large part of the cytotoxicity and DNA damage observed in response to the particles. In contrast, an increase in the release of inflammatory cytokines was observed in response to all welding fumes, independent of Cr(VI)-reduction. The released metal fractions of the welding fumes did not induce similar inflammatory effects as the particles, indicating this endpoint not to be dependent on the release of Cr(VI). In conclusion, these results suggest the release of Cr(VI) to be at least partly responsible for the acute toxicity of standard FCW generated stainless steel welding fumes. This suggests a potential benefit of substituting standard FCWs with Cr(VI)-reduced wires to reduce the toxicity of the welding fumes and improve the occupational environment of welders.

LP-07

How maternal exposure to aflatoxin B1 impacts the development of progeny intestinal immune system

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Exposure to toxic contaminants during early-life is associated with the development of diseases. Individuals are exposed to mycotoxins since early stages of life^[1]. However how maternal exposure to mycotoxins influences the development and function of the offspring's immune system remains largely unexplored. Recently, we showed that *in utero* maternal exposure to micronutrients is critical for the development of the immune system, which sets long term immunity if the progeny^[2]. Here we show that presence of aflatoxin B1 in the diet of pregnant murine females affects the development and function of the intestinal immune system. Notably, maternal exposure to AFB1 promoted an increase of overall T cell population, while it also resulted in a selective reduction of cytokine-producing innate lymphoid cells group 2 (ILC2) population in intestine of the progeny. These alterations were associated with decreased expression of Reg3b, Reg3g and Fut2 by the intestinal mucosa of progeny. Thus, these results indicate that maternal exposure to mycotoxins impacts the development of offspring intestinal immune system. ILC2 are critical in intestinal epithelial repair, whether mice exposed to AFB1

display defective tissue damage response needs to be investigated. Also FUT2-dependent fucosylation is key in host–commensal symbiosis suggesting alterations in the intestinal microbiota. Our work reveals that maternal exposure to dietary contaminants such as mycotoxins alters the normal development of intestinal immune system framework of the progeny and may have impact in their immune function.

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LP-08

Test System of Gonadotoxic Activity Identification is more Sensitive and Informative than 3-generations Reproductive Toxicity Study in the Endocrine-Disruptor Mesotrione Research

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The mesotrione pesticide is one of the three main chemical classes of compounds – 4-hydroxyphenylpyruvate-dioxygenase inhibitors, namely the class of tricetones.

The mechanism of pesticide mesotrione action is determined by a violation of tyrosine metabolism, which during embryo- and fetogenesis periods can lead to eye and skin damage, mental retardation, and impaired motor coordination in children. This pesticide is an explicit endocrine-disruptor and is more dangerous for offsprings than for parental generation animals. Like most endocrine-disruptors that violate the endocrine activity of the thyroid gland, it can induce transgenerational epigenetic effects when exposed during gametogenesis.

In reproductive toxicity studies of mesotrione in three generations rats (1997), a lowest-observed-adverse-effect level (LOAEL) was established for reproductive toxicity at 2500 ppm (297.2 mg/kg body weight). No-observed-adverse-effect level (NOAEL) in this experiment was 100 ppm (11.7 mg/kg body weight). It should be emphasized that such important indicators as sperm parameters in males and the estrous cycle in females were not studied in this experiment.

We conducted studies of the mesotrione reproductive toxicity in the test-system of gonadotoxic activity identification. The test substance was administered to females (10 weeks) and males (11 weeks) before mating with intact animals (untreated). Based on the analysis of the results obtained, it was concluded that at a dose of 12.0 mg/kg body weight when exposed to males, the mesotrione has manifested antiandrogenic activity (a decrease in the total number of sperm, the number and percentage of motile sperm, an increase in the percentage of pathological forms of sperm, a decrease of conception and fertility indexes). When exposed to females, the estrous cycle disorder was observed (a decrease in the estrogen-dependent stage of proestrus).

NOAEL in this experiment is 0.3 mg/kg body weight.

Thus, in this test system, a greater sensitivity of males was found in comparison with females to the endocrine-disruptor effect of mesotrione when exposed during gametogenesis.

LP-09

Isoflucypram: A new fungicide with no thyroid related endocrine disrupting properties and an overall favorable mammalian safety profile

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Isoflucypram is a new cereal fungicide currently undergoing evaluation for European registration. Based on its extensive data package, isoflucypram is not genotoxic nor carcinogenic and has no developmental toxicity potential. Furthermore, although a delay in puberty was observed in the 2-generation study, isoflucypram has no overall impact on fertility or reproduction. As per EU Regulation 283/2013 the endocrine disrupting (ED) potential of isoflucypram was evaluated as part of the safety assessment package. As the liver (all species) and thyroid (rat only) are target organs of isoflucypram, the main focus of the ED evaluation was the mode of action (MoA) responsible for the minimal effects observed on the rat hypothalamus-pituitary-thyroid (HPT) axis and determining the human relevance of the perturbation of this axis.

A combination of standard repeat dose toxicity studies and short-term mechanistic investigations conducted in the rat demonstrated that isoflucypram is a marked hepatic enzyme inducer, most notably of the cytochrome P450s, Cyp3a and Cyp2b, and of UDP-glucuronosyltransferases (UDPGT; including T4-UDPGT). This increased enzyme activity was associated with increased liver weight and hepatocellular hypertrophy. In the same studies, minimal thyroid follicular cell hypertrophy was observed but only at doses where liver stimulation was already established. A slight, statistically significant, increase in thyroid stimulating hormone, with no concomitant decrease in T4, was recorded at the same dose level as that triggering follicular cell hypertrophy. Overall, the profile of observations was indicative of a liver mediated MoA giving rise to the minimal thyroid findings. The homeostasis of the HPT axis could, however, still be maintained as evidenced by the absence of clear effects on T4. Direct thyroid effects via thyroid peroxidase (TPO) and sodium iodide symporter (NIS) inhibition could be excluded providing further support for a liver mediated MoA. An *in vitro* comparison of hepatic enzyme induction conducted in rat and human hepatocytes confirmed the rat *in vivo* profile of increased Cyp3a, Cyp2b and T4-UDPGT activities. In contrast, marked quantitative differences were observed when comparing the rat and human responses, particularly for T4-UDPGT, where the basal and induced levels were 4-fold and up to 11-fold lower respectively in the human hepatocytes compared to those observed in the rat. The *in vivo* rat data demonstrate that the minimal perturbation of the HPT axis induced by isoflucypram was secondary to liver enzyme induction. Furthermore, the *in vitro* comparison of hepatic enzyme induction demonstrated marked species differences thus providing evidence that this MoA for isoflucypram is not relevant for humans. Overall, it can be concluded that this molecule is not an ED via the T-modality for mammals based on the weight of evidence derived from the *in vitro* and *in vivo* standard and mechanistic investigations.

LP-10

Development of a novel QSAR model for predicting the synergistic effect of binary mixtures of estrogen receptor agonists

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Worldwide, there are growing concerns about the potential health threat caused by endocrine-disrupting compounds (EDCs) manufactured in the chemical industry^[1,2]. Estrogenic EDCs can interact with estrogen receptors (ERs) and induce ER-mediated responses. In hu-

mans, estrogen plays a primary role in developing many functions related to reproduction, thyroid hormone, metabolism, etc. [3,4]. Some studies showed that different potential EDCs were detected in the environment and simultaneously used in consumer products. The potential synergistic and antagonistic effects of estrogenic EDCs have also been reported in previous studies [5–7]. To screen the combined effect of EDCs, conducting experimental toxicity tests for every possible EDCs mixture might be unfeasible due to the huge number of mixture combinations [8–10]. To overcome the limitations of toxicity tests, *in silico* methods, such as quantitative structure–activity relationships (QSARs), have been developed for predicting potential estrogenic activity of EDCs to replace or reduce *in vivo/in vitro* assay. In previous studies, the collaborative estrogen receptor activity prediction project (CERAPP) was performed and developed 48 QSAR models for ER activity of a single chemical based on the training set by the US EPA [11]. However, since QSAR models of the CERAPP were developed based on a single chemical, it is not suitable for predicting the synergistic effect of ER agonists in binary mixtures.

In this study, we developed a novel QSAR model to predict the synergistic effect of ER agonists in binary mixtures. From the literature, we collected 511 data on the ER agonist activity in a binary mixture [12–14]. We classified them into potential synergistic (positive value) and non-synergistic (negative value) effects based on the model deviation ratio (MDR) between observed and predicted values by the additive toxicity model. MDR value ranged from 1.0 to 2.0 was considered an indicator of synergistic effect considering the balance of available data and toxicological aspect. Among 511 datasets, we selected a QSAR model dataset containing positive and negative values as a 1:1 ratio using the under-sampling method. The QSAR model was developed using the support vector machine (SVM) algorithm and the extended circular molecular fingerprint (ECFP) as a molecular descriptor [15,16]. Molecular descriptors of the binary mixtures were calculated as composite properties based on the mole fraction of the mixture. The internal validation of a QSAR model by using 10-fold cross-validation and external validation was performed using the external validation dataset. The model's performance was evaluated as accuracy, the area under the curve, sensitivity, specificity, and balanced accuracy. The QSAR model showed good performance with most of the performance indices of > 0.75. Therefore, the developed QSAR model showed the potential to predict the synergistic effect of binary mixtures of ER agonists.

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LP-11

Investigation of mutagenicity of lambda-cyhalothrin in a test battery

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Lambda-cyhalothrin (LC) is one of the common pyrethroid insecticides. A mutagenicity study is required to justify a toxicological assessment for the safe use of pesticides in Ukraine.

The objective was to investigate the mutagenic activity of 2 active LC substances in three tests: in the Ames test *in vitro*, in the test for the induction of chromosome aberrations (CA) and the micronuclear (MN) test *in vivo*.

2 LC samples from various manufacturers were studied in the laboratory – 95.2% and 97.1%.

LC was studied using the Ames test in fluctuation modifications with pre-incubation in the microplate format, on *Salmonella typhimurium* strains TA 98 and TA 100 with and without metabolic activation at concentrations from 0.0008 to 2.5 mg/ml.

The same samples were investigated in the polychromatophilic erythrocyte (PCE) and *in vivo* test for CA induction in mouse bone marrow cells at doses of 5.0; 1.0; 0.2 mg/kg.

The obtained results of the LC study in three tests were compared with negative data and historical controls.

In the Ames test, the data obtained indicated that the studied samples did not significantly exceed the number of revertant colonies of TA98 and TA100 test strains with and without metabolic activation compared to negative controls so that they did not cause a mutagenic effect in all the concentrations studied.

Research results in the CA induction test indicate that LC samples in 5.0; 1.0 and 0.2 mg/kg doses did not reveal a significant increase in CA.

In the MN test, LC samples at doses of 1.0 and 0.2 mg/kg did not show a significant increase of the MN level in PCE. But both samples of LC in doses of 5.0 mg/kg of body weight induced a statistically significant excess of the MN frequency in the PCE of the negative control and compared with the data of the negative historical control.

In the Ames test, the LC samples on the *S. typhimurium* TA 98 and TA 100 strains from and without metabolic activation in all concentrations did not show mutagenic properties. LC samples at all doses did not exhibit genotoxic properties in the CA induction test. LC samples induced a statistically significant genotoxic effect at a dose of 5.0 mg/kg in the MN test.

LP-12**Environmental chemical screening for inhibition of the Sodium-Iodide Symporter (NIS) using both a human and rat radioactive iodide uptake (RAIU) assay**

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The uptake of iodide into follicular cells of the thyroid gland via the NIS is the first step in thyroid hormone synthesis. Chemicals that inhibit NIS can suppress circulating thyroid hormones and perturb critical thyroid-related physiological functions in humans and wildlife. NIS inhibition assays were first developed to screen pharmaceutical chemicals but more recently to screen environmental chemicals as potential thyroid disruptors. Our laboratory developed and validated a human NIS NIS-EPA-HEK293T (hNIS) and rat thyroid follicular (FRTL-5) RAIU assays and used them to screen >1800 environmental chemicals in the U.S. EPA ToxCast Phase 1, 2 and e1k library for potential NIS inhibition. In those screening assays, we identified 130 active NIS inhibitors and recently confirmed some of the more potent inhibitors for *in vivo* effects using a weanling and pubertal rat model. Among the more potent chemicals tested, one of the nitrophenyl herbicides, oxyfluorfen, was found to be effective with a clear dose-responsive suppression of serum T4 in both animal models. Because there are several other chemicals in this nitrophenyl class, we hypothesized that they may also be active NIS inhibitors. Here, we tested three additional nitrophenyl herbicides (Lactofen, Fomesafen and Acifluorfen) and two other broadleaf herbicides of interest (Metazachlor and Fluxapyroxad) in both RAIU NIS inhibition assays. In addition, we tested two chemical energetics (5-aminotetrazole or 5-AT and ammonia borane or AB) that are being considered as potential replacements of perchlorate. Briefly, chemicals were diluted in <1% DMSO and tested in parallel FRTL-5, hNIS and cell viability assays. Test chemicals, perchlorate and positive and negative controls were run at six concentrations (0.001–100 μ M) in three independent biological replicates. Cells were plated in 96 well plates, grown for two days and exposed for 2 h to the test chemicals and radioactive iodide 125. Of the nitrophenyls, only Lactofen clearly inhibited NIS in both cell lines with no effect on cytotoxicity. The only nitrophenyl chemical that demonstrated significant cytotoxicity was fomesafen, which had similar confounding NIS inhibition at 100 μ M in FRTL-5 cells. Metazachlor had no effect on cytotoxicity in either cell line, but inhibited NIS by 20% at 100 μ M in FRTL-5 cells. Fluxapyroxad inhibited NIS activity in both cell lines (IC₅₀ of 28 μ M, FRTL-5), with minimal cytotoxicity at 100 μ M. Neither 5-AT or AB inhibited NIS in either RAIU assay, although ammonium borane inhibited cell viability by 75% in hNIS cells at 100 μ M. In summary, we showed that several of the herbicides inhibited iodide uptake but further *in vivo* evidence of altered thyroid hormones is necessary to predict adverse effects in humans or wildlife. The identification of another nitrophenyl herbicide to inhibit NIS warrants continued investigations into this class of chemicals.

This abstract may not necessarily reflect EPA policy.

LP-13**Avoiding contamination during successive transport of food and dangerous goods**

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Bulk transport of liquid or solid food has a great importance in the successive transport of dangerous goods. An additional hazard of successive transport is the possible contamination arising from the pre-

vious cargoes. Our aim is to control the relevant national and international regulations whether there are requirements on stowage, separation and hygiene of food as bulk goods to protect against contamination and, if necessary, to improve these regulations.

First of all, food and feed complaints should be considered for the protection of human health and examined for systematic contamination in the European Union. In the last few decades non-food contamination was found during random inspections of the food transport units during various food transfer on the road. For instance, in several cases, traces of mineral oil were found in the European Union in sunflower oil, and according to the European Rapid Alert System for Food and Feed (RASFF) it was reported with the concentration of 5790 mg/kg in unrefined sunflower oil in 2008, probably as the result of successive transport. The allowed maximum level of mineral oil is 50 mg/kg. Since 2009, saturated hydrocarbons from mineral oils (MOSH) and aromatic hydrocarbons from mineral oils (MOAH) have been characterized as residues of mineral oils in the analysis of cocoa beans, sunflower oil and packaged foods.

Due to the risks to human and animal health, the previous cargoes must be classified as acceptable or unacceptable depending on the type of food. In case of fats and oils, which are often transported in bulk, the acceptable previous cargoes are listed in various European and German regulations and in the Codex Alimentarius. To avoid contamination, the Scientific Committee on Food (SCF), the Codex Committee on Fats and Oils (CCFO) and the Food and Agriculture Organization of the United Nations (FAO) have compiled selection criteria for acceptable previous cargoes for edible fats and oils. With regard to food, the aim is to protect them from cross-contamination during transport. The regulations are intended to ensure that food that are not transported in their own transport units achieve the same level of safety. If acceptable previous cargoes or at least their selection criteria are defined for each type of food involved in the successive transport, a higher level of the food safety can be achieved during transport.

LP-14**Preliminary toxicity study of Investigational Anti-Hypertensive Small Molecule KSD179019**

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Hypertension is the leading cause of mortality and morbidity around the world. About 10% of hypertensive patients are non-responsive by the current available drug regime, condition known as resistant hypertension. Our lab has dedicated decade of research to study the role of Secretin and Secretin receptor (SCTR) in water homeostasis and hence identified SCTR as novel anti-hypertensive drug target. We recently discovered KSD179019 as Positive Allosteric Modulator (PAM) of SCTR with anti-hypertensive activity in hypertensive animal model.

Safety of a novel drug molecule is of utmost importance. Therefore, we aimed at assessing the potential toxicity of KSD179019 both *in vitro* and *in vivo*. A battery of *in vitro* toxicity studies was designed to evaluate the cell toxicity, and mutagenicity. There was no deleterious effect on cell viability and no mutagenic effect of KSD179019. As these results were encouraging hence, we proceeded to *in vivo* toxicity study according to OECD guideline.

A14 days acute toxicity study of KSD179019 was conducted, male and female mice were treated with KSD179019 via a single oral administration at a dose of 2000 mg/kg (n=6/group/sex). Changes in skin and fur, eyes, abnormal behaviour, body weight, and death were observed for 14 consecutive days to assess the acute toxicity. No fatality was observed at 2000 mg/kg body weight (b.w.) post-acute test. Therefore, to identify the Lethal Dose 50 (LD50) for KSD179019 we

performed a limit test with 5000 mg/kg b.w. There was no fatality in the limit test.

Our preliminary findings demonstrated no toxicity of KSD179019 in both *in vitro* and *in vivo* acute toxicity study. According to OECD guidelines (OECD 423) KSD179019 can be classified under the drug category 5 where LD50 >5000 mg/kg b.w. Further sub chronic and chronic studies will be performed in future to find out the NOEL and BMD of KSD179019 for clinical studies.

LP-15

Safe strain lineage concept for risk assessment: Safety evaluation of *Bacillus subtilis* food enzymes

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New microbially-derived food enzymes should be carefully assessed before their introduction on the market. The safety of microbial enzyme preparations is evaluated on a case-by-case basis by checking the following points: 1) the intrinsic safety of the enzyme, 2) the safety of the production process, and 3) the safety of the production organism. The safety of the production strain, and in particular its toxigenic potential, is considered the primary determinant to evaluate the safety of an enzyme preparation. Strains belonging to the same strain lineage typically have similar metabolic characteristics and food enzymes produced by these strains thus display comparable impurity profiles when manufactured under similar fermentation conditions. The term “safe strain lineage” refers to a group of related strains derived from a single parental strain, where the safety of the derived products was shown via systemic and genetic toxicity studies. Once the safe strain lineage has been established, additional well-characterized genetic modifications that do not give rise to safety concerns can be applied without the need for additional toxicological studies.

DSM started its *Bacillus subtilis* strain lineage from a well-documented non-pathogenic and non-toxigenic *Bacillus subtilis* isolate and used strain improvement via a combination of random mutagenesis and targeted genetic modifications. As demonstrated in cytotoxicity studies, the introduction of strain modifications via either classical mutagenesis or genetic engineering did not lead to toxicity of the DSM *Bacillus subtilis* strain supernatants towards CHO epithelial cells, suggesting that none of the introduced mutations lead to the formation of toxic peptides with cytotoxic properties. Genome sequence analysis of the production strains confirmed the absence of vector sequences and of antibiotic resistance genes in the production strains. In addition, three food enzyme preparations produced by strains within this lineage did not show any genotoxic potential *in vitro* and no adverse effects in 90-day rat oral toxicity studies. These results further demonstrate absence of any safety concern coming from the introduced genetic modifications. The outcome of the toxicity studies can be extended to other enzyme preparations produced by any strain from this lineage through read across. Upon the establishment of the DSM *Bacillus subtilis* safe strain lineage, toxicity studies may be waived on food enzymes produced with strains from this lineage. Consequently, this approach is in line with the public ambition to reduce animal toxicity studies.

LP-16

Development of an autologous human physiologically relevant 3D vessel-on-a-chip cytokine release assay system to predict the safety of novel biologics

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Following the failure to predict the cytokine release syndrome (CRS) induced by TGN1412 in 2006, the need for improved predictive pre-clinical *in vitro* safety assessments has become a priority and a regulatory expectation. Current *in vitro* assays using Peripheral Blood Mononuclear Cells (PBMC) and whole blood utilise standard formats of antibody presentation (dry coat, wet coat or liquid phase). While these are biologically simple to run, they do not accurately reflect human *in vivo* physiology, and the limitations of toxicology models are well documented. Efforts have therefore been made to address this, for example through the development of a co-culture assay using Human Umbilical Vein Endothelial Cells (HUVEC) with PBMCs or whole blood, designed to mimic the vascular environment. While this is more physiologically relevant than standard assays, the heterologous approach results in higher basal levels of pro-inflammatory cytokines as previously described in the literature, which ultimately reduces assay sensitivity. This study looked to advance the assay further by developing a human autologous co-culture system using Blood Outgrowth Endothelial Cells (BOEC) derived from adult peripheral blood, which can be co-cultured with PBMCs or whole blood from the same donor. The performance of the assay model was assessed using a range of clinically understood therapeutic antibodies, such as OKT3, TGN1412, Campath and Trastuzumab, with results showing the successful detection of a range of cytokine responses with minimal background interference. The model was also successfully transferred to a three-dimensional (3D) microfluidics system using both PBMC and whole blood, helping to further mimic the physiological microenvironment of a blood vessel. Whilst cytokine response predictions using PBMCs were comparable in 2D vs. 3D, the whole blood 3D assay alone achieved responses to anti-CD28 and anti-CD3 in line with those seen clinically but not previously predicted using 2D static whole blood *in vitro* assays. This autologous model of human vasculature therefore provides an invaluable tool for predicting cytokine release response to novel biologics in pre-clinical cytokine release studies.

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LP-17

An *in vitro* dermal absorption study ring trial with ¹⁴C-Caffeine according to OECD test guideline 428

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Dermal absorption of chemicals is a key factor in non-dietary human risk assessment with agrochemicals. A ring trial exploring the dermal absorption potential of ^{14}C -Caffeine was investigated in six laboratories under GLP conditions using the OECD TG 428-compliant *in vitro* assay with flow-through cells and dermatomed human skin. Potential sources of variation were reduced by a common protocol, test item and skin source. Particularly, skin samples from the same donors were distributed over two repeats and between labs in a non-random, stratified design.

The data show very similar recovery in the various investigated compartments of the assay between laboratories, repeats and donors, which demonstrates that the assay can be robustly and reliably performed. A low intra-lab variability was demonstrated in all 6 laboratories participating. Further, a low inter-lab variability was notable in five out of six laboratories resulting in mean caffeine absorption estimates of $3.93 \pm 2.96\%$ of the applied dose (4 mg/mL) and a mean overall compound recovery of $99.03 \pm 2.11\%$. In the remaining laboratory presumably due to a Covid-19 driven shipment delay of the skin samples, the skin quality may have been affected since the receptor fluid recovery was substantially higher and to a certain degree did correlate with skin integrity parameters. Overall, the ring trial's results demonstrate that the OECD TG 428-compliant *in vitro* assay can be robustly and reliably performed in different laboratories.

Skin absorption results for caffeine did not present any statistically significant variability, with minor differences in the results across the laboratories (5/6) and indicates the robustness of the methodology and the positive impact of strictly controlling the variables in the study. Understanding the variability in the *in vitro* dermal absorption assay with human skin will continue contributing to the design and conduct of appropriate regulatory studies, and adding further considerations to the existing OECD dermal guidance notes. This will result in greater confidence in the data utilised from these assays in risk assessments by companies and regulators alike.

*Authors 1 & 2 have equally contributed to the publication.

LP-18

***In silico* toxicity prediction using Derek Nexus® for skin sensitization, phototoxicity, hepatotoxicity and *in vitro* hERG inhibition**

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Purpose: Quick screening of compounds during drug discovery in hit-to-lead identification phase is needed. Also, regulatory agencies stress on use of non-animal methods for toxicity testing of chemicals. We performed *in silico* toxicity evaluation using Derek® Nexus 6.1.1/ Derek Knowledge Base 2020 1.0 (Lhasa Limited, UK), aiming for a quicker and cost-effective screening for skin sensitisation, phototoxicity, hepatotoxicity and *in vitro* hERG channel inhibition.

Method: We selected 45 substances including sensitiser and non-sensitiser drugs and chemicals for *in silico* testing for sensitisation potential. LLNA data was primarily used for comparison purposes.

For phototoxicity screening, 63 substances belonging to various classes i.e. phototoxic/non-phototoxic chemicals and drugs, were selected. Published human data was primarily used for comparison to *in silico* results. In case of unavailability of human or animal data for a limited number of substances, *in vitro* data was used for comparison.

For hERG channel inhibition screening, 69 drugs were selected from CredibleMeds list of drugs that prolong QT and induce Torsades

de Pointes (TdP) with known/possible/conditional risk for TdP^[1]. Drugs negative for hERG block were also included.

For screening for hepatotoxicity, 65 drugs from FDA's Drug Induced Liver Injury Rank (DILIrank) dataset of from various categories varying from most/less/no concern for DILI were selected^[2].

While analysing Derek results, prediction of toxicity of various degrees such as probable/plausible/equivocal were considered as positive prediction. A negative result was given in case of doubted prediction or no fired alerts.

Results: For predicting skin sensitisation potential, we got 89% accuracy (40/45) i.e. correct predictions, with sensitivity=93%, specificity=82%, Positive Predictive Values (PPV)=90% and Negative Predictive Value (NPV)=88%. Both LLNA and Derek led to classification of tested substances into similar classes of extreme ($\text{EC}_{50} < 0.1$), strong ($\text{EC}_{50} \geq 0.1$ to < 1), moderate ($\text{EC}_{50} \geq 1$ to < 10), and weak ($\text{EC}_{50} \geq 10$ to < 100) sensitisers as well as similar GHS classification. Derek is validated by OECD (OECD-TG 497) for sensitisation screening^[3].

For phototoxicity, we observed that Derek predicted with 78% (49/63) accuracy of the compounds in our study, with sensitivity=73%, specificity=83%, PPV=83% and NPV=74%.

While screening for hepatotoxicity, Derek predicted 72% (47/65) compounds accurately, with sensitivity=65%, specificity=89%, PPV=94% and NPV=52%. We found that there is further scope of improvement of hepatotoxicity prediction by Derek.

With *in vitro* hERG channel inhibition screening, we found that Derek predicted 80% (55/69) compounds accurately, with sensitivity=74%, specificity=91%, PPV=95% and NPV=63%.

Conclusion: Our results show that Derek can be effectively incorporated in the workflow for assessing skin sensitisation, phototoxicity, hepatotoxicity and *in vitro* hERG channel inhibition.

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LP-19

Evaluation of possible health risks associated with consumption of botanical preparations of *Mitragyna speciosa* (kratom)

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Mitragyna speciosa is a tree native to Southeast Asia, where its leaves have been traditionally used as a herbal medicine. Botanical preparations from *Mitragyna speciosa* (usually called kratom) are increasingly being used in other parts of the world, e.g. as supplements for the self-treatment of opioid withdrawal or for pain management. The popularity of kratom is a subject of international health concern. The aim of the present assessment was to summarize and evaluate the current scientific data regarding health risks related to kratom consumption, taking into account relevant published animal studies and human data, including information from case reports and observational studies.

The alkaloids mitragynine and 7-hydroxymitragynine are assumed to be mainly responsible for the psychoactive effects of kratom, being agonists of the μ -opioid human receptor. In behavioral studies in rodents, oral (acute and sub-acute) administration of mitragynine induced cognitive impairments of learning and memory function. Acute and sub-acute oral administration of *Mitragyna speciosa* preparations caused hepatotoxic and mild nephrotoxic effects

in mice and rats. Additionally, neurotoxicity and pulmonary toxicity were observed in sub-acute rat studies. Human observational studies in which adverse effects of kratom ingestion were investigated are limited. Regular kratom use has been observed to produce dependence and withdrawal syndrome following cessation. Other adverse effects associated with kratom use include “hepatic face” (a possible indication of liver toxicity) and neurological symptoms such as dizziness and drowsiness. A large number of cases involving intoxication and even death following kratom ingestion have been reported. The documented adverse events include herb-induced liver injury, neurological effects (seizures, coma, CNS depression, altered mental status, hallucinations, confusion and drowsiness) cardiovascular effects (palpitations, tachycardia, cardiac arrest), withdrawal syndrome and respiratory dysfunction.

The available animal and human data strongly indicate that kratom consumption, while bearing the potential to cause addiction and withdrawal syndrome, may also lead to other manifestations of toxicity, including further aspects of neuro- and liver toxicity. However, the derivation of any health-based guidance values for kratom preparations or constituents thereof is severely impeded by considerable uncertainties, including the variability in composition of kratom preparations, insufficient information on dose-response relationships or on effects of long-term use. Further information (e.g. 90 day animal toxicity studies, well-designed clinical trials) is required to reduce uncertainties and enable a more refined risk assessment of this botanical.

LP-20

Influence of silver nanoparticles and multi-wall carbon nanotubes on the metastatic potential of MDA-MB-436 human breast cancer cells

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The influence of nanomaterials on mammalian cells is primarily cyto- and genotoxic, probably as a result of the induction of oxidative stress and inflammation. Moreover, nanomaterials may show carcinogenic and prometastatic effects. The potential of cancer cells to migrate and invade is directly related to metastasis. The aim of the research was to determine the effect of silver nanoparticles (AgNPs) and multi-wall carbon nanotubes (MWCNTs) on the level of migration and invasion of MDA-MB-436 human triple negative breast cancer cells. AgNPs and MWCNTs influence on the MDA-MB-436 cells viability was determined by two cytotoxicity assays - the neutral red and the MTT tests. Two sizes of AgNPs (20 nm and 200 nm) and MWCNTs (5-20 nm and 50-80 nm) were used to additionally determine a possible size dependency. Appropriate nanomaterials concentrations were selected for further experiments. The level of migration and invasion of MDA-MB-436 cells treated with AgNPs and MWCNTs was determined using commercially available transwell assays. The conducted experiments showed that all tested nanomaterials were cytotoxic to MDA-MB-436 cells, disturbing both the integrity of cell membranes and the activity of mitochondrial enzymes. The tested nanomaterials increased the migration potential of MDA-MB-436 cells in a concentration dependent manner. For AgNPs, a dependence on the size of the nanoparticles used was also noted. In turn, it was noted that both AgNPs and MWCNTs have only a slight stimulating effect on the level of MDA-MB-436 cells invasion. The conducted research shows for the first time the influence of AgNPs and MWCNTs on the level of migration and invasion in MDA-MB-436 cells. The impact of nanomaterials in the level of migration/invasion of both cancer and normal cells has been examined so far only in several *in vitro* models like BEAS-2B [1-2], Caco2 [3], U937 [4], A549 [5] and MCF7 [6] cells

for AgNPs and A549 [7], MDA-MB-231 [8], MCF7 and HeLa [9] for MWCNTs. However, due to the small amount of research, different cell models, parameters of nanomaterials and divergent results, it is difficult to draw any conclusions at the moment. Summarizing, the results of the conducted experiments indicate that AgNPs and MWCNTs influence the level of migration and invasion of breast cancer cells of the MDA-MB-436 line. The obtained results enrich the existing knowledge on the impact of nanomaterials on mammalian cells. The increasing number of cancer cases and the general exposure of the population to nanomaterials prompts further work on this topic. This study was financed by Ministry of Education and Science resources for young scientists in 2020.

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LP-21

Novel multiple targeting monitoring by label-free binding by mass spectrometry directly in tissue

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Polypharmacology describes the activity of compounds at multiple targets. Current research focuses on two aspects of polypharmacology: firstly, unintended polypharmacology can lead to adverse effects and secondly polypharmacology across several disease-relevant targets can improve therapeutic efficacy, prevent drug resistance, or reduce therapeutic target-related adverse effects¹. In the drug development process, binding assays play a critical role in the assessment of the affinity of test compounds to specific targets². Traditionally, binding assays have been performed using either radioactive, fluorescent, or luminescence labeled ligands. The “gold standard” for binding assay remains the use of radioligands. However, this approach does not allow for the monitoring of multiple targets simultaneously. Our aim was to determine the feasibility of using label-free multiple target monitoring (MTM) to analyze 10 receptors simultaneously: glutamate ion channel, GABAB (B1b/B2), norepinephrine trans-

porter, 5-HT1A serotonin receptor, alpha 1 adrenergic receptor, alpha 2 adrenergic receptor, adrenoceptor D1 dopamine, serotonin transporter, opioid GPCR and muscarinic acetylcholine receptor M1, in the brain cerebral cortex.

The membrane fraction of a rat brain cortex was isolated. For each of the 10 receptors a specific well-documented ligand was selected. The quantification of each of the specific ligands individually or in a mixture was optimized by ultra-high performance liquid chromatography (Infinity 1290 and CTC autosampler, Agilent) using a C18 column coupled to triple quadrupole linear ion traps (QTRAP® 5500, SCIEX).

For the binding assays the methodology was identical to that used for traditional “gold standard” radioligand binding assays. This included incubation of the ligand individually or a mixture of all ten ligands (MTM) and the membrane fraction in the presence of individual test compounds, followed by a filtration and wash steps. The plates were subsequently dried and remaining bound ligands were eluted and quantified by UHPLC-MS/MS. The IC₅₀'s of each test compound was determined for each of the 10 targets individually or simultaneously.

For the test compounds 87% were found to have a binding affinity to at least one of the 10 targets.

For the closely related alpha 1 and 2 adrenergic receptors only phentolamine, a synthetic imidazoline was found to bind to both receptors with a high affinity, whereas another 5 compounds demonstrated selectively to alpha 1 over alpha 2.

A high level of correlation of IC₅₀ values were established between the individual binding by mass and the MTM binding for 27 targets with an IC₅₀ values below 11 000 nM.

Our work clearly demonstrates that using MTM label-free binding by mass spectrometry does allow for the monitoring of multiple targets simultaneously in complex biological matrices and might be an attractive approach for safety/toxicology studies related to polypharmacology issues.

LP-22

Human enterocyte-based quantitative monitoring of foodborne trichothecene-specific inflammatory insult

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Mucosa-associated inflammation is an early frontline response during human and animal exposure to foodborne mycotoxins. Despite various tools for chemical identification, optimized biomonitoring of sentinel response-associated biomarkers is required to assess the specific proinflammatory actions of mycotoxins in the gut epithelial barrier. In the present study, intoxication with 8-keto-trichothecenes in human intestinal epithelial cells was found to trigger early response gene 1 product (EGR-1) that plays crucial roles in proinflammatory chemokine induction. In contrast, epithelial xenobiotic exposure resulted in downregulated expression of nuclear factor NF-kappa-B p65 protein, a pivotal transcription factor, during general inflammatory responses in the gut. Based on the early molecular patterns of expression, the inflammation-inducing activity of mucotoxic trichothecene mycotoxins was quantified using human intestinal epithelial cells with dual reporters for EGR-1 and p65 proteins. EGR-1-responsive elements were linked to luciferase reporter while p65 promoter was bound to secretory alkaline phosphatase (SEAP) reporter. In response to conventional inflammagens such as endotoxins and cytokines, both luciferase and SEAP activity were elevated. However, as expected from the mechanistic evaluation, xenobiotic-exposed dual reporters of luciferase and SEAP displayed contrasting expression patterns. Furthermore, EGR-1-responsive

luciferase activity was improved by deficiency of PSMA3, a subunit of the 20S proteasome core complex for ubiquitin-dependent EGR-1 degradation. This molecular event-based dual biomonitoring in epithelial cells is a promising supplementary tool for detecting typical molecular inflammatory pathways in the food matrix.

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LP-23

Ocular irritation and photo-irritation testing of medical devices and extracts using 3D reconstructed human cornea-like tissue models.

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Medical devices are increasingly used in the population to cope with degenerative diseases, treat traumatic injuries or in personal and dental care. Each medical device used in a patient must be subjected to a structured biological evaluation program within a risk management process described by ISO standards or other national and international regulations. In recent years, the ISO implemented series of highly sensitive *in vitro* methods that help safety assessors in the screening of potential health hazards of medical devices even before conducting animal studies or clinical trials^[1]. Significant success has been achieved with the development and validation of *in vitro* protocol for sub-cutaneous irritation testing of medical devices^[2,3]. The outcome led to the development of a new ISO standard 10993-23.

Building on the experience obtained in the above-mentioned project, an *in vitro* protocol combining the ocular irritation and photo-irritation endpoint has been developed using *in vitro* 3D reconstructed cornea-like tissue model (3D RHC). In the initial experiments we have evaluated the tolerance of the 3D RHC models towards an increasing dose of the UVA and visible light and selected a dose, that is sufficiently high to cause excitation of the tested compounds and in the same time is still non-toxic to the 3D model. In the following experiments, we tested phototoxic benchmark chemical Chlorpromazine and several materials representing medical devices and drugs used in ophthalmology to challenge the protocol. All materials were predicted correctly based on the viability and the proposed prediction model. In addition to the viability, we also evaluated IL1- α and TNF- α as markers of inflammation. The responses supported the *in vitro* predictions based on cytotoxicity. The next phase of the project will aim at the correct detection of a larger set of polymers with extractable irritating impurities to challenge the protocol sensitivity. The development of a protocol for early detection of toxic materials and impurities posing a health risk will help to increase the safety of medical devices for patients. At the same time, *in vitro* methods will help to reduce or even eliminate the need for follow-up tests on animals in selected medical devices categories.

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LP-24

Modification of root microRNAs expression and exudation of wheat in response to bisphenol A contamination

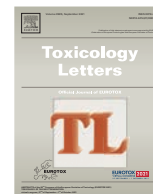
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MicroRNAs (miRs) are recognized as key molecular regulators in most of intracellular processes essential for plant adaptation to environmental changes. microRNAs can mediate regulation of gene expression involved in development, acquisition of nutrients, defense and interactions with soil microorganisms to enhance stress

tolerance of plants. Accumulating evidences indicate that microRNAs are also transferred within cells, organs or whole plant and released in soil as stress signals of the plant-microbiome communication. However, little information is available about how mobile microRNAs are conveyed from plant to the rhizosphere. To understand whether this intriguing phenomenon occurs through root secretion, morphological and physiological changes as well as the expression profiles of developmental and oxidative stress responsive microRNAs were analyzed in root tissues and exudates collected from wheat (*triticum aestivum*) exposed to bisphenol A at low and high concentrations (0.1 and 5000 mg. kg⁻¹ soil). BPA contamination significantly affected aerial and root biomass, antioxidant enzymes activities (catalase and superoxide dismutase) and mir 164, miR 167, and miR 398 expression in roots of the group that received the highest dose. The presence of miR156, miR164, miR167 was detected in root exudates of controls and contaminated plants while BPA exposure increased miR 167 levels exuded at high dose. In summary, this experiment offered first results reporting a participation of the microRNAs regulatory network in the BPA stress response of wheat. The identification of microRNAs as integral component of the root exudates will provide novel insights into chemical signaling in plant-soil microbiome interactions.



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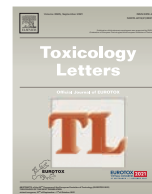
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Keyword Index

B – Bo Holmstedt Memorial Fund Lecture
 CEC – Continuing Education Course
 D – EUROTOX-SOT Debate
 H – HESI CITE Lecture
 LP – Late Poster
 P – Poster
 S – Session
 SOC – Short Oral Communications
 SOT – SOT Merit Award Lecture

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