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JRC Summer School on Non-animal Approaches in Science, May 2021

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Background

"The JRC Summer School on Non-animal Approaches in Science — a flair of holidays and fun. Learning and socialising, intellectual challenge and playful competition." That was our tempting vision, back in 2016, for installing such a training course at EURL ECVAM — and we decided to go for it. We already had some experience in this respect, as every year students from the Karolinska Toxicology Master course programme visited the EURL ECVAM laboratories to learn more about non-animal methods and regulatory toxicology, and to have a closer look on the life in an international research centre such as the Joint Research Centre (JRC).

Our aim was to share knowledge and experience on the latest non-animal approaches in science with young scientists and professionals, to give them new insights and to enable networking and exchange among all participants — students as well as invited experts and ourselves at the JRC. We wanted the experience to be as interactive as possible for the students, hopefully eliciting input from every participant.

With the approval of our hierarchy, a rather small group of brave people started to tackle the preparatory work for the inaugural summer school, which turned out to be quite extensive. Besides the elaboration of the programme itself, there was also a broad range of logistical challenges. Increasingly more people became involved, beyond the staff of our unit — for example, technical, catering, cleaning, transport and security services were all required. Many people had to be transported around (local public transport is, to put it mildly, suboptimal), accommodated and fed. In order to reach as many students as possible to inform them about the event, a range of different communication channels were explored. To limit the participation to our targeted audience, i.e. students or early-career scientists (maximum of four years post-masters or PhD) in relevant fields, and to ensure a good level of existing scientific education, we asked for a motivation letter, a CV and a poster abstract to be submitted along with the application. Based on these pre-defined criteria, these documents were used for the selection of participants.

The inaugural JRC Summer School

In May 2017, our first Summer School — *'Alternative Approaches for Risk Assessment'*, became a reality. Specifically, we wanted to provide comprehensive training on state-of-the-art alternative (non-animal) approaches for use in predictive toxicology, and to promote their use within modern chemical risk assessment practice. One hundred and one students and early-career scientists, from 29 countries worldwide, participated in the four-day programme. In the morning sessions, an overview on the whole process of chemical risk assessment was given, while afternoons were dedicated to interactive activities. We had two poster sessions, each starting with ten flash presentations (the selection was made by the scientific committee out of the submitted abstracts). The three Excellent Poster Award winners, however, were nominated by the summer school participants (each registered person had one vote to cast).

World Café-style sessions allowed for discussion in small groups, with the speakers of the morning sessions. Marketplaces offered hands-on demonstrations of various databases and tools for toxicology, as well as information on career opportunities within the EU and beyond. Further activities were visits to the EURL ECVAM laboratory and to the Visitors' Centre to gain information on the JRC, as well as a JRC Science Quiz. We also prepared an interactive Adverse Outcome Pathway (AOP) game, in which participants had to seek out people possessing the other modules of their assigned AOP and present themselves as one complete AOP on the last day. The splendid weather allowed for breaks outside in the garden, where all could mingle, also joined by JRC staff members who were not directly involved in the Summer School. Other socialising opportunities were an *aperitivo* and a social dinner. A booklet, including the programme, bio-sketches, contacts, lecture and poster abstracts, together with an USB pen with relevant background material, were distributed to the participants; a certificate of attendance was given to all students that attended the whole course.

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This inaugural event was a great success, with participants and organisers happy with having gained new insights, experiences, inspiration and friends. We asked participants for their feedback, which nearly all participants provided. This information was taken very seriously, and provided guidance for the organisation of the following event. We kept what was appreciated and modified the programme according to the feedback, regarding the content and duration. One major complaint concerned the large amount of plastic water bottles used — this prompted us to completely ban plastic for the second Summer School, held two years later.

Building on the lessons learned

The second Summer School, held in 2019, was entitled *'Non-Animal Approaches in Science — Challenges and Future Directions'*. Its aim was to explore the role of the Three Rs in current science and policy, through discussion and debate. Though we already felt more experienced in the organisation, the challenges associated with the preparation and organisation did not appear any fewer than two years before. The application and registration procedures remained unchanged, but we increased the number of participants to 120. This time, students from 34 countries worldwide (including India, Bangladesh, Korea, China, Brazil and Iran) joined us. The programme itself is offered for free, but the travel costs are at the student's expense, and this can be a limiting factor for participation in some cases. Therefore, a number of supporting organisations generously provided travel grants for 13 students.

Essentially, we kept the programme structure the same — with lectures, interactive sessions, poster sessions and lab visits. In addition, we introduced a debate format, for which students were divided in eight groups (with mixed backgrounds and affiliations). Students could get appropriate arguments by listening to various presentations. The debates took place on the last morning to give sufficient time for getting prepared during the course. The level and content of these debates were amazing. Sli.do was used as interactive tool for polls and questionnaires. A wide social programme — including an *aperitivo*, dinner, and a fun-quiz evening — was arranged, with many networking opportunities. The feedback received from the first JRC Summer School, to reduce waste such as plastic water bottles and plastic cutlery, led us to rethink the sustainability and recycling strategies when planning the second event. This effort was rewarded with us being awarded 1st Prize in the *1st Corporate Competition on Sustainable Conference and Events (Category 2 External Events in EC-Premises)*.

More recent events

In June 2020 the first sister Summer School,[1] on *'Innovative Approaches in Science'*, was held in the United States, and then in 2021, we proposed the third JRC Summer School, *'The Three R…evolution'*. When we started the preparation for this third JRC event, more than a year ago, the pandemic was already starting to change our lives, but we could not imagine that this situation would persist for so long, and that it would have such an impact on our event. So, in good spirits, we prepared for another engaging and interactive training course, to be held at the JRC Ispra site. With growing concerns, we finally had to accept the move to a virtual event space at the end of 2020. Suddenly, we were confronted with a new situation — the whole schedule had to be reconfigured into a suitable virtual format, and we also had to consider the different time zones of participants; finding a way to transform the interactive parts into virtual formats was especially challenging. We ended up with a programme of five half-days (to help adjust to the different time zones) and decided to further increase the number of participants to 144. Going beyond this number would have been at the expense of active collaboration possibilities for all participants. As was the case for the first Summer School, selection of the participants was based on their submitted motivation letter, CV and poster abstract. The poster abstracts for the 2021 event are featured here in the *Appendix*.

With support from DG SCIC (the Directorate-General for Interpretation) we were able to use an interactive platform; the specific set-up required took a great deal of time and effort. Content-wise, we kept the lectures, World Café-style sessions for discussion in smaller groups, poster sessions with flash presentations, and the opportunity to vote for the best poster by the participants. Also, the debates were maintained, with topics related to the content of the lectures. We added a market fair presenting various organ-on-a-chip models and a career session to present various career options in the field of NAMs. Instead of travel grants, this time the three supporting organisations (PETA, ERA21 and ESTIV) kindly sponsored poster prizes, which were presented in addition to the three existing Excellent Poster Awards, the latter again being nominated by the summer school participants.

Again, we aimed for an environmentally friendly event and had non-tangible items as give-aways, to avoid the purchase and shipment of gadgets and other such merchandise. We invited all participants to plant trees and the JRC Summer School supported the planting of 2,000 trees in Rondônia, Brazil.[2] We also offered the students a specific training course in scientific writing, to be held in the week following the Summer School. This professional training focused on the writing of motivation letters and abstracts, taking inspiration from examples provided by the students themselves in their Summer School applications, as well as providing instruction in general communication.

Speakers at the 2021 JRC Summer School

As was the case for the previously-held Summer Schools, the speakers at the 2021 event, came from a variety of

disciplines and had a wide range of expertise to share with the participants. The topics covered by each of these speakers in their presentations are outlined below:

Legal obligations and state of play in the European Union

(Susanna Louhimies; European Commission, DG Environment (ENV), Brussels, Belgium)

Since the 1980s, the EU has had legislation in place to protect animals used for scientific purposes. In 2010, the legislation was comprehensively revised making it unique in the world: *Directive 2010/63/EU* sets the full replacement of all animal use for scientific purposes as its ultimate goal and requires, by law, the use of alternative non-animal methods as soon as these become available. The Directive has three key aims: to harmonise the legislation to obtain a level playing field and promote EU research and competiveness; to set up high animal welfare standards and speed up the uptake of Three Rs (*replacement*, *reduction*, *refinement*); and to improve transparency. Transparency facilitates the progress towards full *replacement*. It provides important tools to gain a better understanding of where and how animals are used, and the level of distress and suffering scientific procedures have on animals. This information helps determine where efforts are most needed to have the highest impact on reducing animal numbers and the negative impact on their welfare. The EU has taken a quantum leap in pushing the boundaries of transparency by amending the Directive in 2019. The new transparency measures will be of benefit to all those interested in advancing the Three Rs, whether in the EU or elsewhere in the world. Such tools include detailed statistics on animal use, as well as providing the context of these uses through the requirement for non-technical project summaries of authorised projects. These data will be made available through open access EU databases.

Animals used for scientific purposes: The statistics

(Pierre Deceuninck; European Commission, Joint Research Centre (JRC), Ispra, Italy)

In 2020, the first report of statistical information on the use of animals in procedures became available, in accordance with the provisions of Article 57(2) of *Directive 2010/63/EU* on the protection of animals used for scientific purposes. EURL ECVAM was requested by Directorate-General for Environment to support the preparation of this EC report, based on data provided by Member States in accordance with Article 54(2). For this purpose, EURL ECVAM performed the statistical analysis, providing a comprehensive overview on the use of animals in procedures in the European Union from 2015 to 2017. The first section of this report focuses on the numbers of animals used for the first time, and their origins. These animals can be both conventional animals or those that have been genetically altered (but excludes animals that have been used for the maintenance or creation of new genetically altered animal lines). The second section focuses on the way in which animals are used in scientific procedures, covering both the first and any subsequent reuse, so that a global picture can be drawn of all uses of animals. This section takes into account the nature of the procedures, their legislative context, reuse of animals, their genetic status and the actual severities experienced by the animal having undergone a procedure. The third section focuses on genetically altered animals, providing information on the numbers and types of purpose of genetically altered animals needed to support scientific research in the Union. It reports on the animals used for the creation of new genetically altered animal lines and the maintenance of colonies of existing genetically altered animals.

Predictive toxicology for a more sustainable future

(Andrew Worth; European Commission, Joint Research Centre (JRC), Ispra, Italy)

This presentation discusses the role of predictive toxicology and chemical safety assessment in the context of broad policy challenges faced by the European Union. The state of the European Environment is considered from the perspective of chemical contributions to the burden of disease and ecosystem damage. This sets the scene for highlighting research and innovation opportunities to further develop New Approach Methodologies (NAMs) for assessing the human health and environmental effects of chemicals. Emphasis is placed on the potential contribution of predictive toxicology in supporting one of the six political priorities of the European Commission — 'The European Green Deal' and its zero pollution ambition for a toxic-free environment. The Green Deal sets out an ambitious plan to make the EU the world's first 'climate-neutral' continent by 2050. Of particular relevance is the Chemicals Strategy for Sustainability, adopted in October 2020 under the umbrella of the Green Deal. This strategy sets a pathway toward implementing the vision of a toxic-free environment through a series of actions to support innovation for safe and sustainable chemicals, strengthen the protection of human health and the environment, simplify and strengthen the legal framework on chemicals, build a comprehensive knowledge base to support evidence-based policy making, and set the example of sound management of chemicals globally.[3]

What can regulatory toxicologists learn from jazz improvisation?

(Annamaria Carusi; Department for Science and Technology Studies, University College London (UCL), London, UK)

The distance between regulatory toxicology and jazz improvisation seems very large indeed. What can the creativity

and spontaneity of jazz improvisation have in common with the much more formulaic and standardised nature of regulatory toxicology? This presentation will aim to show that there are useful similarities between them, especially if we want to see change happen in regulatory toxicology, and new methods adopted in it. I claim that regulatory toxicology can learn some tricks from artists such as jazz improvisers — the combination of deep knowledge and understanding of their own instrument, cultivated without ever losing sight of the potentialities of other instruments, and the abilities of other musicians. From this comes the spark of creating something new, that we see in improvisation. Scientific change and innovation happens through collaborations not unlike those that occur in some art forms. But whereas artists spend a huge amount of time training with others, scientists are not trained for collaboration, and they are especially not trained for interdisciplinarity. This talk will explore what such a training for scientists will look like, taking inspiration from the arts. The main topics will be: learning and practicing disciplines, with a view to opening to other disciplines; sharing ways of knowing; and framing common questions and challenges. Various examples from the history of biomedical science and the history of toxicology, and also from the arts, will be used.

Advanced non-animal models in biomedical research: A new collection of JRC models

(Laura Gribaldo; European Commission, Joint Research Centre (JRC), Ispra, Italy)

According to the last report on the use of animals for scientific purposes in the EU, about 70% of animals are used for research and development in the fields of human and veterinary medicine and in biological studies of fundamental nature. Nowadays, a relevant percentage of drug programmes fail to progress, largely due to a lack of efficacy or unexplained toxicity. Although there are several factors underpinning this failure rate, the use of animals to model human biology and disease is coming under increasing scrutiny. For these reasons, EURL ECVAM launched a series of review studies on available and emerging non-animal models in research in seven disease areas: respiratory tract diseases; neurodegenerative disorders; breast cancer; immune-oncology; autoimmunity; cardiovascular diseases; and immunogenicity of advanced medicinal products. These areas were selected because of disease incidence and prevalence, and the amount of animal procedures conducted. The reviews describe both well-established approaches and the ones under development, based on techniques that use cells and tissues (*in vitro* methods), computer modelling and simulation (*in silico*) or cells and tissues explanted from an organism (*ex vivo* methods). Biomedical researchers will be able to use the knowledge base to identify models that might be useful to tackle their specific questions. Educators could use it to provide their students with the latest information on the current state-of-the-art, while funding bodies will be able to consider trends and target promising areas for investment. Furthermore, the knowledge base will be of use to Competent Authorities, to support the process of project evaluation, ensuring that project proposers have properly considered the use of non-animal models in their research proposals.

Biomedical research models in immune oncology

(Lucia Gabriele; Istituto Superiore di Sanità, Rome, Italy)

The expanding field of immuno-oncology relies on the need to depict tumour–immune system interactions driving cancer progression and immunotherapy response. The tumour–immune system interplay is a dynamic and complex phenomenon characterised by patient dependence and variability. The adoption of animal models complementary to advanced 3-D *in vitro* human models are essential to further our understanding of the fine mechanisms shaping cancer–immune system crosstalk, to finally develop personalised immunotherapies. Tumour-bearing immunocompetent mice allow investigation in a complex biological organism, providing valuable information on mechanisms of action and toxicity of immunotherapies. Nevertheless, these models pose a caveat relating to mouse and human disease differences. In light of this, newer studies have implanted privileged human tumours into immunodeficient mice reconstructed with a 'pseudo-human' immune system. However, these models lack the specific organ-dependent tumour microenvironment and thus permit a limited analysis of immune mechanisms. Holistic models of the tumour microenvironment are represented by *in vitro* human organoids, which are advanced 3-D culture systems that recreate the architecture and physiology of human tumours in remarkable detail — with the potential for including stromal cells and diverse immune cell populations of the parental tumours. Despite some limitations in culture and growth features, these models have shown to represent a valuable tool with which to evaluate immunotherapy treatments. In addition, the emerging technology of tumour-on-a-chip, combining cell biology, microfabrication and microfluidics, recapitulates the dynamic interplay between immune cells and tumour cells under controlled physical and biomechanics conditions on an individual patient basis. These systems permit the testing of immunotherapy efficacy by the real-time imaging tracking of autologous immune cells and tumour elimination. Pros and cons of these models will be discussed.

New Approach Methods (NAMs) research: To be or not to be original, that is the question

(Marco Straccia; FRESCI by SCIENCE&STRATEGY SL, Barcelona, Spain)

The development of NAMs based on human biology is key to improving human health research with mechanistic

data, as well as replacing animal models. Within our projects, information on hundreds of human-based models used in biomedical research was retrieved from reviewing the scientific literature. However, only a few of these models were deeply characterised and exploited to provide mechanistic data and thus provide a robust model. In addition, it was clear that when models were used in similar experimental paradigms, previous works published by others in the same field were ignored, as were publications featuring the use of the model in different applications. How can we help inform you? If you have developed, or want to develop, a human-based model, we suggest firstly focusing on the better characterisation and refinement of already available models, instead of looking for further original applications of a poorly characterised model. In this regard, a systematic review is the first step to undertake in order to get a good overview of the current state-of-the-art of your topic. It also helps you choose the right approach to keep expanding your biomedical research field. Being original may give you a high Impact Factor publication — however, the two main goals in your research career are: to answer the relevant biological question behind a certain health problem; and to transfer your solution from the bench into the real world. It is key to start measuring research success through its real impact on society, and not through journal Impact Factor rating. We need better scientists and better solutions to make a better world (see https://ec.europa.eu/jrc/en/eurl/ecvam/knowledge-sharing-3rs/life-science-research).

Organ-on-chip technology and its application in immunological research

(Lorna Ewart; Emulate, Cambridge, UK)

Organ-on-chip technology is an exciting, developing technology that is inspired by biology and uses engineering to create specific microenvironments for cells such that they can live in a 'home away from home'. Cells within the body experience cues that are biochemical and biophysical in nature and organ-on-chip technology aims to recapitulate these cues through the involvement of microfluidics, mechanical strain (in organs where this is relevant), 3-D shape and chemical gradients. It is widely regarded that by creating a physiologically relevant model, the data generated will translate well into human risk assessment or therapeutic efficacy determination. Many of the organ-chip models that have been built to date focus on recreating healthy organ-function, but more recently attention is turning toward the ability to model aspects of disease. To this end, Emulate is focusing on developing models that enable immune cell recruitment and studying the effect of this recruitment on biological function. During this presentation, I will cover the design principles of the Emulate organ-chip and show data on how these models can be used in immunological research.

Biomedical research, translational failures and indicators to monitor the impact of EU-funded research

(Francesca Pistollato; European Commission, Joint Research Centre (JRC), Ispra, Italy & Janine McCarthy; Physicians Committee for Responsible Medicine (PCRM), Washington DC, USA)

Animal models have been traditionally used in biomedical research to recapitulate human disease features and develop new drugs, as they are generally purported to resemble some of the major hallmarks of human diseases.[4,5] However, these animals do not develop the disease as it occurs in humans, and their use has not paved the way toward the development of drugs effective in human patients.[4–6] Indeed, despite conspicuous research and economical endeavours, the clinical failure rate in drug development still remains very high, with an overall likelihood of approval from Phase I of about 9.6%. On the other hand, the expanding toolbox of non-animal methods — such as induced pluripotent stem cells derived from patients, next-generation sequencing, 'omics' and integrated computer modelling — can be used to study human diseases in human-based settings, identify new potential druggable targets and evaluate treatment effects.[7,8] The rise of new technological tools and models in life science, and the increasing need for multidisciplinary approaches, have encouraged many research initiatives and the launch of new EU calls for proposals. Research proposals based on the use of both animal and/or non-animal approaches have been extensively funded at European level. Nowadays, it is becoming pivotal to define and apply indicators suitable to measure the social impact of research funding strategies, monitor contribution to innovation, retrospectively assess public health trends, and readdress funding strategies when needed.[8] Here we discuss such issues, describing a list of indicators to measure the impact and innovation of biomedical research.

Adverse Outcome Pathways — Inflammation as a hub in AOP networks

(Brigitte Landesmann; European Commission, Joint Research Centre (JRC), Ispra, Italy)

An AOP depicts how an injury at the molecular level — the molecular initiating event — propagates through the levels of biological organisation up to an adversity at organism or population level, by describing a sequential series of Key Events (KE; change of a biological state) and their causal relationships (KER; key event relationship). Initially, the AOP framework was built to support chemical risk assessment based on mechanistic reasoning. Meanwhile, in

addition to chemicals, nanoparticles, low-dose radiation and microbes have also been considered as potential stressors for initiating an AOP. AOPs combine and integrate data from various methods and disciplines and are an ideal platform for interdisciplinary collaboration and exchange. The modular structure of AOPs, consisting of KEs and KERs, allows sharing of common elements among multiple AOPs and subsequently the building of AOP networks from independently described AOPs. AOP networks, defined as sets of AOPs sharing at least one common element, can more realistically describe the complexity of biology by providing information on interactions between AOPs and potentially revealing links between different biological pathways. Some biological processes are common to many pathways and thus represent highly connected central nodes within the global AOP network. Inflammation is an important and central biological process, and three hub KEs were identified that represent salient features common to the inflammatory process across tissues, thus facilitating the interconnection of various AOPs.

Probing the human immune system during aging in health and disease

(David Furman; Buck Institute, Novato, California, USA)

In addition to its function in the protection against infections, the immune system plays a chief role in the development of chronic disease. However, metrics with which to identify those at risk are lacking. We have developed a pipeline to study the human immune system, utilising an unbiased approach to deconstruct a human blood sample in large cohorts and applying deep analytics — including artificial intelligence and advanced machine learning methods. This 'systems immunology' approach to aging and chronic disease has led to fundamental discoveries in cardiovascular senescence and the identification of interventions.

Lack in regulation of inflammation as a key factor in fluoroquinolone-induced liver injury: Translation from in vivo *to* in vitro

(Raymond Pieters; HU University of Applied Sciences, Utrecht, the Netherlands)

Idiosyncratic drug-induced liver injury (iDILI) is an example of an adverse immunotoxicological outcome with a poorly understood pathogenesis. iDILI is often associated with inflammatory stress signals, both in human patients and animal models. In some of these models, but particularly in the established iDILI-mouse model with the fluoroquinolone trovafloxacin (TVX), macrophages and neutrophils appear crucial. Since the exact role of these and other leukocytes remained to be defined, we set out to study the kinetics of immunological changes that occur in the liver during the development of TVX-induced iDILI in mice. *In vivo* studies show that the TNF-induced liver influx of leukocytes, in particular macrophages and neutrophils, was disturbed by TVX. TVX not only reduced the TNF-induced influx but also delayed the recovery to the normal situation, that in case of TNF-only occurred within 4 hours after induction. Unlike the controls, mice receiving TVX + TNF displayed severe hepatotoxicity within these 4 hours, with clear pathology and apoptosis, coagulated hepatic vessels and increased alanine aminotransferase levels and interleukin 6/10 ratios. In line with these *in vivo* mouse data, we also found that TVX inhibits *in vitro* migration of human neutrophils. Overall, findings indicate that TVX causes DILI by interfering with regulation of inflammation, rather than by inducing inflammation. This apparently contradictory finding may represent an additional KE, fitting into the already identified KEs representing the process of inflammation.[9] This new KE may help to predict certain cases of DILI, by using selected *in vitro* methods for assessing cellular migration.

Using non-animal models to understand SARS-CoV-2 and Covid-19

(Maria-Joao Amorim; Instituto Gulbenkian de Ciencia, Oeiras, Portugal)

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the virus responsible for the worldwide pandemic of coronavirus disease 2019 (Covid-19) that has caused the death of over 2 million people (as of 25 January, 2021) in only one year. SARS-CoV-2 emerged as a novel zoonotic viral infection in humans for which the most basic aspects of infection were unknown. Strategies to control and prevent the infection worldwide were implemented and adapted to reflect the knowledge as it was being acquired. Scientists mobilised in unprecedent manner to understand many different aspects of the infection, including its origin, epidemiology, disease, therapies, evolution, prevention and detection. Many of these aspects have relied on animal models, but for this presentation I will focus on how non-animal models were used to further our understanding of SARS-CoV-2 and Covid-19.

CIAO Project — Integration of knowledge based on the AOP concept

(Penny Nymark; Karolinska Institutet, Solna, Sweden and the CIAO consortium, www.ciao-covid.net)

The AOP framework provides a structured approach for systematic organisation of research data and knowledge. The five key principles of the framework allow for broad application and data integration across a range of diverse disciplines related to human health — including toxicology, pharmacology, virology and medical research. The CIAO

project, '*Modelling the pathogenesis of Covid-19 using the Adverse Outcome Pathway framework'*, aims at a holistic assembly of knowledge to deliver a truly transdisciplinary description of the entire Covid-19 physiopathology, starting with the initial contact with the SARS-CoV-2 virus and ending with a variety of adverse outcomes in various organs. In January 2021, more than 50 scientists from numerous organisations around the world met in the 2nd CIAO AOP Design Workshop, to discuss the depiction of the Covid-19 disease process as a series of key events (KEs) in a network of AOPs. Seventy-four KEs forming thirteen AOPs were identified, covering Covid-19 manifestations that affect the respiratory, neurological, liver, cardiovascular, kidney and gastrointestinal systems. In addition, modulating factors influencing the course and severity of the disease, as well as possible framework extensions beyond purely biological phenomena, were addressed. This lecture will provide an overview of the concept underlying application of the AOP framework to Covid-19, and the expected outcomes and research support that it provides, as well as a brief glance at the preliminary results obtained in the project.

Multiclonal antibodies as animal-free replacements for polyclonal antibodies

(Stefan Duebel; TU Braunschweig, Institute of Biochemistry, Braunschweig, Germany)

While phage display, the premier animal-free antibody generation method, is well established for the generation of therapeutic antibodies, most antibodies for research and diagnostics are still made by using animals. This presentation reviews the achievements and prospects of recombinant *in vitro* antibody generation, demonstrating how animal-derived antibodies could be complemented or replaced in a large number of typical current research applications. Examples of further advantages of *in vitro* antibody generation will be presented, with respect to their predesigned features and sequence-defined quality. This will also include how polyclonal animal-derived antibodies, which are widely used as secondary antibodies in countless assays, can be replaced by recombinant multiclonal antibodies. The successful generation of multiclonal antibodies for use as replacements for typical secondary antibodies used in research, as well as replacements for horse sera used for therapy by passive vaccination, will be presented.

Room on 'Noah's Ark' — High-throughput screening and New Approach Methodologies for ecotoxicology

(Daniel Villeneuve; United States Environmental Protection Agency, US EPA)

In recent decades, significant progress has been made in development of high-throughput screening and other non-animal assays for evaluating chemical safety. However, to date, these efforts have focused almost exclusively on human and mammalian cells, proteins, and human-oriented exposure and toxicokinetic models. Nonetheless, most regulatory and product stewardship programmes are charged with considering not only safety to humans, but also safety to ecosystems and the diversity of non-human wildlife within those ecosystems. While it is neither feasible, nor desirable, to develop NAMs for all the species we aim to protect, consideration of evolutionary diversity, conservation and divergence of genes and pathways, and life history attributes that influence exposure, can inform the design and application of ecologically-focused NAMs. For example, growing databases of protein sequence information were used to identify molecular targets for which taxon-specific assays may be needed, and to identify which assemblage of species may best reflect diversity in intrinsic susceptibility. Likewise, high-throughput transcriptomic assays are being explored as an efficient and effective — while not overly conservative — means to detect chemical impacts on pathways for which there are no orthologs in humans or traditional mammalian test organisms. Together with an established history of using NAMs to fill the substantial ecotoxicological data gaps that exist, current efforts to develop eco-focused NAMs aim to ensure that the non-human residents of our planet are not left behind in 21st century safety assessments. The contents of this presentation abstract neither constitute, nor necessarily reflect, official US EPA policy.

Endocrine Disruptors and the thyroid validation project

(Sharon Munn & Sandra Coecke; European Commission, Joint Research Centre (JRC), Ispra, Italy)

Criteria to identify substances with endocrine disrupting properties have been recently established in the EU under both the plant protection and biocidal products regulations. In order to be able to apply the new criteria, mechanistic methods are needed 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 methods to detect chemicals that can interfere with the estrogenic, androgenic, thyroid and steroidogenesis (EATS) pathways. However, *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 conducting a multi-laboratory study aimed at validating a number of *in vitro* 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. Characterising and validating these methods are important steps towards their regulatory use and international adoption. The methods and their potential regulatory uptake within the context of the implementation of the EU criteria for ED identification will be discussed.

OECD guidance on in silico *methods (QSAR and PBK)*

(Andrew Worth & Alicia Paini; European Commission, Joint Research Centre (JRC), Ispra, Italy)

Computational toxicology is a fast developing field of science that integrates information and data from a variety of sources (e.g. biology, chemistry) that allows the development of mathematical and computer-based models to predict the exposure, fate and interactions of a chemical leading to adverse health effects. The uptake of these mathematical models in chemical risk assessment is still minimal. Driven by good modelling practices, the Organisation for Economic Co-operation and Development (OECD) has published several guidance documents to support the use of computational models in chemical risk assessment. The first OECD Guidance Document covers the Quantitative Structure–Activity Relationships (QSAR) domain.[10] To keep QSAR applications on a solid scientific foundation, principles for QSAR models were established, covering how to develop, validate and use QSARs for regulatory needs.[11,12] The second set of guidance is on the characterisation, validation and reporting of Physiologically-based Kinetic (PBK) models for regulatory purposes.[13] In this case, the goal is to increase confidence in the use of PBK models parametrised with data derived solely from *in vitro* and *in silico* methods and how these models can be validated. The use of scientifically valid QSAR and PBK models will allow chemical assessment to rely on the use of these approaches for toxicity predictions, rather than *in vivo* data derived from animal studies.[13] This presentation will introduce the principles and criteria captured in these Guidance Documents and also illustrate the application of computational toxicological models in risk assessment.

Chemical assessment and risk management

(Elisabet Berggren & Federica Madia; European Commission, Joint Research Centre (JRC), Ispra, Italy)

Within the EU as well as in all other parts of the world, management measures are in place to protect consumers, workers and the environment from potential adverse effects caused by chemical exposure. Hazard or risk assessment of a chemical and its intended use are the bases for the risk management. In this lecture, we will present how this is currently performed especially within the EU legal framework. In addition, we will explore how the assessment of chemicals could be further improved and made more efficient, based on known underlying biological mechanisms rather than on information on adverse effects in animal studies. Focusing on long-term health effects, we will identify different modes of action contributing to the adverse outcome. These could then build the bases for integrated approaches to testing and assessment and make use of human relevant information.

References

1. Physicians Committee for Responsible Medicine. Summer School on Innovative Approaches in Science, https://www.ascctox.org/innovativescience2020 (undated, accessed 12 August 2021).
2. reforestACTION. 2,000 trees planted by the JRC Summer School on Non-animal Approaches in Science, https://www.reforestaction.com/en/european-commission (undated, accessed 12 August 2021).
3. Worth AP. Computational modelling for the sustainable management of chemicals. *Comput Toxicol* 2020; 14: 100122.
4. Archibald K, Tsaioun K, Kenna JG, et al. Better science for safer medicines: The human imperative. *J R Soc Med* 2018; 111: 433–438.
5. Kramer LA and Greek R. Human stakeholders and the use of animals in drug development. *Bus Soc Rev* 2018; 123: 3–58.
6. Pound P and Ritskes-Hoitinga M. Is it possible to overcome issues of external validity in preclinical animal research? Why most animal models are bound to fail. *J Transl Med* 2018; 16: 304.
7. Marshall LJ, Austin CP, Casey W, et al. Recommendations toward a human pathway-based approach to disease research. *Drug Discov Today* 2018; 23: 1824–1832.
8. Pistollato F, Bernasconi C, McCarthy J, et al. Alzheimer's disease, and breast and prostate cancer research: Translational failures and the importance to monitor outputs and impact of funded research. *Animals (Basel)* 2020; 10: 1194.
9. Villeneuve DL, Landesmann B, Allavena P, et al. Representing the process of inflammation as key events in adverse outcome pathways. *Tox Sci* 2018; 163: 346–352.
10. OECD. (Q)SARs: Guidance documents and reports, http://www.oecd.org/env/ehs/risk-assessment/guidancedocumentsandreportsrelatedtoqsars.htm (undated, accessed 12 August 2021).
11. OECD. *OECD Series on Testing and Assessment: Number 49. The Report from the Expert Group on (Quantitative)*

Structure–Activity Relationships [(Q)SARs] on the Principles for the Validation of (Q)SARs [ENV/JM/MONO(2004) 24]. Paris: Organisation for Economic Co-operation and Development, 2004, 206 pp.
12. OECD. *Guidance Document on the Validation of (Quantitative)Structure–Activity Relationships [(Q)SAR] Models. [ENV/JM/MONO(2007)2]*. Paris: Organisation for Economic Co-operation and Development, 2007, 154 pp.
13. OECD. *Series on Testing and Assessment: No. 331. Guidance Document on the Characterisation, Validation and Reporting of Physiologically Based Kinetic (PBK) Models for Regulatory Purposes*. Paris: Organisation for Economic Co-operation and Development, 2021, 103 pp.

Appendix: Abstracts submitted by the participants

*The JRC Summer School participant is indicated by * and, where different, the corresponding author is underlined. The abstracts do not necessarily reflect the policies of the participants' respective employers.*

Non-animal Testing in the Cosmetic Industry: An *In Vitro* Evaluation of the Antioxidant Efficacy and Dermal Absorption of Bioactive Substances in Comparison with Synthetic Antioxidants

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Human exposure to environmental free radicals and antioxidant stress is associated with the development of a number of serious diseases.[1] New bioactive substances (astaxanthin, crocin and ubiquinol) have been investigated in terms of their antioxidant effects in comparison with traditional synthetic antioxidants (α-tocopherol, butylhydroxytoluene (BHT), butylhydroxyanisole and gallic acid) which are currently used in the cosmetic industry for skin and product protection. As a part of the move away from *in vivo* testing, *in vitro* methods of antioxidant activity detection by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and via Ferric Reducing Antioxidant Power (FRAP) have been applied.[2] Additionally, the DCFH-DA fluorescence assay was used to evaluate whether the substances reduced free radicals in *ex vivo* pig ear skin. The antioxidant activities of astaxanthin, as well as ubiquinol, were higher than the synthetic antioxidants in the tested concentration range (0.1–1 mM) with the DPPH as well as with the FRAP method, except in the case of gallic acid. Due to the different principles of the *in vitro* methods employed, the antioxidant activity of crocin reached significantly lower ($p < 0.05$) values compared to other antioxidants measured by using the DPPH method, while high and very similar activity to BHA was achieved with the FRAP method. BHT showed significantly low ($p < 0.05$) redox properties with the FRAP method as well as with the DPPH method. In the follow-up experiments, in view of the potential endocrine-disrupting effect of BHT, its dermal absorption will be studied *in vitro* with *ex vivo* porcine ear skin, in accordance with the OECD Test Guideline 428.[3] Subsequently, the systemic exposure dose and margin of safety for BHT will be calculated.

References

1. Carocho M, Morales P and Ferreira ICFR. Antioxidants: reviewing the chemistry, food applications, legislation and role as preservatives. *Trends Food Sci Technol* 2018; 71: 107–120.
2. Ácsová A, Martiniaková S and Hojerová J. Selected *in vitro* methods to determine antioxidant activity of hydrophilic/lipohilic substances. *Acta Chim Slov* 2019; 12: 200–211.
3. Organisation for Economic Co-operation and Development. *Test No. 428: Skin Absorption:* In Vitro *Method*. OECD Guidelines for the Testing of Chemicals, Section 4. Paris: OECD Publication Office, 2004, 8 pp.

Immunomodulatory Effect of PFOS in Intestinal Inflammation in Genetically-susceptible Hosts

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Inflammatory bowel disease (IBD), which encompasses Crohn's disease and ulcerative colitis, is characterised by a chronic inflammation of the gastrointestinal tract.[1] Although the aetiology is not completely understood, it is regarded as a multifactorial disease that is believed to develop in genetically susceptible hosts as an active immune response to environmental triggers.[2,3] However, suitable *in vivo* models to investigate the effect of the interaction of multiple factors involved in IBD progression are lacking.[4] Among environmental factors, per- and polyfluoroalkyl substances (PFAS), such as perfluorooctanesulfonic acid (PFOS), are a group of persistent organic pollutants that have been used globally in industrial and commercial products.[5] Exposure to PFAS has been associated with adverse health effects, including increased incidence of ulcerative colitis.[6] In this study, we examined the impact of the interaction of genetics (IBD risk genes) and environmental exposures (PFOS) in intestinal inflammation. Here, we utilised a chemically-induced model of intestinal inflammation in zebrafish larvae harboring mutations in IBD risk genes (e.g. il10, il23r) that had been crossed with a neutrophil-specific transgenic larvae Tg(lysC:DsRED2), and analysed neutrophil recruitment to the intestine as a hallmark of intestinal inflammation. In summary, our project proposes a platform to understand how these environmental pollutants, together with genetic susceptibility, shape the development of intestinal inflammation, which remains a major challenge in the field.

References

1. Sanchez-Muñoz F, Dominguez-Lopez A and Yamamoto-Furusho JK. Role of cytokines in inflammatory bowel disease. *World J Gastroenterol* 2008; 14: 4280–4288.
2. Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat Clin Pract Gastroenterol Hepatol* 2006; 3: 390–407.
3. Ng SC, Shi HY, Hamidi N, et al. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: A systematic review of population-based studies. *Lancet* [Internet]. 2017; 390: 2769–2778.
4. Westling M. *A zebrafish-based system to study the impact of environmental factors in Inflammatory Bowel Disease (IBD).* Sweden: Karolinska Institutet, 2020, 40 pp.
5. Kotthoff M, Müller J, Jürling H, et al. Perfluoroalkyl and polyfluoroalkyl substances in consumer products. *Environ Sci Pollut Res* 2015; 22: 14,546–14,559.
6. Steenland K, Zhao L, Winquist A, et al. Ulcerative colitis and perfluorooctanoic acid (PFOA) in a highly exposed population of community residents and workers in the mid-Ohio valley. *Environ Health Perspect* 2013; 121: 900–905.

Development of Ecological and Molecular Biomarkers with Model and Non-model Invertebrate Species for the Evaluation of the Toxic Response

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Contamination of the environment by toxic pollutants has become widely recognised as an environmental concern. These compounds are often present at low concentrations, making them difficult to detect.[1] They can also cause ecological issues, according to their nature (heavy metals, pesticides, endocrine disruptors, etc.). In recent years, the use of molecular techniques has provided a good opportunity to achieve an in-depth knowledge of the mechanisms underlying these harmful effects. These tools include gene expression and enzyme activity studies, among other targets.[2] Since toxicological studies on model and non-model organisms complement traditional approaches and provide powerful information in terms of natural ecosystems, these represent a breakthrough in ecotoxicology. However, few models offer the opportunity to perform an integrated study with multiple approaches, from molecular variations to physiological consequences. Further study and refinement of these techniques, in combination with the use of invertebrate test models, could serve to reduce the number of animal experiments currently needed to carry out the toxicological evaluation of chemicals. Such tools would permit analysis of the toxicogenomic effects of genes that are directly or indirectly involved in hormonal pathways, reproduction and development, and further our understanding of the mechanisms of action of environmental pollutants, thus improving environmental risk assessment.

References

1. Barouki R. Endocrine disruptors: revisiting concepts and dogma in toxicology. *Comptes Rendus Biologies* 2017; 340: 9–10.
2. Kim HJ, Koedrith P and Seo YR. Ecotoxicogenomic approaches for understanding molecular mechanisms of environmental chemical toxicity using aquatic invertebrate, *Daphnia* model organism. *Int J Mol Sci* 2015; 16: 12,261–12,287.

Swedish Government Agencies Are Important Contributors/Partners in National and International Three Rs Development

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In 2016, the Swedish government commissioned six Swedish government agencies — that all, to a varying extent, use data from animal studies in their work — to draw up strategic Three Rs plans. The Swedish 3Rs Centre (S3RC) supported this work and compiled these Three Rs strategies in a report.[1] The different Three Rs strategies include both overall strategic goals and more specific tasks. The Swedish Chemicals Agency (KEMI), the Swedish Medical Products Agency and the National Food Agency are mainly focusing on method use and Three Rs development in regulatory toxicity and risk assessment. The Swedish Agency for Marine and Water Management and the Swedish Environmental Protection Agency apply the Three Rs in their environmental and wildlife management and research, as well as in questions regarding handling and marking of wild animals. The Swedish Veterinary Institute is following the Three Rs in wildlife research and management, laboratory animal use, diagnostics and vaccine development. In order to increase awareness of the Three Rs, all six agencies disseminate information internally and/or externally. The importance of collaboration with other agencies is highlighted and the collaboration with S3RC is expressed as valuable and fruitful. International co-operation is deemed to be important and different contexts of international participation are mentioned. The S3RC has invited the government agencies to Three Rs seminars and workshops, and all agencies have been invited to discuss Three Rs and possible collaborations. One fruitful example is the collaboration with KEMI, resulting in the appointment and financing of Swedish experts in the OECD Test Guideline programme. More collaborations are planned for the future.

References

1. Ljung PE, Bornestaf C, Zidar J, et al. Work with 3R related questions at the Swedish governmental agencies: the Swedish Agency for Marine and Water Management, the Swedish Chemicals Agency, the Swedish Medical Products Agency, the Swedish Environmental Protection Agency, the National Food Agency and the Swedish Veterinary Institute. [Published in Swedish] *The Swedish Board of Agriculture* 2020; DNR 5.2.17-10717/2018, https://www2.jordbruksverket.se/download/18.58eb179c1754a145d97737f7/1603405030639/ovr565.pdf (accessed 11 August 2021).

Development of Canine Intestinal Acute Dysbiosis *In Vitro* Model

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Improvement of the intestinal health of companion animals is an emerging research field. Thus, the Simulator of the Canine Intestinal Microbial Ecosystem (SCIME™) was recently developed and validated with *in vivo* data.[1] SCIME consists of a four-stage reactor composed of a stomach and a small intestine, linked to proximal and distal colon compartments. This semi-continuous gastrointestinal tract model allowed a long-term, region-dependent and pH-controlled simulation of the canine colon-associated microbial community, with physiological retention times.[1] The current study presents a further improvement of this model, by using an antibiotic to induce canine acute dysbiosis conditions in the SCIME. Intestinal dysbiosis and acute diarrhoea are important health concerns among dog owners and evidence shows an important correlation between the gut microbiota and host health.[2,5] During our SCIME experiment, distal colon vessels were treated with amoxicillin and clavulanic acid (for three days)[3,4,6] and concentrations of short-chain fatty acids (SCFAs), major end products of bacterial carbohydrate fermentation in the intestinal tract, were quantified by sampling the SCIME lumen. Concentrations of propionic acid were significantly decreased in SCIME vessels challenged with amoxicillin and clavulanic acid treatment, as reported in the literature.[2] Microbial community composition is under investigation, to assess the model's reliability compared to *in vivo* cases of acute diarrhoea in dogs. Furthermore, the SCIME acute dysbiosis model exhibits

interesting application potential, not only as an *in vitro* model in research related to the gastrointestinal health of dogs, but also of other mammals.

References

1. Duysburgh C, Ossieur WP, De Paepe K, et al. Development and validation of the Simulator of the Canine Intestinal Microbial Ecosystem (SCIME). *J Anim Sci* 2020; 98: skz357.
2. Guard BC, Barr JW, Reddivari L, et al. Characterization of microbial dysbiosis and metabolomic changes in dogs with acute diarrhea. *PLoS One* 2015; 10: e0127259.
3. Ichim TE, Kesari S and Shafer K. Protection from chemotherapy- and antibiotic-mediated dysbiosis of the gut microbiota by a probiotic with digestive enzymes supplement. *Oncotarget* 2018; 9: 30,919–30,935.
4. Joly C, Gay-Quéheillard J, Léké A, et al. Impact of chronic exposure to low doses of chlorpyrifos on the intestinal microbiota in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) and in the rat. *Environ Sci Pollut Res* 2013; 20: 2726–2734.
5. Minamoto Y, Minamoto T, Isaiah A, et al. Fecal short-chain fatty acid concentrations and dysbiosis in dogs with chronic enteropathy. *J Vet Intern Med* 2019; 33: 1608–1618.
6. Réquilé M, Gonzàlez Alvarez DO, Delanaud S, et al. Use of a combination of *in vitro* models to investigate the impact of chlorpyrifos and inulin on the intestinal microbiota and the permeability of the intestinal mucosa. *Environ Sci Pollut Res Int* 2018; 25: 22,529–22,540.

Synovial Cells Secrete a Temperature-stable Protein that Inhibits Hypertrophic Differentiation and Induces Articular Cartilage Differentiation of Chondrocytes *In Vitro*

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Osteoarthritis (OA) is a degenerative joint disease resulting from articular cartilage disruption, with limited therapeutic options. From the original cartilaginous template, all but the chondrocytes closest to the synovial joint undergo hypertrophic differentiation, indicating that the articular chondrocytes are protected from hypertrophic differentiation and remodelling into bone. We hypothesised that the synovial microenvironment inhibits hypertrophic differentiation and promotes articular cartilage formation. We cultured epiphyseal chondrocytes in high-density pellets, with or without synoviocyte-conditioned medium, and quantified and localised expression of differentiation markers by using quantitative RT-PCR and *in situ* hybridisation. Chondrocytes in regular culture medium underwent reproducible hypertrophic differentiation, with increasing expression of collagen type 10 (*Col10)* ($p < 0.05$), alkaline phosphatase (*Alp*) ($p < 0.001$) and Indian hedgehog (*Ihh*) ($p < 0.01$), whereas articular cartilage-marker lubricin *(Prg4)* remained low. However, in synoviocyte-conditioned medium, hypertrophic markers remained low (*Col10*, *Ihh*, $p < 0.05$; *Alp*, $p < 0.001$), whereas *Prg4* increased (18-fold; $p < 0.05$). We next aimed to characterise the synovial factor(s) that promote articular cartilage formation and inhibit hypertrophic differentiation. We used an *in vitro* bioassay that quantifies hypertrophic and articular differentiation and found that the putative factor is inactivated by proteinase K digestion ($p < 0.01$), is heat-resistant (90°C; $p < 0.001$) and has a size of between 50–100 kDa. Our findings show that chondrocytes cultured in pellets undergo a sequential differentiation programme similar to chondrocytes *in vivo,* and suggest that synoviocytes secrete a heat-resistant, large protein that inhibits hypertrophic differentiation while promoting articular cartilage differentiation. This finding may have important implications for articular cartilage development, OA pathogenesis and treatment, as well as for articular cartilage tissue engineering.

Toward the Replacement of Animal-derived Reagents in *In Vitro* Methods Assessing Thyroid Signalling Disruption

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Certain chemicals present in the environment, food, medicinal or consumer products cause disruption of the thyroid hormone signalling pathway, which can lead to serious health issues. There is a need for a validated set of methods to assess

the toxicological effects of chemicals on multiple stages of thyroid hormone signalling[1] and to support policy making. Motivated by the EU *Directive 2010/63/EU*,[2] such methods should, where possible, avoid using live animals. To this end, a network of validation laboratories across the EU, in liaison with method developers and Unit F.3 of the Joint Research Centre, has been working toward the validation of 17 *in vitro* mechanistic methods for assessing the disruption of the key events in thyroid hormone signalling. These methods include animal-derived ingredients, such as serum, enzymes and antibodies. Their replacement by chemically defined animal-free ingredients, recommended by the Guidance Document on Good *In Vitro* Method Practices,[3] could improve method reliability and reproducibility, enhance the relevance for human physiology and reduce the number of animals used. The aim of this project is to systematically map animal-derived ingredients present in the *in vitro* methods assessing thyroid signalling disruption, to evaluate the availability of animal-free alternatives to those ingredients, and to plan the implementation of such refined protocols. The results of this investigation will be relevant to test systems and assays beyond those focusing on thyroid signalling disruption, thus contributing to the global shift toward fully animal-free *in vitro* methodologies in biomedical research and regulatory testing.

References

1. OECD. New scoping document on *in vitro* and *ex vivo* assays for the identification of modulators of thyroid hormone signalling. OECD Series on Testing and Assessment, No. 207. Paris: OECD Publishing, 2017, 148 pp.
2. European Commission. *Directive 2010/63/EU* of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Off J Euro Union* 2010; L276, 20.10.2010: 33–77.
3. OECD. Guidance document on Good *In Vitro* Method Practices (GIVIMP). OECD Series on Testing and Assessment, No. 286. Paris: OECD Publishing, 2018, 206 pp.

New Approach Methodologies (NAMs) to Advance Points of Departure (PoDs) Estimation

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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 explored a novel gene co-expression method to investigate pathway–dose–response relationships and estimate PoDs. Here we employed the decompositional matrix-based pathway-level information extractor (PLIER)[2] in combination with the Reactome database, to identify latent variables (LVs) describing relevant biological activity of seven compounds of interest. Concentration–response transcriptomic profiles were evaluated in HepG2 cells at six time points. We first investigated LVs dose–response relationships and computed PoD by using the ToxCast pipeline (tcpl).[3] Then, LVs associated with relevant biological activity were further investigated to identify key genes driving pathway perturbation. These results represent a first step toward 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 and drugs using NAMs.

References

1. Baltazar MT, Cable S, Carmichael PL, et al. A next-generation risk assessment case study for coumarin in cosmetic products. *Toxicol Sci* 2020; 1: 236–252.
2. Mao W, Zaslavsky E, Hartmann BM, et al. Pathway-level information extractor (PLIER) for gene expression data. *Nat Methods* 2019; 16: 607–610.
3. Filer DL, Kothiya P, Setzer RW, et al. tcpl: The ToxCast pipeline for high-throughput screening data. *Bioinformatics* 2017; 33: 618–620.

Tumour Engineering 3-D Approaches as More Predictive *In Vitro* Preclinical Models

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The failure of conventional therapies for osteosarcoma is mainly due to the lack of specificity for the cancer stem cell (CSC) subpopulation[1–3] and to the inadequate use of 2-D *in vitro* culture systems that show a poor *in vitro–in vivo* translation ability.[4,5] Additionally, species differences between animal models can limit their predictivity for clinical translation.[6] To address these limitations, and thus create more-predictive *in vitro* platforms for drug testing and biological studies, we developed 3-D *in vitro* models of the osteosarcoma CSC niche by exploiting two tumour engineering strategies.[5,7] Firstly, two different hydroxyapatite-based bone mimicking scaffolds were used to recapitulate the *in vivo* microenvironment. Secondly, a well-established sphere-forming culture method was applied to human osteosarcoma cell lines (MG63 and SAOS-2) as a CSC enrichment method.[3,8] CSC enrichment was successfully demonstrated by morphological analysis and expression of stemness genes (SOX-2, OCT-4 and NANOG) on scaffold-free spheroids compared to parental cells. Fluorescence, SEM and histological analyses showed good cell–biomaterial interaction, with the maintenance of specific phenotype in spheroids and parental cells on the two types of scaffolds. The statistically significant expression of stemness and niche-related genes (NOTCH-1, IL-6 and HIF-1α) in scaffold-based spheroids, as compared to scaffold-free spheroids, was shown by using immunofluorescence analysis and qRT-PCR, confirming the greater *in vivo* mimetism of 3-D conditions. Further investigations on the design of scaffolds, tumour cell population heterogeneity, drug resistance and testing are necessary for better recapitulating the pathological conditions of osteosarcoma. Future projects planned include the expansion of this bioengineering method to other tissues and pathologies.

References

1. Abarrategi A, Tornin J, Martinez-Cruzado L, et al. Osteosarcoma: cells-of-origin, cancer stem cells, and targeted therapies. *Stem Cells Int* 2016; 3631764.
2. Kamal AF, Pranatha DY, Sugito W, et al. Isolation, culture and characterization of cancer stem cells from primary osteosarcoma. *Open Stem Cell J* 2018; 5: 1–13.
3. Brown HK, Tellez-Gabriel M and Heymann D. Cancer stem cells in osteosarcoma. *Cancer Lett* 2017; 386: 189–195.
4. Horvath P, Aulner N, Bickle M, et al. Screening out irrelevant cell-based models of disease. *Nat Rev Drug Discov* 2016; 15: 751–769.
5. Yamada KM and Cukierman E. Modeling tissue morphogenesis and cancer in 3D. *Cell* 2007; 130: 601–610.
6. Hartung T and Daston G. Are *in vitro* tests suitable for regulatory use? *Toxicol Sci* 2009; 111: 233–237.
7. Brancato V, Oliveira JM, Correlo VM, et al. Could 3D models of cancer enhance drug screening? *Biomaterials* 2020; 232: 119744.
8. Tang QL, Zhao ZQ, Li JC, et al. Salinomycin inhibits osteosarcoma by targeting its tumor stem cells. *Cancer Lett* 2011; 311: 113–121.

Development of a Human Innervated Skin Model to Identify Skin Sensitising Substances

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The skin is permanently in contact with our surroundings, from cosmetics to clothing and materials of our everyday life. New products have to be tested for their potential to cause skin sensitisation. Identifying skin sensitisers relies on an Adverse Outcome Pathway (AOP) with certain key events, which can be investigated via *in vitro* activation of keratinocytes, dendritic cells and *in vivo* activation of T-cells, as well as the actual adverse outcome, 'allergic contact dermatitis', which can so far only be investigated via poorly representative animal experiments.[1] In this project, an *in vitro* 3-D skin model that includes sensory neurons and Schwann cells derived from human induced pluripotent stem cells (iPSCs) is under development. This will be used to quantify increased neurite outgrowth, which is equivalent to pruritus — a key symptom of allergic contact dermatitis.[2] This assay could add to the so far existing AOP and could allow a significant reduction in the translational gap between *in vitro* experiments and *in vivo* induced allergic contact dermatitis. To date, we have established a protocol for the differentiation of sensory neurons[3] and Schwann cells[4] from iPSCs. These cells were characterised via RT-qPCR and immunocytochemistry for the expression of sensory neuron markers (Brn3a, TrkA, TRPV1 and Substance P), and Schwann cell markers (Sox10 and GFAP), respectively. The next steps involve the coculture of sensory neurons with Schwann cells and seeding onto a sponge containing fibroblasts and keratinocytes to create a 3-D, innervated skin model.

Activating the keratinocytes with skin sensitising substances will induce neurite outgrowth into the epidermis, which can then be tracked to quantify the sensitisation potential of a substance *in vitro*.[2]

References

1. OECD. *The adverse outcome pathway for skin sensitisation initiated by covalent binding to proteins*. OECD Series on Testing and Assessment, No. 168. Paris: OECD Publishing, Paris, 2014, 105 pp.
2. Kinkelin I, Mötzing S, Koltzenburg M, et al. Increase in NGF content and nerve fiber sprouting in human allergic contact eczema. *Cell Tissue Res* 2000; 302: 31–37.
3. Muller Q, Beaudet MJ, De Serres-Berard T, et al. Development of an innervated tissue-engineered skin with human sensory neurons and Schwann cells differentiated from iPS cells. *Acta Biomater* 2018; 82: 93–101.
4. Kim HS, Lee J, Lee DY, et al. Schwann cell precursors from human pluripotent stem cells as a potential therapeutic target for myelin repair. *Stem Cell Reports* 2017; 8: 1714–1726.

QSAR Prediction of *In Vitro* Biotransformation in Mammals

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Over the last 20 years, our research group has developed many validated Quantitative Structure–Activity Relationships (QSARs) to estimate different physical-chemical properties and biological activities of chemicals from their molecular structure.[1] Our most recent models are the result of an international collaboration for the CEFIC-LRI ECO44 'Integrating Bioaccumulation Assessment Tools for Mammals (iBAT-Mam)' project, and focus on the prediction of the *in vitro* biotransformation of heterogeneous organic chemicals in mammals. These models are particularly relevant in the context of chemical hazard and risk assessment, where reliable toxicokinetics data can be used to evaluate the exposure to chemical substances in support of regulatory decisions.[2] For instance, it has been shown that clearance data measured *in vitro* can be scaled using *in vitro–in vivo* extrapolation (IVIVE) models and used to parameterise physiologically-based biokinetic models for various chemical assessment contexts (some examples are listed[3–5]). In this study, we propose a new approach based on Multiple Linear Regression QSAR models for the prediction of intrinsic hepatic clearance measured *in vitro* in human and rodent liver. To create these QSARs, chemicals were grouped according to their most likely reaction pathway based on predictions of their CYP-450 mediated reactivity. This work demonstrates how the new reactivity-based QSAR approach can be applied to profile the potential *in vitro* biotransformation of chemicals, which is relevant to clarify their toxicological behaviour and improved assessment of the potential risk they may pose.

References

1. Gramatica P, Papa E and Sangion A. QSAR modeling of cumulative environmental end-points for the prioritization of hazardous chemicals. *Environ Sci Process Impacts* 2018; 20: 38–47.
2. ECHA. Chapter R.11: PBT/vPvB assessment. In: *Guidance on information requirements and chemical safety assessment*. Helsinki: ECHA, 2017, pp. 66–68.
3. Paini A, Leonard JA, Joossens E, et al. Next generation physiologically based kinetic (NG-PBK) models in support of regulatory decision making. *Comput Toxicol* 2019; 9: 61–72.
4. Coecke S, Pelkonen O, Leite SB, et al. Toxicokinetics as a key to the integrated toxicity risk assessment based primarily on non-animal approaches. *Toxicol In Vitro* 2013; 27: 1570–1577.
5. Wilk-Zasadna I, Bernasconi C, Pelkonen O, et al. Biotransformation *in vitro*: an essential consideration in the quantitative *in vitro*-to-*in vivo* extrapolation (QIVIVE) of toxicity data. *Toxicology* 2015; 332: 8–19.

Differentiated Neuroblastoma F-11 Cells as an Alternative *In Vitro* Model to Dorsal Root Ganglion Neurons

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Animal models have been used in scientific research for decades. However, these models are often expensive, difficult to set up and subject to ethical issues. For these reasons, we tried to develop an alternative to animal models by using the F-11 cell line, a hybridoma derived from embryonic rat dorsal root ganglion (DRG) neurons and mouse neuroblastoma,[1] whose cryopreservation and thawing are simple and economic. F-11 could be differentiated into functional neurons by their maintenance on biocompatible substrates,[2] but their properties as sensory neurons remain so far unknown. In order to verify whether they could show functional similarities to DRG neurons, we induced differentiation by incubating them in serum-deprived medium for 10–14 days and then performed an electrophysiological investigation. By using the patch clamp technique, we recorded the typical electrical activity of a mature sensory neuron and the voltage-dependent Na^+, Ca^{2+} and K^+ channels they express. We also used capsaicin, substance P, neurotransmitters and acidic solutions to verify the expression of ion channels typical of sensory neurons. Differentiated F-11 cells showed Na^+ currents similar to those exhibited by primary DRG neurons and the percentage of cells responsive to acetylcholine was similar to that of neurons recorded in rat DRGs. Glutamate was effective on non-NMDA receptors, as is the case of embryonic DRG neurons. Moreover, an acidic solution induced desensitising currents through proton-activated cation channels expressed also in DRG neurons. Results shows that differentiated F-11 cells express some ion channels and an electrical activity typical of sensory neurons. Our data demonstrate that these cells could represent a simple and economic alternative to DRG neurons and, for this reason, they might be employed for studying mechanisms involved in the detection and transmission of noxious stimuli and sensory inflammatory pain.[3]

References

1. Platika D, Boulos MH, Baizer L, et al. Neuronal traits of clonal cell lines derived by fusion of dorsal root ganglia neurons with neuroblastoma cells. *Proc Natl Acad Sci USA* 1985; 82: 3499–3503.
2. Russo L, Sgambato A, Lecchi M, et al. Neoglucosylated collagen matrices drive neuronal cells to differentiate. *ACS Chem Neurosci* 2014; 5: 261–265.
3. Yu Y-Q, Chen X-F, Yang Y, et al. Electrophysiological identification of tonic and phasic neurons in sensory dorsal root ganglion and their distinct implications in inflammatory pain. *Physiol Res* 2014; 63: 793–799.

Organoids in Precision Medicine and Cancer Biology

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Precision medicine is a treatment approach that seeks to exploit patient-specific individualised therapeutic strategies. Due to their ability to capture patient diversity, organoids are well suited for the development of personalised therapeutic approaches. An organoid is a miniaturised 3-D representation of an organ that accurately replicates the histological and functional aspects of *in vivo* tissue.[1] They can be used as models in basic and translational approaches (e.g. disease modelling) and in drug screening. In fact, in the latter context, they are also used in the fight against cancer.[2] There are many models to study tumours and, in this regard, conventional cell cultures or animal models fail to accurately predict drug responses in humans because they do not properly mimic the complexity of the cancer microenvironment.[3] Tumour organoids closely resemble the patient's cancer and they are an improvement over generating patient-derived cell lines.[4] A clear advantage is the usefulness of a fully autologous *ex vivo* model with cancer, immune, and stromal cells. These models provide insights into how T-cells interact with tumours, allowing neo-antigen discovery on an individual basis, besides having the potential to advance T-cell therapy.[5] Organoids contribute to the reduction of the need for animal testing, supporting the Three Rs principles of *Replacement*, *Reduction* and *Refinement*. Moreover, experiments are less expensive than animal ones, as well as faster, ethically acceptable, and potentially more predictive.

References

1. Dye BR, Hill DR, Ferguson MA, et al. *In vitro* generation of human pluripotent stem cell derived lung organoids. *eLife* 2015; 2015: 1–25.
2. Drost J and Clevers H. Organoids in cancer research. *Nat Rev Cancer* 2018; 18: 407–418.

2. Dutta RC and Dutta AK. Human-organoid models: accomplishments to salvage test-animals. *J Biomed Eng Medical Devices* 2016; 01(02): 1–8.
4. Campaner E, Zannini A, Santorsola M, et al. Breast cancer organoids model patient-specific response to drug treatment. *Cancers* 2020; 12: 1–19.
5. Tsai S, McOlash L, Palen K, et al. Development of primary human pancreatic cancer organoids, matched stromal and immune cells and 3D tumor microenvironment models. *BMC Cancer* 2018; 18: 1–13.

Short-term Exposure to Nanoplastic-containing Aerosol Induces Inflammatory Cytokine Production *In Vitro* in a 3-D Healthy Human Primary Airway Epithelium Model

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As environmental pollutants, microplastics and nanoplastics are now detected in ocean and freshwater ecosystems. According to the Guidance on Monitoring of Marine Litter in European Seas of the EU Marine Strategy Framework Directive: Technical Subgroup on Marine Litter, microplastics can be divided into groups based on their size of either 1–5 mm or 20 μm–1 mm.[1] However, it is also known that microplastics manufactured at these macro and microscopic scales, inevitably break down into finer particles measuring less than 100 nm, termed nanoplastics.[2] Recent evidence suggests that these microplastic (MP) and nanoplastic (NP) particles become airborne in aerosols generated by natural water body motion.[3] While the inhalation exposure to aerosolised MP and NP particles is grossly understudied, occupational exposure to various MP and NP particles suggest airway irritation, neutrophilic inflammation, translocation, increased risk of lung carcinoma and chronic respiratory disease such as asthma, and even respiratory failure.[4,5] In this study, we exposed a fully differentiated 3-D airway epithelium derived from 14 healthy donors to 50 nm polystyrene NP-containing aerosol for just three minutes a day for three days. Concentrations of NP particles ranged from 2.5 μg/ml to 2500 μg/ml before nebulisation. Although there were no phenotypic alterations in tissue integrity, cell survival, mucociliary clearance or cilia beating frequency, a significant abundance of neutrophil chemoattractant molecule CCL3 (5-fold) was detected in epithelium conditioned-media of NP-exposed tissues, as compared to vehicle-exposed. These preliminary findings suggest that neutrophilic inflammation may result from environmental exposure to NP-containing aerosol and warrant further investigation.

References

1. MSFD Technical Subgroup on Marine Litter. *Guidance on monitoring of marine litter in European seas*. JRC Scientific and Policy Reports. Luxembourg: Publications Office, 2013. p. 98.
2. Jeong CB, Kang HM, Lee MC, et al. Adverse effects of microplastics and oxidative stress-induced MAPK/Nrf2 pathway-mediated defense mechanisms in the marine copepod *Paracyclopina nana*. *Sci Rep* 2017; 7: 41323.
3. Trainic M, Flores JM, Pinkas I, et al. Airborne microplastic particles detected in the remote marine atmosphere. *Commun Earth Environ* 2020; 1: 64.
4. Amato-Lourenco LF, dos Santos Galvao L, de Weger LA, et al. An emerging class of air pollutants: Potential effects of microplastics to respiratory human health? *Sci Total Environ* 2020; 749: 141676.
5. Prata JC. Airborne microplastics: Consequences to human health? *Environ Pollut* 2018; 234: 115–216.

Xenoestrogens Induce GPER Receptor-dependent Centrosome Amplification and Chromosomal Instability in Colorectal Cancer Cells

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Endocrine disruptive chemicals (EDC) refer to a group of natural or synthetic substances with hormone or hormone-like actions. They are ubiquitous to humans, as they are present in pesticides and industrial by-products. They are able to interfere with many aspects of the endocrine system, thereby causing diverse health effects including cancer. As the third most common cancer type worldwide, colorectal cancer (CRC) belongs to one of the most widespread diseases. Importantly, recent work suggests a link between synthetic EDC with oestrogenic activity (xenoestrogens) and an increased

risk of CRC through effects on cell division processes or through stimulating metastasis. However, the molecular mechanism of this xenoestrogen-induced risk potential is still unclear. In order to uncover these cellular and molecular mechanisms, many animal experiments are carried out — this drives a growing need to develop alternative and complementary methods to animal testing. Our preliminary results demonstrate that xenoestrogens induce chromosomal instability (CIN) in non-transformed colon epithelial and colorectal cancer-derived cell lines. CIN is a hallmark of CRC and is referred to as persistent segregation errors of whole chromosomes during mitosis; it is also strongly associated with tumorigenesis. A central mechanism of CIN and human cancers is supernumerary centrosomes, the microtubule organisation centres of the cell. Remarkably, our results showed that xenoestrogen-mediated CIN is induced by centrosome amplification (CA). We could show that xenoestrogen-induced CA leads to defects in chromosome alignment and the generation of lagging chromosomes representing an important precursor of CIN. Our studies further indicate that CA seems to be dependent on the alternative oestrogen receptor GPER1, which prompts us to focus on the identification of centrosomal targets of GPER1. Understanding the molecular mechanisms of xenoestrogen-mediated colon carcinogenesis enables the development of new *in vitro* alternative methods to animal testing and might help to specify CRC therapies.

Metabolic Changes of Mature Adipocytes Exposed to Endocrine Disrupting Chemicals (EDCs)

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Metabolic disrupting chemicals (MDCs) are a sub-category of endocrine disrupting chemicals (EDCs). Over the last decade, they have been shown to contribute to the immense increase in obesity and obesity-related diseases, like type-2 diabetes (T2D). In one of our group's projects working on the generation of novel testing methods for MDCs, we studied whether previously identified MDCs could initiate insulin resistance (IR), a recognised T2D precursor, in the target adipose tissue. For this goal, human mesenchymal stem cells (hMSCs) were differentiated into mature adipocytes over a 14-day period and later exposed to various concentrations of MDCs for three days. Effects on lipid storage, and adipocyte size and number, were studied with high content analysis, and cytotoxicity was determined by measuring membrane permeability. Glucose uptake from the medium was studied by using a luminescence assay. Lastly, the expression of genes characteristic of adipocyte development and of glucose utilisation were investigated via qPCR. The project is currently still ongoing and, until now, only limited MDC-related changes have been observed in mature adipocytes.

Alanine Scanning of Dinitroaniline Binding Site on Malaria *Plasmodium* and Human α-Tubulin

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Human malaria is a complex disease caused by such *Plasmodium* species as *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*.[1] Previous studies have shown that dinitroaniline compounds, which specifically depolymerise plant microtubules, are also active against *P. falciparum*, and may act as antimalarial drugs.[2] At the same time, they are not highly specific for human tubulin. Therefore, these compounds are considered to be one of the most promising sources for the design of new antimalarial agents.[3] Our investigation focused on the *in silico* identification of amino acid residues and interactions, predetermining the existence of a common site and similar interaction of α-tubulin from different *Plasmodium* species with dinitroaniline compounds, including newly synthesised compounds with lower cytotoxicity to human cells. Alanine scanning mutagenesis indicated that two key (Arg2, Val250) and one minor (Glu3) amino acid residues of α-tubulin are involved in the binding of dinitroanilines. At the same time, it was revealed that two minor residues (Asp251, Glu254) of *Plasmodium* α-tubulin interact only with some members of dinitroanilines. Our data indicate that, in the case of dinitroaniline binding, such differences may predetermine much stronger interaction with *Plasmodium* α-tubulin in comparison with human tubulin. This finding opens an additional possibility for the design of new antimalarial drugs with low human toxicity.

References

1. Karpov PA, Demchuk OM, Ozheredov SP, et al. Conservation of dinitroaniline/phosphorothioamidate site of α-tubulin in *Plasmodium* species distributed in India. *Fakt Eksp Evol Org* 2019; 24: 327–332.
2. Corral MG, Leroux J, Stubbs KA, et al. Herbicidal properties of antimalarial drugs. *Sci Rep* 2017; 7: 45871.
3. Fennell BJ, Naughton JA, Dempsey E, et al. Cellular and molecular actions of dinitroaniline and phosphorothioamidate herbicides on *Plasmodium falciparum*: tubulin as a specific antimalarial target. *Mol Biochem Parasitol* 2006; 145: 226–238.

Embryonic Zebrafish (ZF4) Cells: An Alternative Model for Nano(eco)toxicity Studies

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Fish cells have been widely used as an alternative aquatic testing model for many years, providing a valuable, quick, and cost-effective toxicity screening tool in many nanotoxicological studies.[1,2] Despite the large variety of commercially available fish cells, there is a constant need to further explore the role of nanoparticles (NPs) in new and different aquatic *in vitro* models. Hence, in this study, we explored the use of commercial embryonic zebrafish cells (ZF4) to evaluate the biological responses after exposure to three representative AgNPs sizes (10, 30 and 100 nm) and concentrations (2.5, 5 and 10 μg/ml), including $AgNO_3$ as its ionic counterpart (1, 1.5 and 2 μg/ml), for 24 hours. The results demonstrated that ZF4 cells were able to trigger biological responses linked to the exposure concentration, size and form (ionic) of the AgNPs. AgNP-induced toxicity was displayed through different cell death modalities, such as apoptosis, necrosis and autophagy, including other stress-induced signalling pathways such as mitochondrial permeabilisation and oxidative damage, as a cellular attempt to overcome the induced damage. Finally, the results presented in this study elucidate further insights into the biological mechanisms activated in ZF4 cells, highlighting the complexity and interplay between AgNPs and induced intracellular pathways, contributing to the development of the Adverse Outcome Pathway (AOP) framework. This work also aligns with the Three Rs principles of replacement, reduction and refinement of animal experimentation, as part of a safer, sustainable and 21st century nanotoxicological assessment approach.[3]

References

1. Fent K. Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. *Toxicol In Vitro* 2001; 15: 477–488.
2. Castaño A, Bols N, Braunbeck T, et al. The use of fish cells in ecotoxicology. The report and recommendations of ECVAM Workshop 47. *Altern Lab Anim* 2003; 31: 317–351.
3. Quevedo AC, Lynch I and Valsami-Jones E. Silver nanoparticle induced toxicity and cell death mechanisms in embryonic zebrafish cells. *Nanoscale* 2021; 13: 6142–6161.

Cell Membrane Capacitance — A Non-invasive Alternative to Predict Ocular Irritancy on Reconstructed Human Corneal Epithelia

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Cell membranes display the ultrastructure of a natural capacitor due to the dielectric properties of the phospholipid bilayer. This means that cell capacitance can be related to cell membrane integrity, and this could be altered after chemical exposure[1] and might lead to ocular irritation. In this work, changes in cell membrane capacitance in reconstructed human corneal epithelia were evaluated following the Standardised Operational Procedures detailed in OECD TG 492 for identifying chemicals not requiring classification and labelling for eye irritation. Briefly, cell membrane capacitance was evaluated by using coupled electrodes connected to a HAMEG® LCR meter prior to chemical exposure at different frequencies. Thirty chemicals (15 irritants and 15 non-irritants), including liquids and solids, were then applied in duplicate for 30 minutes or 6 hours, respectively. After a PBS rinse, cornea models were incubated at 37°C for 2 hours for liquids or 18 hours for solids. Finally, cell capacitance was evaluated again, and cell viability was assessed by using the MTT assay. A prediction model was developed based on changes to cell membrane capacitance and compared to the classification obtained with the MTT

assay. Standard classification according to the MTT resulted in 93.3% sensitivity (14/15), 66.6% specificity (10/15) and 80% accuracy (24/30). The cell capacitance prediction model at 600 Hz resulted in 93.3% sensitivity (14/15), 66.6% sensibility (10/15) and 80% accuracy (24/30). Our results show that by evaluating cell capacitance, the ocular irritation potential of chemical products could be assessed in a non-invasive assay while complying with OECD TG 492 requirements,[2] representing a novel alternative method to assay ocular damage in reconstructed human corneal epithelia.

References

1. Lee RM, Choi H, Shin JS, et al. Distinguishing between apoptosis and necrosis using a capacitance sensor. *Biosens Bioelectron* 2009; 24: 2586–2591.
2. OECD. *Test No. 492: Reconstructed human Cornea-like Epithelium (RhCE) Test Method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage*. OECD Guidelines for the Testing of Chemicals, Section 4. Paris: OECD Publishing, 2019, 43 pp.

High-content Imaging of Drug-induced Effects on Cell Health and Morphological Features in Lung Cell Lines Integrated Within our Ongoing Respiratory Strategy

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The novel respiratory drug development process aims to identify and mitigate potential safety issues and concerns, in order to deliver safer drugs to patients with respiratory diseases. As part of the strategy, this project will combine biological systems approaches and human *in vitro* models to predict clinical safety and collect related mechanistic toxicity data. Recently, a confocal imaging assay that allows highly resolved observations of epithelial barrier integrity and cellular differentiation has been developed. Such an innovative assay will provide an opportunity to mechanistically predict drug-induced airway irritancy. The project's goal is to further develop the capability of the imaging assay to quantitatively assess different mechanisms of respiratory irritancy and aspects that could be predictive of clinical toxicity, in a high-content and high-throughput format. In addition, the project will focus on retrieving generated data from previous studies to fully develop an imaging-based cellular safety assay. In this case, currently established *in vitro* assays include reactive oxygen species (ROS) production, mitochondrial disfunction, cytoskeletal reorganisation and caspase-3 apoptotic signalling. For the next steps, primary screening of a compound set to identify the best assay parameters followed by a larger validation screen to calculate the predictive capacity of the assay will be carried out. Finally, a secondary analysis of the images based on machine learning might be included, if time allows. The aim would be that through artificial intelligence analysis of the image set, additional cell phenotypic markers and combinations could help to identify, develop and improve predictive toxicology in future cell-based assays.

Hypothalamic *In Vitro* Model to Evaluate Circadian Rhythm Dysfunction-induced Metabolic Stress

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The hypothalamus is involved in the regulation of various physiological functions, such as eating, body temperature, stress response and circadian rhythm. This brain region is key to the pathogenesis of obesity.[1] Obesity leads to hypothalamic dysfunction, promoting metabolic deregulation and the disruption of circadian rhythm. Resetting the circadian clock on obesity, *per se*, can prevent metabolic dysfunction.[2] Thus investigating new ways to manipulate the circadian clock might lead to new therapeutic targets for obesity. The aim of this work was to use a hypothalamic cell model to implement a model of metabolic stress with circadian rhythm alterations, to study the effect of a carotenoid

compound as an anti-obesogenic. For this study, a hypothalamic cell line was incubated with palmitate (metabolic stressor) and treated with the carotenoid. Cells were collected at different timepoints throughout the day for protein (Western blotting) and gene expression (qRT-PCR) analyses of metabolic and circadian rhythm molecular intermediates. Cells incubated with palmitate showed increased mTOR signalling and dampened circadian rhythms of core clock genes, such as PER2. The carotenoid treatment was able to decrease mTOR levels and RPS6K (mTOR substrate). Furthermore, the treatment prevented PER2 alterations induced by palmitate. The results show that palmitate can induce metabolic and circadian dysfunction *in vitro*, similar to obesity, and that the carotenoid tested can prevent palmitate-induced dysfunction.[3,4]

References

1. Carmo-Silva S and Cavadas C. Hypothalamic disfunction in obesity and metabolic disorders. *Adv Neurobiol* 2017; 19: 73–115.
2. Froy O. Circadian rhythms and obesity in mammals. *ISRN Obes* 2012; 2012: 437198.
3. Greco JA, Oosterman JE and Belsham DD. Differential effects of omega-3 fatty acid docosahexaenoic acid and palmitate on the circadian transcriptional profile of clock genes in immortalized hypothalamic neurons. *Am J Physiol Regul Integr Comp Physiol* 2014; 307: R1049–R1060.
4. Li S, Ren X, Wang Y, et al. Fucoxanthin alleviates palmitate-induced inflammation in RAW 264.7 cells through improving lipid metabolism and attenuating mitochondrial dysfunction. *Food Funct* 2020; 11: 3361–3370.

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Phytochemical Composition and Biological Activities of Different Grape Extracts

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The grape (*Vitis Vinifera* L. ssp sativa) is a fruit cultivated worldwide, and since it represents a rich source of polyphenols, it is related to numerous health benefits.[1] Our research team focuses on the antioxidants present in grape extracts, as well as their relation to biological activities. We aimed to optimise the extraction process, in order to obtain the highest recovery of these valuable compounds, as well as the highest stability of the extracts. The main purpose of the optimisation process was the election of the solvent. We examined whether there was a difference in extraction capacity between volatile organic solvents and natural deep eutectic solvents, which have been promoted as an optimal and sustainable alternative for conventional solvents.[2] The biological activities of the extracts obtained were investigated *in vitro*. Antioxidant activity was evaluated by different spectrophotometric assays, while the broth microdilution method was used to determine antimicrobial activity. Cytotoxicity was investigated on three different cell lines. Natural deep eutectic solvents, especially choline chloride:citric acid, outperformed acidified aqueous ethanol, with regard to total polyphenol content and antioxidant activity. Nevertheless, there were some discrepancies in extraction affinity toward different classes of polyphenols. Antimicrobial and cytotoxic studies also prompted the use of choline chloride:citric acid as the most promising solvent. Further, there is a need to distinguish the specific compounds responsible for the biological activities, which will be the basis of our future research. Finally, it is worth mentioning that, although great variability between varieties was observed, international grapes did not surpass Serbian autochthonous ones concerning parameters of interest.

References

1. Nassiri-Asl M and Hosseinzadeh H. Review of the pharmacological effects of *Vitis vinifera* (Grape) and its bioactive constituents: an update. *Phytother Res* 2016; 30: 1392–1403.

2. Radošević K, Ćurko N, Srček VG, et al. Natural deep eutectic solvents as beneficial extractants for enhancement of plant extracts bioactivity. *LWT* 2016; 73: 45–51.

Lung Tumour Spheroids to Assess Immunological Assets of the Tumour Microenvironment

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Anti-tumour immunotherapy aims to boost tumoricidal interactions between tumour and immune cells within the complex multicellular tumour microenvironment (TME). Most research relies on mouse tumour models, posing three issues: a) *in vivo* real-time measurements of tumour–immune cell interactions go alongside costly imaging instruments; b) most mouse models only partially recapitulate human immunity; and c) as tumour immunotherapy is claiming its place as a first-line treatment option, expanding research in this field leads to an increase in laboratory animal use. There is an unmet need for novel animal-sparing, yet clinically relevant, tumour models that can be exploited to evaluate the dynamics of innate and adaptive immune responses in the TME. To comply with this current need, we have developed fully histocompatible 3-D tumour spheroids. As a proof-of-concept, we generated lung cancer spheroids by using two murine lung cancer cell lines and one human lung adenocarcinoma line. In addition, a stromal component was provided via a murine or human fibroblast line. Aside from their ease to generate and evaluate, we report on their potential to measure: T-cell infiltration and motility; interaction of tumour cells with tumour-associated macrophages; tumour target cell specific killing; and efficiency of immune checkpoint inhibitors. Moreover, the model allows the functional evaluation of patient-derived myeloid cells. We believe that our lung cancer 3-D spheroids can serve as a blueprint for a Three Rs-minded immunological anti-tumour assay for other solid cancer types. In this respect, the use of laboratory animals can be further reduced, with a concomitant increase in the adoption of human-relevant and patient-related immunological assays.

Altered Metabolism of Heparan Sulphate Leads to Developmental Dopaminergic Abnormalities Responsible for Autistic-like Symptoms in Lysosomal Storage Disorders

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Lysosomal storage disorders (LSD) characterised by altered metabolism of heparan sulphate (HS), including mucopolysaccharidosis (MPS) III and MPS-II, exhibit lysosomal dysfunctions leading to neurodegeneration and dementia in children. In LSD, dementia is preceded by severe and therapy-resistant autistic-like behaviour symptoms (ALBSs) of unknown cause. ALBSs, including stereotyped behaviours and changes in sociability, have dramatic impact on children and parents' life and are resistant to behavioural and classic antipsychotic therapies. Despite this, currently the diseases' mechanisms leading to ALBSs remain unexplored. In this study, we identified endophenotypes of ALBSs in young male MPS-IIIA mice, including social interaction impairment, increased stereotyped behaviours and hyperactivity. We then found that the identified ALBSs are associated with increased expression of mesencephalic tyrosine hydroxylase (TH) positive neurons appearing early during development and followed by increased striatal DA content. These changes in TH expression can be reproduced in cellular models of the disease (mesencephalic primary neurons; reprogrammed dopaminergic neurons; SH-SY5Y CRISP/Cas9). Using

different behavioural pharmacological approaches, we identified at least two compounds that can rescue ALBSs in MPS-IIIA. These findings identify, for the first time, embryonic dopaminergic neurodevelopmental defects due to defective function of HS leading to ALBSs in LSD, and support evidence showing that altered HS-related genes function are causative of autism.

Mechanistic Understanding of PFAS Penetration Across Human Blood–Brain Barrier and Neurotoxicity Using an IVIVE–PBPK Framework

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Perfluoroalkyl substances (PFAS), especially PFOS (perfluorooctane sulphonate) and PFOA (perfluorooctanoic acid), are widely used in manufacturing and ubiquitously spread in the environment throughout the world. Recent studies have claimed that the accumulation potential of PFOS and PFOA in the brain is linked to various neurological disorders. However, research regarding the penetration of these chemicals across the blood–brain barrier (BBB) is still lacking due to ethical concerns, suggesting the need for an integrated *in vitro* and *in silico* framework. The objective of this study is to develop a mechanistic model describing the penetration of PFAS across the human BBB, utilising the data generated from *in vitro* experiments, and then to investigate the risk based on the brain tissue dosimetry physiologically-based pharmacokinetic (PBPK) model. The *in vitro* experiments include quantification of PFAS permeability, interaction with uptake and efflux transporters proteins, and PFAS-induced expression of these transporters at the transcript level in human-derived *in vitro* cell lines. Further, a brain-specific PBPK model is being developed, incorporating a BBB penetration mechanism based on *in vitro* to *in vivo* extrapolation (IVIVE) of data and dividing the brain into different sub-compartments (such as the cortex, hippocampus and rest of the brain). It is proposed that this model is used to predict maximum concentration (C_{max}), area under curve (AUC) for brain tissue regions, in order to associate it with various neurological disorders. Such a mechanistic brain PBPK model, combined with *in vitro* experiments, can provide a quantitative estimation of the pharmacokinetics of PFAS in the human population. This model could improve the understanding of the toxicological profile of PFAS at target sites and its association with various neurological disorders.

The Chorioallantoic Membrane — *In Vivo* Alternative Model for Studying Biocompatibility

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The chorioallantoic membrane (CAM) is an alternative animal model that is commonly used to study angiogenesis and its inhibition.[1] It can also be used as an *in vivo* pre-screening tool for determining scaffold biocompatibility. We studied the angiogenic response of the quail CAM (*Coturnix coturnix japonica*) after the implantation of a biopolymer composite prepared on a polyhydroxybutyrate and chitosan base (PHB/CHIT, MARSH). We observed differences in the angiogenic response, depending on the porosity of the material and the addition of vascular endothelial growth factor (VEGF-A). On embryonic day 6 (ED6), the tested biomaterials were placed on the CAM alone, or soaked with VEGF-A, at an application dose of 1 μg and 25 ng. Implantation of a piece of biopolymer scaffold on the CAM resulted in a vascular reaction, documented by stereomicroscopy on ED9. We observed and evaluated the formation of vessels in the area surrounding the scaffold, as well as inside the implant. In terms of the increase in blood vessels, the highest angiogenic potential was shown by the MARSH scaffold (66.50%), while the angiogenic effect of the VEGF-A soaked MARSH scaffold was slightly decreased at application dose of 25 ng (65.48%). The angiogenic potential of PHB/CHIT scaffold was lower than MARSH (61.65%), as well as in combination with 25 ng VEGF-A (61.15%). The addition of 1 μg VEGF-A to both types of materials had a weaker angiogenic effect: MARSH (46.48%) and PHB/CHIT (40.69%). For the visualisation of the vascular network inside the implants we used the QH1 and WGA endothelial cell markers. Differences in angiogenic response may be due to the pore size of biomaterials. In the PHB/CHIT scaffold, up to 90% of pores do not reach more than 30 μm. The same percentage of pores in the MARSH scaffold reach a size up to 80 μm. The CAM model is a cost-effective and easy way of monitoring the biocompatibility of porous biomaterials with respect to the Three Rs. Detection of changes in the angiogenesis process makes this alternative animal model a suitable system for rapid screening of porous biomaterials and their biocompatibility, especially in the field of tissue engineering.

Reference

1. Mangir N, Dikici S, Claeyssens F, et al. Using *ex ovo* chick chorioallantoic membrane (CAM) assay to evaluate the biocompatibility and angiogenic response to biomaterials. *ACS Biomater Sci Eng* 2019; 5: 3190–3200.

Acknowledgements

This work was supported by Project No. 2018/14433: 1-26C0, which is supported by the Ministry of Education, Science, Research and Sport of the Slovak Republic within the provided incentives for research and development from the state budget in accordance with Act No. 185/2009 on incentives for research and development, and Grants APVV-17-0110 and VEGA 1/0050/19.

A Human-relevant, Biomimetic, Micro-engineered Platform to Study Wound Biofilms

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Microbial infections are a major cause of chronic, non-healing wounds. Bacteria colonise the wound bed and form biofilms. Infections involving biofilms are recalcitrant to conventional antibiotics.[1] Alternative antimicrobial therapeutics for wound biofilms are being investigated — however, there is a lack of high-throughput testing methods to advance them through the drug development pipeline. In addition to ethical concerns, *in vivo* animal models, such as mouse models, do not represent human wound healing processes. Wound biopsies used as *ex vivo* models are difficult to obtain, and are limited by throughput. Existing *in vitro* models, although suitable for high-throughput testing, may not have all the relevant components of the wound infection microenvironment. We propose a 3-D human-relevant, biomimetic *in vitro* model that recapitulates the wound infection microenvironment. The model consists of two components: a) a coculture of human dermal fibroblasts and epidermal keratinocytes grown on a collagen-coated transwell membrane; and b) an *in vitro* wound milieu (IVWM), consisting of an in-house composition of simulant wound fluid. We have demonstrated that the prominent wound pathogens, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, form biofilms in the IVWM.[2] Further, we have established a human-relevant wound bed by developing reduced-serum methods to coculture human dermal fibroblasts and epidermal keratinocytes.[3] We are now adapting the wound bed to the IVWM. Using our platform, we will explore how biofilms are developed in the cocultured wound bed, and further validate the model for preclinical testing of novel and potential antimicrobials.

References

1. Schultz G, Bjarnsholt T, James GA, et al. Consensus guidelines for the identification and treatment of biofilms in chronic nonhealing wounds. *Wound Repair Regen* 2017; 25: 744–757.
2. Kadam S, Madhusoodhanan V, Bhide D, et al. Milieu matters: an *in vitro* wound milieu to recapitulate key features of, and probe new insights into, polymicrobial biofilms. *Biofilm* 2021; 3: 100047.
3. Kadam S, Vandana M and Kaushik KS. Reduced serum methods for contact-based coculture of human dermal fibroblasts and epidermal keratinocytes. *Biotechniques* 2020; 69: 347–355.

An *In Vitro* Study for Exploring the Cytostatics Conditioning Prior to Mesothelin CAR T-Cell Therapy for the Treatment of Advanced Ovarian Cancer

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To reduce animal testing in medicine, there is a need to test the adverse effects of drugs preliminarily *in vitro*. Here, the best combination for cytostatic conditioning, without affecting the anti-tumour activity of chimeric antigen receptor (CAR) T-cell therapy for the treatment of advanced ovarian cancer, was determined *in vitro*. In our study, the cytostatics fludarabine and treosulfan were employed, and their anti-tumour effects on ovarian cancer cells were tested by ELISA in a hypoxic environment.[1] To test the effect of the cytostatics on the anti-tumour activity of CAR T-cells, the mitochondrial metabolism of CAR T-cells, the secretion of various cytokines, and the ability to differentiate into CD4 T-cells and CD8 T-cells, were tested by FACS under drug exposure.[2] The combination of cytostatics, having good anti-tumour activity but minimal effects on CAR T-cells, was selected and was validated against different ovarian cancer cell lines and T-cells from different donors.

The use of an *in vitro* cytostatic dose determination method such as the one described, could significantly reduce the numbers of animals used for this type of medical testing.

References

1. Xu J, Wang Y, Shi J, et al. Combination therapy: a feasibility strategy for CAR-T cell therapy in the treatment of solid tumors. *Oncol lett* 2018; 16: 2063–2070.
2. Das RK, O'Connor RS, Grupp SA, et al. Lingering effects of chemotherapy on mature T cells impair proliferation. *Blood Adv* 2020; 4: 4653–4664.

UVA-induced Damage and Replicative Senescence in Human Dermal Fibroblasts: *In Vitro* Models of Skin Ageing

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Senescent cells are heterogenous populations of cells that have undergone phenotypical changes due to various stimuli (e.g. oxidative stress, oncogene activation, or telomere attrition).[1] Regardless of the stress inducer, senescent cells may accumulate over time, thus contributing to or causing the ageing phenotype of skin[2] — i.e. disruption of the extracellular dermal matrix, loss of tensile strength and elasticity, impaired wound healing, loss of skin tone and formation of wrinkles and age spots.[3] We are studying two different models of ageing in human dermal fibroblasts (HDFs) by inducing DNA damage and cell cycle arrest via: a) UVA irradiation causing stress-induced premature senescence; and b) telomere shortening and exhaustion of proliferative capacity (replicative senescence). The overall aim is to investigate how premature and replicative senescence of fibroblasts impacts the metabolism of skin keratinocytes. Our results confirm DNA damage (increase of γ-H2AX foci) and positivity for several markers of senescence: enlarged cell morphology, loss of proliferation, increased expression of senescence-associated beta-galactosidase (SA-β-gal) in both models. UVA-induced premature senescence provides a useful model to study physiology and molecular mechanisms involved, to prevent, slow down or reverse skin ageing. Our UVA-induced premature senescence model shows a temporary cell cycle arrest in HDFs. Moreover, comparison of genes associated with senescence and metabolism via Fluidigm Real-Time PCR between the two models reveals activation of different cellular pathways. Next steps would involve investigating the effects of senescent fibroblasts at different stages on primary keratinocytes, in terms of their viability, proliferation and metabolism.

References

1. Campisi J and d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* 2007; 8: 729–740.
2. Baker DJ, Wijshake T, Tchkonia T, et al. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* 2011; 479: 232–236.
3. Lephart ED. Skin aging and oxidative stress: equol's anti-aging effects via biochemical and molecular mechanisms. *Ageing Res Rev* 2016; 31: 36–54.

Human Progeria Fibroblasts in Culture to Study New Strategies to Delay Ageing

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Hutchinson–Gilford progeria syndrome (HGPS), or progeria, is a rare premature ageing syndrome caused by a mutation within the LMNA gene, producing an abnormal lamin A protein, termed progerin. The accumulation of progerin causes

nuclear abnormalities and cell cycle arrest, ultimately leading to early cellular senescence. We have previously shown that neuropeptide Y (NPY) increases autophagy in the hypothalamus,[1] a brain area already identified as a central regulator of whole-body ageing and mediator of caloric restriction-induced autophagy. These results are in accordance with other studies suggesting that NPY may act as a caloric restriction mimetic and play a role in ageing regulation and lifespan.[2] Therefore, our aim was to investigate whether NPY could be a relevant strategy to delay ageing in progeria fibroblasts. As a cellular ageing research model, we used primary cell cultures of dermal fibroblasts derived from progeria patients. Cells were exposed to NPY and its effects on several cellular ageing hallmarks were evaluated. In progeria cells, NPY increased autophagic flux and decreased progerin levels. NPY also rescued nuclear morphology and decreased γH2AX foci, a marker of DNA damage. In addition, NPY increased the proliferative capacity of HGPS cells, decreasing the well known cell cycle inhibitors, p53 and p21. Moreover, NPY decreased the number of SA-β-Gal positive cells, indicating that NPY slowed down the cellular senescence progression. Altogether, our study shows that NPY rescues several hallmarks of cellular ageing in progeria fibroblasts, suggesting that NPY modulation can be considered as a stepping-stone to future genetic/pharmacological interventions to counteract HGPS progression and age-related deteriorations.

References

1. Aveleira CA, Botelho M, Carmo-Silva S, et al. Neuropeptide Y stimulates autophagy in hypothalamic neurons. *Proc Natl Acad Sci USA* 2015; 112: E1642–E1651.
2. Botelho M and Cavadas C. Neuropeptide Y: an anti-aging player? *Trends Neurosci* 2015; 38: 701–711.

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The Future of EPA's Toxicity Reference Database, ToxRefDB

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A component of building scientific confidence in new approach methodologies (NAMs) for toxicology is comparison to results from *in vivo* studies. The EPA's Office of Pesticide Programs (OPP) requires registrants to submit *in vivo* studies to evaluate potential human health and environmental effects. Data evaluation records (DERs) from the OPP provide a review of the studies conducted in accordance with EPA testing guidelines for pesticide registration, and represent enormous time, financial, and animal resources. Each DER summarises methods, results, as well as evaluator conclusions. These data remain largely cached in an image-based format and, therefore, are largely under-utilised for NAM evaluation and development. The Toxicity Reference Database (ToxRefDB) takes advantage of this resource through manual curation of nearly 6000 studies for over 1000 chemicals to date.[1] The objective of ongoing development work for ToxRefDB is to increase the chemical and study data coverage to enable comparative analyses of NAM performance. A new data collection tool (DCT) is being developed for curation of additional documents with enhanced quality control. The first proof-of-concept project using the DCT includes developmental toxicity DERs from approximately 50 pesticide submissions. The DCT application will capture basic study design metadata, dose–response, treatment-related and critical effects, and endpoint testing status information. A graphical user interface will be used to visualise these legacy *in vivo* study data and as a prototype for future public interfaces. ToxRefDB expansion increases its utility as a resource for retrospective analyses that lay the foundation for acceptance of NAMs, as well as development of new predictive tools.

[This abstract does not necessarily reflect U.S. EPA policy.]

References

1. Watford S, Ly Pham L, Wignall J, et al. ToxRefDB version 2.0: Improved utility for predictive and retrospective toxicology analyses. *Reprod Toxicol* 2019; 89: 145–158.

PFAS Interference with Lipid Metabolism and Phase I and II Biotransformation Enzymes in Human Liver Cells

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Human exposure assessments for perfluorooctanoate (PFOA) and perfluorooctane sulphonate (PFOS) have been mostly limited to the quantification of these chemicals in different environmental matrices, but only a few studies have addressed adverse biological aspects associated with them. Significant attention has been given to the potential of these compounds to disrupt lipid homeostasis at the cellular level. Similarly, while the high chemical stability of many PFAS prevent them from being metabolised, previous reports have described abnormal biotransformation activity in exposed organisms. In the present study, the dysregulation of lipid metabolism and potential alterations to biotransformation pathways in human liver cells were examined through a quantitative metabolomic approach, and by the measurement of expression and activity of phase I and II biotransformation enzymes, respectively. PFOA, but not PFOS, significantly increased total lipids in hepatocytes, with diglycerides and triglycerides being the most prominent, and led to significant lipid bioaccumulation at environmentally relevant concentrations. In terms of biotransformation, the expression of phase I enzymes (CYP1A2, CYP2C19 and CYP3A4) was significantly reduced from exposure to both PFOA and PFOS, with CYP3A4 presenting the lowest activity. Among phase II conjugation enzymes, the expression of UGT was significantly reduced by PFOA, yet no significant alterations in its activity were observed. Besides the significant contribution to the applicability of animal alternatives in toxicology, the results from these mechanistic assessments suggest that PFOA/PFOS-driven interferences could contribute to the onset of lipid-related diseases and to a number of adverse outcomes resulting from the inability of biotransformation pathways to function as required.

Use of Bioanalytical Tools for the Screening of Fishes Naturally Contaminated with Ciguatoxins

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Ciguatera fish poisoning (CFP) is one of the most relevant seafood-borne diseases worldwide. It is caused by the ingestion of fish containing ciguatoxins (CTXs), lipophilic marine toxins produced by microalgae of the genera *Gambierdiscus* and *Fukuyoa*[1] that accumulate in fish flesh and through the food webs. CFP is characterised by severe neurological, gastrointestinal and cardiovascular disorders, and affects approximately between 50,000 and 500,000 consumers annually worldwide.[2] The real incidence of CFP is difficult to ascertain, due to under-reporting and misdiagnosis. The mouse bioassay (MBA) has been the most widely used method to detect CTXs over the years. However, due to its insufficient detection capability and ethical concerns, other methods have been developed, like liquid chromatography and cell-based assays (CBAs). Here, the first electrochemical immunosensor for the detection of CTXs is presented. Three different monoclonal antibodies (mAbs) — two capture mAbs (3G8, 10C9)[3,4] and a detector MAb (8H4)[3] — were merged in a sandwich configuration for the combined detection of two main groups of CTX congeners (CTX1B and CTX3C). Initially, the applicability of the immunosensor has been demonstrated with the analysis of fish samples coming from La Réunion island, providing results that correlate with those of CBAs and MBAs previously performed on the same extracts. Also, liquid chromatography confirmed the presence of CTX1B in the fish extracts. Subsequently, fish originating from Mediterranean waters were also analysed, giving promising results. This bioanalytical tool is user-friendly, does not require the sacrifice of animals, and can help to mitigate ciguatera risk.

References

1. Litaker RW, Holland WC, Hardison DR, et al. Ciguatoxicity of *Gambierdiscus* and *Fukuyoa* species from the Caribbean and Gulf of Mexico. *PLoS One* 2017; 12: e0185776.

2. Friedman MA, Fernandez M, Backer LC, et al. An updated review of ciguatera fish poisoning: clinical, epidemiological, environmental, and public health management. *Mar Drugs* 2017;15: 72.
3. Tsumuraya T, Fujii I, Inoue M, et al. Production of monoclonal antibodies for sandwich immunoassay detection of ciguatoxin 51-hydroxyCTX3C. *Toxicon* 2006; 48: 287–294.
4. Tsumuraya T, Takeuchi K, Yamashita S, et al. Development of a monoclonal antibody against the left wing of ciguatoxin CTX1B: thiol strategy and detection using a sandwich ELISA. *Toxicon* 2012; 60: 348–357.

Metal Exposure During Pregnancy and Telomere Length and mtDNA in the Placenta

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Through our diet we are exposed to various toxic metals, which may be of concern during pregnancy as many metals have been associated with developmental toxicity, although the underlying modes of action are largely unknown. There are indications that the accumulation and transport of toxic metals may affect placental function which, in turn, may cause adverse effects on the developing fetus. This study aims to elucidate if toxic metal exposure in pregnancy impacts telomere length (TL) and mitochondrial DNA (mtDNA) in the placenta. This study involved 419 mothers from the longitudinal mother–child cohort NICE in Northern Sweden. The exposure to several metals — arsenic (As), cobalt (Co), cadmium (Cd) and mercury (Hg) — was assessed by determining blood and urine concentrations in the third trimester of pregnancy, and in placentas after delivery, by using Inductively Coupled Plasma-Mass Spectrometry. Placental DNA was extracted and qPCR was performed to quantify telomere repeats and mtDNA. The median concentrations of As, Co, Cd and Hg in the placenta were 1.77, 3.19, 2.29 and 1.82 ng/g. In preliminary multivariable-adjusted regression models, placental concentrations of Co, Cd and Hg were inversely associated with relative mtDNA, while As was associated with increased relative mtDNA. Besides this, exposure to As was positively associated with relative TL. In conclusion, our findings suggest that placental accumulation and transport of toxic metals may affect placental function.

Investigation of Natural Agents as Novel Anti-cancer Agents in an *In Vitro* Model of Kidney Cancer

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The use of the TNF-related apoptosis-inducing ligand (TRAIL) in cancer therapy has been considered an attractive option to target different types of tumours with minimal toxicity to normal cells. However, many cancer cells, including kidney cancer, are resistant to TRAIL, limiting its therapeutic application in the clinical setting.[1] The natural agent curcumin has been studied for its anti-cancer properties alone and in combination with TRAIL, but its low bioavailability reduces its therapeutic potential.[2] Our study focuses on the ability of curcumin analogues to sensitise kidney cancer cells to TRAIL and eradicate carcinoma cells. IC_{10} concentrations of curcumin and its derivatives were estimated in cytotoxicity assays performed on a healthy kidney cell line, and then tested on a renal cell carcinoma cell line (ACHN) to assess their potential to induce cell death and their potential synergy with TRAIL. As an alternative to *in vivo* xenograft models, *in vitro* scratch migration assays were used to determine whether these compounds, individually and in combination with TRAIL, reduce the migratory potential of ACHN. Western blot analysis was used to investigate the potential effects of these compounds on different pro- and anti-apoptotic molecular targets. It was established that curcumin and derivatives sensitised ACHN cells to TRAIL-induced apoptosis, as well as reducing the migration potential *in vitro*. Therefore, the use of curcumin and other natural compounds as TRAIL sensitisers, in combination with TRAIL therapies, may prove to be a useful cancer therapeutic strategy.

References

1. de Miguel D, Lemke J, Anel A, et al. Onto better TRAILs for cancer treatment. *Cell Death Differ* 2016; 23: 733–747.
2. Obaidi I, Cassidy H, Gaspar VI, et al. Curcumin sensitizes kidney cancer cells to TRAIL- induced apoptosis via ROS mediated activation of JNK-CHOP pathway and upregulation of DR4. *Biology* 2020; 9: 92.

Students' Demographics Influence their Opinion on the Use of Animals for Educational Experiments in Medicine and Veterinary Medicine

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Human–animal interaction is dated back to ancient times when animals played an important role as a source of food, guardianship, traction power and warfare. During the Renaissance, and in more recent times, animals were the 'resource' used for the development of science through animal experimentation. Nowadays, humankind is still dependent on the use of animals for scientific purposes, with aims such as discovering treatments for serious diseases, developing vaccines against emerging viruses, progressing biotechnology, among others. Non-human animals are also used for teaching purposes for gaining practical skills in some professions, like human medicine and veterinary medicine. The present work studied the demographic profile of veterinary and medical students and its influence on their perceptions on the use of animal experiments for training. Gender,[1] geographical location and personal experience of pet-keeping were found to be among the factors which contribute to the formation of human–animal relationship and thus predetermine respondents' attitudes toward animal experiments.[3] The adoption of a vegetarian or vegan diet[2] was also found to impact the student's position on animal exploitation, even during the course of their training.

References

1. Herzog HA. Gender differences in human-animal interactions: a review. *Anthrozoös* 2007; 20: 7–21.
2. Mesarčová L, Skurková L, Kottferová J, et al. The influence of dietary preferences of veterinary medicine students on opinions forming in various ethical dilemmas. In: Springer S and Grimm H (ed) *Professionals in food chains*. Wageningen: Wageningen Academic Publishers, 2018, pp. 505–511.
3. Sandgren EP, Streiffer R, Dykema J, et al. Assessing undergraduate student and faculty views on animal research: what do they know, whom do they trust, and how much do they care? *PLoS One* 2019,14: e0223375.

Benchmark Dose Analysis in Toxicology and its Support of the Three Rs Principles

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By definition, the benchmark dose (BMD) is considered a dose that results in a small change in effect in comparison with the control. The BMD approach was first introduced by Crump in 1984 for analysis of quantal data. Later, its use was extended to also include continuous data, and the approach was recognised as an advanced scientific method to obtain a point of departure. So far, two software packages are highly recommended for BMD calculations: Benchmark Dose Software (BMDS), created by the US Environmental Protection Agency; and PROAST software, created by the Netherlands National Institute for Public Health and the Environment.[1] This methodology represents a substitute for the No Observed Adverse Effect Level (NOAEL) approach and a significant advancement from the standpoint of the Three Rs, as applied to the use of laboratory animals. It allows the use of existing datasets from earlier experiments for evaluation of a dose–response relationship, rather than conducting new experiments.[2] Moreover, for novel toxicological endpoints, alternatives should be performed, including contemporary *in silico* and *in vitro* methods. The European Food Safety Authority (EFSA) supports the use of epidemiological toxicological data for dose-response analyses and determination of the BMD.[1] In conclusion, the BMD approach could be counted as a significant step forward regarding the Three Rs principles and a major step in avoiding the use of laboratory animals wherever possible.

References

1. EFSA Scientific Committee, Hardy A, Benford D, et al. Update: Use of the benchmark dose approach in risk assessment. *EFSA J* 2017; 15(1): e04658.
2. Slob W. Benchmark dose and the Three Rs. Part I. Getting more information from the same number of animals. *Crit Rev Toxicol* 2014; 44(7): 557–567.

Qualification of Microsampling in the Metabolites in Safety Testing (MIST) Analysis

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Upon administration, drugs are usually transformed to metabolites, which may be associated with toxic effects. To ensure that human metabolites have been tested in toxicity studies — i.e. adequately covered in the preclinical safety studies — a metabolite in safety testing (MIST) guidance was introduced by the Food and Drug Administration in 2008.[1] A key aspect of the MIST is to identify metabolites that are disproportionate or unique in humans, as compared to the animals used for toxicity testing. This is performed through a multi-step process, from *in vitro* cross-species comparison to human ADME metabolite profiling and characterisation. Part of the process is the crucial MIST analysis, where exposures to human metabolites are compared between human and preclinical safety species. In this analysis, samples from all study species are used for AUC pooling and comparative analysis.[2] The overall aim of this work is to qualify the use of small volumes of plasma, i.e. microsamples, for this approach. This will be done by generating relevant samples *in vitro* and by comparing both quantitative and qualitative accuracy of the analysis of microsamples *versus* conventional larger volume samples.

References

1. FDA. *Safety testing of drug metabolites for industries, guidance for industry*. Silver Spring, MD: Center for Drug Evaluation and Research, 2020, 11 pp.
2. Cheung BWY, Cartier LL, Russlie HQ, et al. The application of sample pooling methods for determining AUC, AUMC and mean residence times in pharmacokinetic studies. *Fundam Clin Pharmacol* 2005; 19: 347–354.

Health Hazards of Detergents, Degreasers and Disinfectants Available on the Swedish Market

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There is an ever-growing and extensive usage of cleaning agents worldwide. This causes humans to be frequently exposed to a wide range of chemicals used in cleaning products. To evaluate the acute health hazards of cleaning agents on the Swedish market, we applied three methods. First, we collected data on marketed volumes of cleaning agents from the Swedish Chemicals Agency. Second, we evaluated 224 safety data sheets (SDS) from a major retailer in Sweden. Third, using calls to the Swedish Poisons Information Centre (PIC) during 2015–2019, accidents with cleaning products — noting the exposure route, cause, hazard category and product group — were studied. Health hazard statements that occurred commonly among the SDS of cleaning products and their recurrent combinations were visualised via social network analysis for estimation of the hazard potential. The PIC data were evaluated with cross tabulation and graphing. Hazard statements associated with the eyes were found to be the most recurrent among cleaning products. Ocular injuries were also the most frequently found in the records of cases handled by the Swedish PIC. Out of a total 17,622 cases, 35% concerned ocular exposure, 33% ingestion and 16% inhalation, as the route of exposure. The cases occurring via several exposure routes were most frequently judged to pose a clear risk to the persons exposed. Harsh cleaning agents were found to be responsible for the highest percentage of clear risk incidents (28%). The analysis of Swedish PIC call records revealed that the general public was 4.33 times more likely to call about cleaning agent exposures at home than professionals in occupational settings. However, accidents at work were more likely to be judged as posing a clear risk (27%) to the exposed workers than those occurring among the general public (7%). Our findings suggest additional risk management measures are needed to protect cleaning agent users from ocular injuries.

Toxicological Profiling of Environmental 2-, 3- and 4-Nitrophenols Using Human Lung Cells

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Nitrophenols are important environmental pollutant tracers, including in agricultural residues, rainwater, industrial wastes, toluene emissions and benzene emissions.[1,2] The current study highlights the toxicological profiling, in the lung normal bronchial epithelial (BEAS-2B) and alveolar epithelial cancer (A549) cell lines, of three important atmospheric nitrophenols, including 2-, 3-, and 4-nitrophenols and their equimolar mixture. The aim of the study was to determine the effects on the lung cells following inhalation. The IC_{50} was found to be highest following treatment with 2-nitrophenol, while lowest after treatment with 4-nitrophenol. The comparative toxicology profile of the three individual nitrophenols and the equimolar mixture revealed that the mixture was more cytotoxic. A higher percentage of cell death was observed following exposure to the mixture, as determined through viability analysis in the lactate dehydrogenase (LDH) assay and by calcein-AM/propidium iodide staining. Furthermore, the percentage population of late apoptotic/necrotic BEAS-2B and A549 cells increased with 3-nitrophenol, 4-nitrophenol and the nitrophenol mixture, after the 24-hour and 48-hour treatments. The 2-nitrophenol treatment did not cause changes in cellular proliferation rate or viability following exposure, as observed through annexin-V/FITC analysis via flow cytometry. Finally, the cells were analysed for changes in reactive oxygen species (ROS) accumulation. An increase in general ROS led to cell death post-exposure. The 3- and 4- nitrophenols and the nitrophenol mixture caused an increase in mitochondrial superoxide signal post-exposure in the BEAS-2B cells only. Furthermore, the cells were analysed for changes in mitochondrial membrane potential through the use of TMRM dye. Decrease in the TMRM signal was significant in BEAS-2B only, predominantly with the 4-nitrophenol treatment. This shows that 4-nitrophenol exposure significantly altered ROS regulation in the cell lines, while 2-nitrophenol was less toxic. These data are important to help promote the setting up of air pollution control policies by regulatory authorities. They further highlight the imminent need to control the anthropogenic pollution associated with the emission of nitrated aerosol particles, as these particles are likely to contribute to lung-associated pathologies.

References

1. Rudziński KJ and Szmigielski R. Aqueous reactions of sulfate radical-anions with nitrophenols in atmospheric context. *Atmosphere* 2019; 10: 795.
2. Lüttke J, Scheer V, Levsen K, et al. Occurrence and formation of nitrated phenols in and out of cloud. *Atmos Environ* 1997; 31: 2637–2648.

Comparative Study on Arsenic Effects in Selected Species of Earthworms

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Arsenic (As) is widely spread in soil and is poisonous to plants, animals and humans. Arsenic contamination from both anthropogenic and natural origins are one of the biggest problems around the globe. The present research aimed to assess the bioaccumulation of arsenic in selected species of earthworms. To examine comparative bioaccumulation, *Pheretima posthuma* and *Lumbricus terrestris* were exposed to arsenic-contaminated soil having doses of 210, 270 and 330 mg arsenic per 3 kg of soil. Two control experiments, each for one species, and six treatment experiments (all with three replicates) were carried out for a one month duration. The accumulation of metals in the digested samples was analysed by using atomic absorption spectrophotometry. The size of the earthworms, morphological characteristics and weight were examined and contrasted between the different species of earthworm, along with the initially recorded parameters. The biomass (length and weight) of the earthworms increased in the control group and decreased in the treated groups. Dark-coloured patterns were observed on the skin, which indicates the accumulation of metals. Cocoon production was reduced in the treated groups. The arsenic compounds showed more toxicity toward the *Lumbricus* species, as compared to the *Pheretima* species. All the parameters

used in this study indicated arsenic toxicity in both species of earthworms. No changes were seen in the behaviour of the earthworms after the trial, and no mortality occurred during this period. Treatments were statistically evaluated by using ANOVA and the *t*-test. It was inferred that the bioaccumulative ability of earthworms was directly proportional to the concentration of arsenic in the habitat soil.

Development of a Microphysiological Platform for the Integration of Primary Human Cervical Tissue

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The cervix uteri acts as a barrier to prevent infection of the uterus. However, the cervix itself can be infected by viruses such as the human papilloma virus (HPV). HPV infections are the most common sexually transferable infection, i.e. 80% of sexually active people are infected at least once in their lifetime. In most cases the infection will clear itself, yet, depending on the strain, genital warts or cervical intraepithelial neoplasia (CIN) can develop. Upon a persistent infection, CIN may progress to a squamous cell carcinoma, which is the fourth most common cancer in women worldwide leading to 310,000 deaths. Due to the high clinical relevance of cervical cancer, a 3-D *in vitro* model of the cervix in a microfluidic platform (Cervix-on-Chip, CoC) is being developed. The CoC consists of a tissue chamber for the integration of primary human cervical cells and a channel for media supply. For fabrication of the CoC, layers of polymethyl(meth)acrylate (PMMA) and polycarbonate were laser cut and thermally bonded together. Isolation and culture of human patient-specific keratinocytes and stromal cells from biopsies using optimised protocols result in proliferative, viable cells that can successfully be cultured in the CoC platform. Processes for the seeding of the primary keratinocytes into the CoC were developed and media supply optimised. In the future, the CoC has the potential to serve as a basis for prospective disease modelling and as a testing platform for treatment options.

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Mechanically Stimulated 3-D Endothelial Intestine-on-Chip Device to Study Gut–Microbiome Interactions

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The human gut microbiome constitutes the most abundant and diverse ecosystem, as compared to the other areas of the body.[1] Growing attention is devoted to the bacterial balance and make-up in human intestines, which changes over time.[2,3] These bacteria play a significant role in the response to cancer immunotherapy.[4] Current cell culture and animal models present substantial limitations to the investigation of this effect.[5] 3-D multi-compartment microfluidic cultures may overcome these obstacles, mainly due to their ability to mimic elaborate multicellular architectures and niches, while maintaining high levels of experimental control.[6,7] Among the various gut-on-chip designs, the distinction can be made between 2-D and 3-D cellular microenvironments, either mechanically stimulated or not. Mechanical actuation and 3-D niche recapitulation proved to be critical in the maturation and translation of models from *in vivo* to *in vitro*. Here, we present the intestine-on-chip device with endothelium captured in 3-D under mechanobiological stimulation. The device integrates actuation on a three-cell type coculture, which includes human intestinal epithelial cell lines (Caco2, HT29) and human micro-endothelial cells (HMEC1), connected by a collagen-based extracellular matrix compartment. Multi-parametric assessment of cell viability and function at different time points describes the influence of mechanobiological stimuli on the maturation of the gut endothelium. The mechanically stimulated 3-D intestine-on-chip provides an appealing platform to study how microorganisms modulate the crosstalk between epithelial and endothelial compartments in the gut, and thus represents a valuable alternative model for preclinical studies.

References

1. Quigley EMM. Gut bacteria in health and disease. *Gastroenterol Hepatol* 2013; 9: 560–569.

2. Wu GD, Chen J, Hoffmann C, et al. Linking long-term dietary patterns with gut microbial enterotypes. *Science* 2011; 334: 105–108.
3. Alcock J, Maley CC and Aktipis CA. Is eating behavior manipulated by the gastrointestinal microbiota? Evolutionary pressures and potential mechanisms. *BioEssays* 2014; 36: 940–949.
4. Gopalakrishnan V, Spencer CN, Nezi L, et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science* 2018; 359: 97–103.
5. Robinson NB, Krieger K, Khan F, et al. The current state of animal models in research: a review. *Int J Surg* 2019; 72: 9–13.
6. Low LA, Mummery C, Berridge BR, et al. Organs-on-chips: into the next decade. *Nat Rev Drug Discov* 2021; 20: 345–361.
7. Zhang B, Korolj A, Lai BFL, et al. Advances in organ-on-a-chip engineering. *Nat Rev Mater* 2018; 3: 257–278.

Impact of Fetal Growth Restriction on the Developing Brain Using an *In Vitro* Neurosphere Model

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Intrauterine growth restriction (IUGR) is defined as a significant reduction in the fetal growth rate. Placental insufficiency, the main cause of IUGR, reduces placentary blood flow leading to fetal development under chronic hypoxia, which is associated with neurodevelopmental damage, cognitive dysfunctions and cardiovascular adverse outcomes. The characterisation of neurostructural changes in fetuses with IUGR is essential to design therapeutic strategies directed to limit its deleterious effects. We have established for the very first time an *in vitro* model based on primary rabbit neuronal progenitor cells (NPCs) cultured as neurospheres.[1] Neurospheres can mimic basic processes of fetal brain development like proliferation, migration and differentiation.[2] We successfully evaluated further relevant endpoints like neurite branching and synaptogenesis. By comparing the functionality of control and IUGR neurospheres we identified that rabbit NPCs from IUGR individuals have a significantly reduced ability to form oligodendrocytes. To find a neuroprotective therapy preventing/reverting the adverse effects of IUGR, we tested six different compounds at increasing concentrations — docosahexaenoic acid (DHA), choline, lactoferrin, melatonin, zinc, and 3,3′,5-triiodo-L-thyronine (T3) — in the neurosphere model. Basic processes of neurogenesis were assessed to determine the maximum tolerated concentration (MTC) and the effective concentration (EC). DHA (MTC = 10 μM; EC = 1 μM), melatonin (MTC = 3 μM; EC = 1 μM) and T3 (MTC = 30 nM; EC = 0.1 nM) have been selected as the most promising therapies due to their promoting effect in oligodendrogenesis. The *in vitro* model allows us to evaluate different processes of neurogenesis in a fast, economic and ethical way, and contributes to a better understanding of IUGR induced neuronal damage and the selection of new neuroprotective therapies.

References

1. Barenys M, Illa M, Hofrichter M, et al. Rabbit neurospheres as a novel *in vitro* tool for studying neurodevelopmental effects induced by intrauterine growth restriction. *Stem Cells Transl Med* 2021; 10: 209–221.
2. Kühne BA, Puig T, Ruiz-Martínez S, et al. Comparison of migration disturbance potency of epigallocatechin gallate (EGCG) synthetic analogs and EGCG PEGylated PLGA nanoparticles in rat neurospheres. *Food Chem Toxicol* 2019; 123: 195–204.

Students' Perceptions on Animal Experiments for Training in Medicine and Veterinary Medicine

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Research has proved that animals are sentient beings which have the ability to feel pain[1] and suffer. Current European and Bulgarian legislation have set provisions for safeguarding animal welfare with the main five freedoms, ensuring that non-human animals are humanely treated when used for breeding, food production, research, entertainment, etc. At the same

time, non-human animals are widely used for experimentation, including for educational purposes,[2] and at the end of some experiments the animals have to be euthanised. We investigated the opinion of medical and veterinary students, in order to understand their perceptions of the use of animals for practical training. The results pointed toward the existence of completely opposing groups, some stating that their competences are dependent on animal experiments, the others arguing that alternative non-animal approaches are enough for professional training.[3] Opinions differed again regarding the animal species being used. Students were more disturbed by experimentation on mammals, while other vertebrates — such as amphibians or fish — did not receive so much compassion.

References

1. Sneddon LU, Elwood RW, Adamo SA, et al. Defining and assessing animal pain. *Anim Behav* 2014; 97: 201–212.
2. Baumans V. Alternatives to the use of laboratory animals in veterinary education. *ALTEX* 2006; 23 Suppl: 68–70.
3. Kawareti PK, Gedam PM and Ganguly S. Alternative sources of animals in veterinary education: a review. In: Ganguly S and Kawareti PK (ed) *Advances in veterinary sciences*. Delhi: AkiNik Book Publications, 2018, pp. 123–129.

Experimental Models to Evaluate Hypothalamic Insulin Signalling in Metabolic Dysfunctions

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The consumption of high calorie diets leads to obesity and may evolve to more severe pathologies, such as insulin resistance. Obesity is characterised by an impairment in body energy homeostasis, which occurs not only in peripheral tissues but also in the central nervous system, particularly in the hypothalamus.[1] It was shown that targeting hypothalamic dysfunction leads to an amelioration of insulin sensitivity in peripheral organs. Identifying new targets to improve hypothalamic insulin sensitivity may contribute to the prevention and/or treatment of metabolic diseases. This study aims to develop experimental approaches to evaluate hypothalamic insulin resistance. We used three different experimental models to study insulin signalling, with palmitate used as a metabolic stressor in conjunction with different insulin concentrations and incubation times. Insulin signalling was measured in the three models through assessment of AKT phosphorylation (phospho-AKT). In the hypothalamic primary culture model, we did not observe a decrease in phospho-AKT upon fatty acid palmitate incubation. Furthermore, we observed a decrease in cell viability, as evaluated via the lactate dehydrogenase (LDH) assay. In the mHypoN42 cell line model, palmitate induced insulin resistance, as determined by a reduction in phospho-AKT. Our work provides additional information on the use of different models to screen new molecules as inducers of insulin sensitivity or to target insulin signalling, which may lead to the discovery of different approaches to treat metabolic dysfunction.

References

1. Cavadas C, Aveleira C, Souza G, et al. The pathophysiology of defective proteostasis in the hypothalamus — from obesity to ageing. *Nat Rev Endocrinol* 2016; 12: 723–733.

Acknowledgements

European Regional Development Fund (ERDF), through the Centro 2020 Regional Operational Programme, under project CENTRO-01-0145-FEDER-000012-HealthyAging and through the COMPETE 2020 — Operational Programme for Competitiveness and Internationalisation and Portuguese national funds via FCT — Fundação para a Ciência e a Tecnologia, under projects PTDC/MED-FAR/30167/2017 (POCI-01-0145-FEDER-030167), UID/NEU/04539/2019, SFRH/BD/121923/2016 fellowship.

Visualising Drug-induced Toxicity in 3-D Microtissues

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Drug-induced liver injury (DILI) from use of xenobiotics remains a common cause for both acute and chronic liver damage with compound toxicity often not detected during *in vitro* safety assays. The current safety assays are still not optimised to bridge the gap between *in vitro* and *in vivo* models. However, recent research is hoping to improve toxicity prediction by using 3-D microtissues which have shown to increase sensitivity to hepatotoxins and increase the physiological relevance of *in vitro* assays.[1] High-content imaging (HCI) provides the possibility of monitoring multiple cellular parameters while providing insight into the mechanisms of drug-induced toxicity and cellular health.[2] The aim of this study was to optimise cellular imaging techniques of 3-D microtissue staining on the C3A cell line to help visualise hepatotoxicity. The cells were grown in a 3-D conformation in ultra-low attachment 96-well plates from a density of 500 cells/well and exposed to a variety of model hepatotoxic compounds. Comparison of the sensitivity between monolayered and 3-D cultured cells was determined via an ATP assay and LIVE/DEAD staining. Furthermore, mitochondrial integrity and apoptosis were measured using MitoTracker and CellEvent staining, respectively.

References

1. Gaskell H, Sharma P, Colley HE, et al. Characterization of a functional C3A liver spheroid model. *Toxicol Res(Camb)* 2016; 5: 1053–1065.
2. Li S and Xia M. Review of high-content screening applications in toxicology. *Arch Toxicol* 2019; 93: 3387–3396.

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 formalise 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, limiting their application in risk assessment. To advance this aspect of the field, we quantify how frequently and confidently cellular events precede adverse histopathology serving 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 repeat-dose studies in rats across eight timepoints, ranging from three hours to four weeks. After inferring dysregulated cellular processes from gene expression, we found that some known key events in DILI[3] are highly specific but precede adverse histopathology only rarely, such as LXR signalling. In contrast, others are found frequently at the expense of lower specificity, e.g. mitophagy. Among all derived events, the most frequent pathways point to RNA metabolism and EIF2AK4 (GCN2), a known regulator of liver fibrogenesis in mice.[4] The most frequent transcription factors, inferred from the expression of downstream genes, suggest increased activity of E2f2, Nr1h3, Atf4 and Nfe2l2, as well as decreased activity of Srebf1 and Srebf2. Among those, changes on the expression level are only found for Atf4 and Nfe2l2, highlighting the role of post-transcriptional regulation. Overall, we derive mechanistic information on events preceding DILI, including their strength of association and longitudinal order, and demonstrate how time-resolved transcriptomics can be used to contribute toward the development of quantitative AOPs.

References

1. Ankley GT, Bennett RS, Erickson RJ, et al. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ Toxicol Chem.* 2010; 29: 730–741.
2. Igarashi Y, Nakatsu N, Yamashita T, et al. Open TG-GATEs: a large-scale toxicogenomics database. *Nucleic Acids Res* 2015; 43(D1): D921–D927.
3. Andrade RJ, Chalasani N, Björnsson ES, et al. Drug-induced liver injury. *Nat Rev Dis Prim* 2019; 5: 1–22.

4. Arriazu E, De Galarreta MR, López-Zabalza MJ, et al. GCN2 kinase is a key regulator of fibrogenesis and acute and chronic liver injury induced by carbon tetrachloride in mice. *Lab Investig* 2013; 93: 303–310.

Building a Toolbox to Mitigate Drug-induced Pancreatic Injury

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In drug discovery, it is crucial to assess potential toxicity in the early phases, in order to select the compounds with the highest chance of success. Screening against toxicities linked to major organ systems, such as the liver and cardiovascular system, is accommodated within the safety strategy of most pharmaceutical companies.[1] Even though pancreatic toxicity has low prevalence in drug discovery, there are certain drugs which possess an inherent risk for damaging the pancreatic beta cells.[2,3] Currently, there is a lack of simple *in vitro* models to predict clinical pancreatic toxicity, and such toxicity in preclinical species does not translate well to humans. Since the evaluation of toxicity to beta cells is important for compounds with a hypothetical risk of causing endocrine pancreatic toxicity, it would be of great value to identify *in vitro* assays which could be used for pancreas risk mitigation during early drug discovery. The aim of this project is, therefore, to develop a toolbox of *in vitro* assays for assessing compounds with pancreatic injury potential. The human pancreatic cell line EndoC-βH1 and the Min6c4 mouse will be treated with known pancreatic toxic compounds to assess structural and functional endpoints, including viability, membrane integrity and glucose-stimulated insulin secretion, through the use of high-content imaging and ELISA. Qualitative markers for beta cell integrity will be assessed by qRT-PCR. Possibilities for growing cells as pseudoislets will also be explored. This toolbox of beta cell assays will be valuable for preclinical safety evaluation of drugs with endocrine pancreatic injury potential at AstraZeneca.

References

1. Johansson J, Larsson MH and Hornberg JJ. Predictive *in vitro* toxicology screening to guide chemical design in drug discovery. *Curr Opin Toxicol* 2019; 15: 99–108.
2. Lindner P, Christensen SB, Nissen P, et al. Cell death induced by the ER stressor thapsigargin involves death receptor 5, a non-autophagic function of MAP1LC3B, and distinct contributions from unfolded protein response components. *Cell Commun Signal* 2020; 18: 12.
3. Axten JM. Protein kinase R(PKR)-like endoplasmic reticulum kinase (PERK) inhibitors: a patent review (2010–2015). *Expert Opin Ther Pat* 2017; 27: 37–48.

In Vitro Approach to Evaluate Genotoxicity of Agrochemicals Used in Grapevine Diseases (Peronospora and Oidium) at Environmentally Relevant Concentrations

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The genotoxic effects of two fungicides, zoxamide (ZM) and mancozeb (MZ), representative of two different agronomical protocols used in Northern Italy to control the main grapevine diseases Peronospora and Oidium, were comparatively evaluated by using the comet assay and the micronucleus test combined with immunofluorescent CREST staining, to detect possible aneugenic and clastogenic effects. The *in vitro* tests were performed on two human cell lines representative of the main target tissues, namely human hepatocellular carcinoma (HepG2) and human pulmonary adenocarcinoma (A549). Cytotoxicity was evaluated by using the MTS assay and the CyQUANT® assay, with a range of 10-fold serial dilutions starting at the concentration commonly used in the field (ZM 463.4–0.004634 μM; MZ 295.7–0.002957 μM). Reactive oxygen species (ROS) evaluation, as well as the expression analysis of genes implicated in apoptosis/necrosis modulation (BAX and BCL2), redox transcription factor regulation (NRF2) and in DNA repair systems (ERCC1, OGG1) were also carried out. ZM and MZ were cytotoxic at the highest tested concentrations in both assays. A549 cells were more resistant to fungicide action than were HepG2 cells, and MZ was more cytotoxic than ZM. The comet assay data indicated no direct DNA damage due to ZM treatment for both cell lines; MZ caused genotoxic effects. Increased micronuclei formation was registered in both cell lines

treated with both fungicides. Our results showed an increase in the number of positive micronuclei (entire chromosome loss), supporting the hypothesis of aneugenic effects for ZM and an aneugenic potential also for MZ. The results of the project will help elucidate the mode of action of both fungicides in target human cells, and thus support more reliable hazard identification.

uHeart: A Beating Heart-on-a-Chip for Culturing 3-D Cardiac Microtissues and Recording Online Cardiac Electrophysiology

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Organs-on-chip reproduce tissue-specific microenvironments leading to the development of representative *in vitro* preclinical models, overcoming animal experimentation limitations.[1] We present uHeart, a heart-on-chip able to provide 3-D microtissues with mechanical stimulation and capable of monitoring cardiac electrophysiology.[2] We exploited uHeart for developing both animal and human functional *in vitro* cardiac models to perform drug cardiotoxicity screening. uHeart integrates two main technologies: a) uBeat® for uniaxial mechanical strain (i.e. 10–12%, 1Hz); and b) micro-Electrode Channel Guide (μECG), recording electrophysiological signals. Animal (primary rat cardiomyocytes) and human (iCell® cardiomyocytes with fibroblasts) cells were embedded in fibrin gel at 100–125 × 10^6 cells/ml and cultured for 5–10 days. Electrophysiological measurements were performed to assess functional parameters (e.g. beating period, spike amplitude, field potential duration-FPD). Compounds with different cardiotoxicity levels (e.g. verapamil, terfenadine, sotalol) were administered at incremental concentrations to screen drug-induced FPD changes. DMSO was used as a vehicle, while aspirin as the negative control. Rat microtissues spontaneously beat after 24 hours and showed synchronised electrical activity after four days of culture. Human cardiac microtissues spontaneously beat after seven days of culture, showing a RR of 1.7 ± 0.45 seconds and a FPD of 0.6 ± 0.2 seconds. DMSO and aspirin did not affect the repolarisation of the microtissues. Conversely, verapamil shortened, and terfenadine and sotalol prolonged, the FPD of human microtissues. Of note, verapamil caused the expected FPD prolongation in rat microtissues. These cardiac microtissues responded to drugs in a physiological manner, accounting for species-related specificity, presenting uHeart as a useful tool for direct on-chip cardiotoxicity drug studies.

References

1. Sosa-Hernández JE, Villalba-Rodríguez AM, Romero-Castillo KD, et al. Organs-on-a-chip module: a review from the development and applications perspective. *Micromachines* 2018; 9: 536.
2. Visone R, Ugolini GS, Cruz-Moreira D, et al. Micro-electrode channel guide (μECG) technology: an online method for continuous electrical recording in a human beating heart-on-chip. *Biofabrication* 2021, DOI: 10.1088/1758-5090/abe4c4.

ThermoTargetMiner: A Target Database for Lung Cancer Drugs Under Clinical Trial

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Lung cancer remains the leading cause of cancer death in both men and women. Therefore, there is an urgent need to identify the targets and mechanism of action of promising drugs against this disease. This information can help to characterise the area of compound efficacy and potential side effects in clinical trials. Here, we employed our high-throughput chemical proteomics tool, the Proteome Integral Solubility Alteration (PISA) assay, which is a high-throughput version of Thermal Proteome Profiling (TPP),[2] both with living cells and with cell lysate,[1] to identify the targets of 67 drugs against lung cancer that are under different phases in clinical trials. The binding of drugs to their targets and downstream protein–protein interactions leads to a shift in protein solubility/stability. By comparing the soluble protein abundance in drug-treated or vehicle-treated groups after heating, direct targets and downstream proteins can be screened. We used non-small (A549) and small (H69) lung cancer cells as models in parallel, circumventing the need for the use of laboratory animals. The results not only showed the known targets for such compounds, but also revealed a large number of novel targets, some of which can be associated with clinical adverse effects. We envisage that our study will help stratify clinical

trials against lung cancer, and that our database can serve as a resource for the scientific community, in extrapolating these results to other types of cancer.

References

1. Gaetani M, Sabatier P, Saei AA, et al. Proteome integral solubility alteration: a high-throughput proteomics assay for target deconvolution. *J Proteome Res* 2019; 18: 4027–4037.
2. Savitski MM, Reinhard FBM, Franken H, et al. Tracking cancer drugs in living cells by thermal profiling of the proteome. *Science* 2014; 346: 1255784.

Effect of Combustion-derived Particles on Genotoxicity and Telomere Length: A Study on Human Cells and Exposed Populations

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Exposure to particulate matter has been associated with oxidative stress to DNA and different types of disease.[1] In our study, we collected particles from a passenger cabin of a diesel-fuelled train and at a firefighting training exercise. Effects on oxidative stress biomarkers, genotoxicity measured by the comet assay and telomere length in particle-exposed A549 cells were compared with the genotoxicity and telomere length in peripheral blood mononuclear cells (PBMCs) from human volunteers exposed to the same particle source. The particles did not cause genotoxicity in the comet assay in A549 cells, while elevated levels of DNA strand break and oxidatively damaged DNA were observed in PBMCs from exposed humans. Moreover, A549 cells showed telomere length shortening after a four-week exposure to particles, which is in line with the slightly shorter telomere length observed in PBMCs from exposed humans (not statistically significant). Our results indicate that genotoxicity measured by telomere length assay may be a novel indicator of genotoxic stress in cell cultures and humans, and that genotoxicity measured by the comet assay in A549 cells may not predict the effects.

References

1. Stone V, Shaw J, Brown DM, et al. The role of oxidative stress in the prolonged inhibitory effect of ultrafine carbon black on epithelial cell function. *Environ Health Perspect* 1998; 12: 649–659.

Transplanting GRPs Into an Animal Model of Cytogenesis Ablation: Uncovering the Interplay Between Neuro and Gliogenesis

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The continuous generation of new neurons and glial cells — adult cytogenesis — is highly impaired in several neuropsychiatric disorders, disturbing cognitive and emotional domains. Abnormal function of glial cells has also been implicated in several mood disorders. Therefore, knowledge of glial cells might be crucial to understand overall brain circuitry. Glial-restricted precursor cells (GRPs) can generate cells derived from the glial lineage and might help elucidate whether new glial cells are able to modulate neuronal networks and behaviour. Moreover, growing evidence has revealed that neurotrophic factors and vesicles released by cells, i.e. the secretome, are important components of different cell-mediated effects. Transplanting GRPs and their secretome to the hippocampal dentate gyrus of rats, using a rat model with

dysfunctional cytogenesis — the transgenic animal model GFAP-tk — further showed that these cells induced reversion of anxiety-like traits affected by cytogenesis ablation. Transplanted GRPs were able to survive, proliferate and differentiate within the hippocampus, accompanied by enhanced cell proliferation. These results highlight GRPs as a promising therapeutic approach for specific behavioural domains known to be affected by mood disorders, such as depression. Although we have explored the role of GRPs in animal models, the incorporation of one additional translation step might allow the use of data and information generated in non-animal models, as inputs to risk assessment. We aim to use these cells *in vitro* and derive them from iPSCs to explore their mechanisms and translate the data to the clinic, overcoming the ethical problems related to the use of animal models.

Development of Self-disinfecting Paints and *In Vitro* Assessment of their Cytotoxicity on Human Cells

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Healthcare-associated infections remain a severe problem across the world. The high number of hospital-acquired infections is related not only to person-to-person transmission of pathogens but also to contact with contaminated surfaces. Traditional cleaning and disinfection processes are proving insufficient in this respect, so new methods are being developed to prevent the propagation of microorganisms. Self-disinfecting surfaces are a good example. This PhD project aims to develop, test and validate a self-disinfecting paint, to be applied in areas with a high propensity for infection spreading, such as healthcare facilities, schools or other public spaces, to help reduce infection risks. To do so, triclosan and isoborneol, substances with proven antimicrobial properties, were incorporated at different concentrations in a conventional paint to achieve an effective formula, conjugating good antimicrobial activity with safety of use and handling. The paint's antibacterial efficacy was evaluated according to international standards ISO 22196 and JIS Z 280, against bacteria well-known for being associated with hospital-acquired infections, such as *Staphylococcus aureus, Escherichia coli* and *Klebsiella* spp.[1,2] To guarantee the paint's safety for commercial use, its cytotoxicity is being evaluated for the most probable routes of exposure: skin contact with HaCaT cells (human keratinocytes); and inhalation with pulmonary cells A549 (human alveolar epithelial cells). Following ISO 10993, direct contact and extract-based tests were performed.[3] Potential genotoxicity will be evaluated with the comet assay and micronucleus assays in the same cell models.

References

1. International Organisation for Standardisation. *ISO 22196: Measurement of antibacterial activity on plastics and other non-porous surfaces*. Geneva: International Organisation for Standardisation, 2011, 15 pp.
2. Situ Biosciences. JIS Z 2801: Antibacterial products: test for antibacterial activity and efficacy, https://www.situbiosciences.com/product/jis-z-2801-test-for-antimicrobial-activity-of-plastics/ (2010, accessed 17 August 2021).
3. International Organisation for Standardisation. *ISO 10993-5: Biological evaluation of medical devices. Part 5: Tests for* in vitro *cytotoxicity*. Geneva: International Organisation for Standardisation, 2006, 34 pp.

Development of a New *In Vitro* Device for Risk Assessment of Inhaled Xenobiotics: Lung/Liver

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Atmospheric pollution has proven to have adverse effects on human health.[1] European regulatory acceptance of the Three Rs testing approaches challenge scientific investigations. To meet these guidelines, the development and use of novel *in*

vitro methods is accelerating.[2] Multi-organ systems prove their relevance by capturing fundamental *in vivo* physiological conditions, such as dynamic inter-tissue interactions.[3] Here, we aim to develop a lung–liver-on-a-chip platform by microfluidically connecting bronchial tissue to a metabolically functional liver biochip. Both compartments were characterised separately through referenced hepatotoxic exposures to acetaminophen[5] (APAP) by monitoring various cell parameters. We found that, in spite of imposed exposures, lung and liver compartments remained functional and showed steady differentiation and viability under stress: cohesive bronchial tissues showed elevated transepithelial electrical resistance (TEER) values and main tight junction architectures, while basal and xenobiotic-related hepatic metabolism levels remained strong. To better mimic *in vivo*-like interactions between both metabolisms, hepatic biochips and bronchial tissues were united into a closed dynamic-circuited platform.[6] Preliminary results showed a sensitivity of our device to APAP exposure, both lung and liver compartments displayed signs of adverse effects to the passage and transit of APAP through the system. Ongoing characterisation is confirming these early results, highlighting that our developed lung–liver-on-a-chip could open the way toward new physiologically relevant applications for toxicological research, such as pesticide investigation.

References

1. WHO. Ambient air: A global assessment of exposure and burden of disease. Geneva: World Health Organisation, 2016, 121 pp.
2. Bouvier d'Yvoire M, Bremer S, Casati S, et al. ECVAM and new technologies for toxicity testing. In: Balls M, Combes RD and Bhogal N (eds) *New technologies for toxicity testing. Advances in experimental medicine and biology.* Amsterdam: Springer, 2012, pp. 154–180.
3. Bhatia SN and Ingber DE. Microfluidic organs-on-chips. *Nat Biotechnol* 2014; 32: 760–772.
4. Prot JM, Bunescu A, Elena-Herrmann B, et al. Predictive toxicology using systemic biology and liver microfluidic 'on chip' approaches: application to acetaminophen injury. *Toxicol Appl Pharmacol* 2012; 259: 270–280.
5. Bricks T. Développement d'un dispositif microfluidique ayant pour objectif l'étude des effets de premiers passages intestinaux et hépatiques. Thesis de l'Université de Technologie de Compiègne. Compiègne: Université de Technologie de Compiègne, 2014.

Current Use of Preclinical *In Vitro* Models in Cancer Research: The First Global Survey

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Although substantial efforts have been made over recent years to develop complex *in vitro* tumours, there are still challenges in developing realistic *in vitro* models that reproduce the multifaceted architecture of tumour tissue and stromal support.[1,2] To improve research models, we must first determine what models are used throughout the world, and why. Thus, the aim of this study was to perform the first global survey of currently used preclinical *in vitro* models in cancer research. We developed our survey in Typeform® and, after obtaining ethics approval, an email invitation to complete an electronic survey form was circulated extensively to cancer research centres and was also shared via social media for the maximum breadth of coverage. A total of 101 responses were collected from individual researchers, of whom 96% were in academia. The most widely used *in vitro* model was the monolayer/2-D culture model (88.1%), with 30.7% of respondents using a 3-D culture model, 2% using a 2.5-D culture model and 3% using other models. The three main reasons reported for not using 3-D culture models were: lack of experience (47.1%); the associated additional costs (34.3%); and, finally, lack of access to such models (24.3%). Approximately 56% of researchers use *in vivo* models, but only 59.6% of those researchers using *in vivo* models indicated that their research was predominantly based on such studies. This survey offers a snapshot of the different *in vitro* models used currently in cancer research, and will help to identify the trends and practices in cancer research for future standardisation.

References

1. Katt ME, Placone AL, Wong AD, et al. *In vitro* tumor models: advantages, disadvantages, variables, and selecting the right platform. *Front Bioeng Biotechnol* 2016; 4: 12.
2. Edmondson R, Broglie JJ, Adcock AF, et al. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay Drug Dev Technol* 2014; 12: 207–218.

Early-life Arsenic and Cadmium Exposure and Reproductive and Growth-related Hormones in Adolescents

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Epidemiological studies have indicated that early-life exposure to toxic metals via food and water may adversely affect levels of different hormones in children, but data are still limited and inconclusive. The aim of the present study was to elucidate any association between prenatal/childhood exposure to arsenic and cadmium, and reproductive and growth-related hormones during mid-adolescence. The aim was also to determine whether this association differed by gender. We studied 1222 adolescents (n = 569 boys and n = 653 girls) in the longitudinal MINIMat cohort in rural Bangladesh. Concentrations of arsenic and cadmium were measured in urine of the mothers during early pregnancy and in the children at age 10 years by using Inductively Coupled Plasma-Mass Spectrometry. In plasma at age 15 years, sex hormone-binding globulin (SHBG) was measured by using the Meso Scale Discovery Platform, and free thyroxine (T4) and triiodothyronine (T3), and thyroid-stimulating hormone (TSH) were measured by using Roche immunoassays. The children's median urinary concentrations of arsenic and cadmium were 62 μg/l (range 34–140) and 0.24 μg/l (range 0.16–0.38), respectively, at age 10 years. The mothers had higher corresponding median concentrations (87 μg/l and 0.61 μg/l, respectively) during pregnancy. Preliminary results indicate that the children's urinary cadmium concentration was inversely associated with TSH levels in girls, but not in boys, at age 15 years. Maternal urinary arsenic in pregnancy, but not children's urinary arsenic, was positively associated with free T3 levels in boys only. In conclusion, exposure to toxic metals early in life appears to impact the endocrine system in adolescence. Further studies are needed to confirm the present findings.

Non-animal Approaches in Pesticide Safety Testing

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Glyphosate, known as Roundup™, is considered to be the most used herbicide worldwide as it has been administered heavily since the introduction of genetically modified organisms (GMOs), specifically pesticide-tolerant crops.[1] As public concerns were raised about its safety, a number of studies were conducted over the years in order to assess glyphosate's potential risks. Surprisingly, they resulted in conflicting outcomes. Indeed, the International Agency for Research on Cancer (IARC), classified glyphosate as Group 2A; a probable human carcinogen.[2] However, the Joint Meeting of the Food and Agriculture Organisation of the United Nations/World Health Organisation (FAO/WHO) on Pesticide Residues (JMPR), responsible for assessing the risk of pesticides in food in the Codex, and the US Environmental Protection Agency (US EPA), did not find evidence for carcinogenicity in humans.[3,4] Our research project consisted of identifying genetically modified sequences associated to glyphosate tolerance in several types of food samples collected from the Lebanese market. Positive samples were analysed to quantify their glyphosate content. Based on our preliminary results, which showed high glyphosate levels in food samples, and due to inconsistent results regarding glyphosate's toxicity, carcinogenicity and other safety concerns, we plan to proceed with the next stage of our study and determine the pesticide's safety. We are interested in applying non-animal approaches, such as *in silico* or *in vitro* methods, to determine the safety of glyphosate and other pesticides, while limiting potential harm to animals by the unnecessary use of *in vivo* models.

References

1. Benbrook CM. Trends in glyphosate herbicide use in the United States and globally. *Environ Sci Eur* 2016; 28: 1–15.
2. IARC. *IARC monograph on glyphosate*, https://www.iarc.who.int/featured-news/media-centre-iarc-news-glyphosate/ (accessed 23 March 2021).
3. Food and Agriculture Organisation of the UN and World Health Organisation. *Joint FAO/WHO meeting on pesticide residues,* Geneva, 17–26 September 2019, Summary Report, https://www.who.int/foodsafety/areas_work/chemical-risks/Sep_2019_JMPR_Summary_Report.pdf (accessed 18 August 2021).
4. US EPA. *Glyphosate*, https://www.epa.gov/ingredients-used-pesticide-products/glyphosate (accessed 18 August 2021).

A Multicellular Model Depicting Implantation Processes in Mice: An *In Vitro* Approach

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In vitro models are gaining relevance as approaches to reduce and replace animal experiments in the life sciences. Especially in the field of embryonic development, *in vitro* models might be a useful tool to investigate unanswered questions and unravel underlying mechanisms. Thus, we aim to generate a more complex *in vitro* murine implantation model to mimic the process of implantation in the mouse. First, two separate models (the endometrium and embryo-like structures (ELS)) need to be developed and subsequently unified, implementing a detailed characterisation by using qPCR, immunohistochemistry and ELISA. To enable development of the ELS, we combined embryonic stem cells (ESC), GFP-tagged trophoblast stem cells (TSC-G) and extra-embryonic endoderm stem cells (cXEN) and let them self-assemble into multicellular aggregates. The second model, mimicking the endometrium, is based on primary epithelial and stromal endometrial cells isolated from the murine uterus. Initially, we attempted two different strategies to generate the endometrial model: coculture in hydrogel matrices and a scaffold-free coculture in micromoulds (endometrial organoids). Moreover, we generated ELS with the desired morphology, i.e. compartmentalisation of the ESC (visualised by Oct3/4) and trophoblast stem cells surrounded by a cXEN layer (visualised by GATA4). Nevertheless, the investigation of various culture parameters (e.g. culture medium and the extracellular matrix) is mandatory before merging the two models, in order to ensure cell viability and to support attachment and invasion of the ELS into the endometrium. In summary, this complex *in vitro* system, mimicking implantation in the mouse, could potentially replace animal experiments in developmental research.

Development and Validation of Novel *In Vitro* Models for Adverse Effects on the Human Neurovascular Unit, Including Compromised Barrier Properties, Inflammatory Processes and Changed Cellular Transcriptional Profiles

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The development of drugs targeting the central nervous system is challenging due to low success rate and poor animal to human translation of the results obtained in current *in vivo* animal experiments.[1] One reason for such results is the paucity of reliable *in vitro* models of the neurovascular unit, in which vascular and neural cells closely interact.[2] The purpose of this work is to develop and validate a novel *in vitro* model of the neurovascular unit (NVU) based on human induced pluripotent stem cells (hiPSCs).[3] This approach will increase the relevance of the model to humans, thereby offering possibilities for personalised treatment of patients with a specific NVU-related disorder. Moreover, toxicity endpoints for novel and/or already approved pharmaceuticals can be identified. Therefore, the use of such models may increase the predictive power in terms of CNS toxicity effects. Thus, hiPSC-based *in vitro* models, combined with microfluidic technology, may pave the way toward patient-oriented therapeutics and offer a possible alternative to animal testing.

References

1. Kesselheim AS, Hwang TJ and Franklin JM. Two decades of new drug development for central nervous system disorders. *Nat Rev Drug Discov* 2015; 14: 815–816.
2. Pardridge WM. The blood–brain barrier: bottleneck in brain drug development. *NeuroRx* 2005; 2: 3–14.
3. Nikolakopoulou P, Rauti R, Voulgaris D, et al. Recent progress in translational engineered *in vitro* models of the central nervous system. *Brain* 2020; 143: 3181–3213.

Improving Dermal Delivery of Active Substances Using Nanoemulsion Combined with Iontophoresis: A Case Study with Curcumin

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It is well-known that most active pharmaceutical ingredients possess low solubility, poor permeability through biological membranes, and low (bio)availability as common properties.[1] Consequently, targeting the specific site of action remains a hurdle, despite proven *in vitro* efficacy. Therefore, along with the development of innovative drug carriers, increased interest has been noted surrounding alternative means of drug administration. In this context, the skin as an application site has been considered. However, skin penetration is a challenge, due to the dense and hydrophobic nature of its superficial layers, acting as an almost impermeable barrier.[2] Therefore, the aim of this work was to apply a carefully developed low-energy nanoemulsion (MCT as the oil phase; polysorbate 80 and lecithin as stabilisers) in combination with a iontophoretic patch, as an innovative topical delivery system for curcumin (a model API). Physicochemical properties of the curcumin-loaded nanoemulsion (3 mg/ml) were: *Z-ave* = 146.3 ± 2.3 nm; PDI = 0.173 ± 0.017; and pH = 5.7 ± 0.05. The *in vitro* MTT cytotoxicity assay indicated significant activity toward cancer cells (HeLa: IC_{50} = 22.89 ± 2.09 μg/ml; MRC-5: IC_{50} = 37.87 ± 7.09 μg/ml), and a good safety profile toward normal cells (HaCaT; Fem-X). Antigenotoxicity was proven with the comet assay. In an *in vivo* study with human volunteers, an iontophoretic patch developed previously in-house (voltage: 9 V; 15 minutes of application, controlled by Arduino software) was used to deliver curcumin into the skin more efficiently. After comparison with the 'bare' nanoemulsion, concentrations two times higher were determined in the stratum corneum after iontophoresis (6.89 ± 0.2 μg/cm^2 *versus* 3.49 ± 0.40 μg/cm^2), demonstrating that the combination of a nanocarrier with an iontophoretic patch may be a successful strategy.

References

1. Boyd BJ. Past and future evolution in colloidal drug delivery systems. *Expert Opin Drug Deliv* 2008; 5: 69–85.
2. Atkinson JP, Maibach HI and Dragicevic N. Targets in dermal and transdermal delivery and classification of penetration enhancement methods. In *Percutaneous penetration enhancers, Chemical methods in penetration enhancement*. Berlin, Heidelberg: Springer, 2015, pp. 93–108.

Comprehensive Chemical Proteomics for Identification of Protein Targets and Action Mechanisms of Approved and Prospective Covid-19 Drugs and Host-directed Therapies

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Mutations in the SARS-CoV-2 virus threaten the efficacy of vaccines to protect against new variants, pressurising efforts for effective therapeutics. Besides drugs against SARS-CoV-2, another promising solution is to use host-directed therapies.[1] Therefore, characterisation of the host interaction with prospective or approved drugs against SARS-CoV-2 is of fundamental importance, since these drugs might be used on millions of patients. Here, we employed parallel chemical proteomic tools to comprehensively characterise host interactions with promising drugs against SARS-CoV-2. To achieve this, we used the Proteome Integral Solubility Alteration (PISA) assay,[2] which is a high-throughput version of Thermal Proteome Profiling (TPP),[3] as well as Functional Identification of Targets by Expression Proteomics (FITExP)[4] and ProTargetMiner.[5] Twelve approved or prospective drugs against SARS-CoV-2 were profiled by using the above methods, in both lung (A549) and immune cells (THP-1), thus circumventing the need for animal models. We validated select targets of both repurposed and investigational drugs, showing that chemical proteomic tools are a powerful tool in drug target deconvolution. Optimal results were obtained when these tools were used in parallel with each other, which is a finding similar to that obtained in our previous efforts.[6] The results of this study provided some potential suggestions for use in the fight against the current SARS-CoV-2 pandemic, as well as potential future outbreaks.

References

1. Reuschl AK, Thorne L, Alvarez LZ, et al. Host-directed therapies against early-lineage SARS-CoV-2 retain efficacy against B.1.1.7 variant. *bioRxiv* [Preprint] 2021; DOI: 10.1101/2021.01.24.427991.
2. Gaetani M, Sabatier P, Saei AA, et al. Proteome integral solubility alteration: a high-throughput proteomics assay for target deconvolution. *J Proteome Res* 2019; 18: 4027–4037.
3. Savitski MM, Reinhard FB, Franken H, et al. Tracking cancer drugs in living cells by thermal profiling of the proteome. *Science* 2014; 346: 1255784.
4. Chernobrovkin A, Marin-Vicente C, Visa N, et al. Functional Identification of Target by Expression Proteomics (FITExP) reveals protein targets and highlights mechanisms of action of small molecule drugs. *Sci Rep* 2015; 5: 11176.
5. Saei AA, Beusch CM, Chernobrovkin A, et al. ProTargetMiner as a proteome signature library of anticancer molecules for functional discovery. *Nat Commun* 2019; 10: 5715.
6. Saei AA, Gullberg h, Sabatier P, et al. Comprehensive chemical proteomics for target deconvolution of the redox active drug auranofin. *Redox Biol* 2020; 32: 101491.

Determination of Mixture Neurotoxicity Using the Hyper- and Hypoactivity Behaviour of Zebrafish Embryos in the Spontaneous Tail Coiling Test

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Risk assessment of chemicals is usually conducted for individual chemicals, whereas mixtures of chemical occur in the environment. Considering that neuroactive chemicals are a group of contaminants that dominate in the environment, it is thus imperative to understand the combined effects of such mixtures.[1] The commonly used models to predict mixture effects, namely concentration addition (CA) and independent action (IA), are thought suitable for mixtures of similarly acting components or dissimilarly acting components, respectively. One important challenge is, therefore, to clarify whether the grouping of neuroactive substances should be based on similar mechanisms of action (e.g. the same molecular target) or rather on similar toxicological response (e.g. hyper- or hypoactivity behaviour; effect direction). We addressed this by using the spontaneous tail coiling of zebrafish embryos, which represents the earliest observable motor activity in the developing neural network, as a model to elucidate the link between mechanism of action and toxicological response. Two questions were asked: a) Can the additivity models CA or IA be used to predict combined effects for neuroactive chemical mixtures when the components share a similar mode of action (hyper- or hypoactivity) but show a different mechanism of action?; and b) Will a mixture of chemicals where the components show opposing effect direction result in an antagonistic, combined effect? Using the CA and IA models, the results indicated that the toxicity of chemical mixtures such as propafenone and abamectin, and chlorpyrifos and hexaconazole, that are known to show different mechanisms of action but similar effect directions, were additive and predictable. This could be interpreted with the convergence of effects on the neural level leading to either a collective activation or inhibition of synapses.[2] We also found antagonistic effects for mixtures containing substances with opposing effect direction. Finally, we consider how the spontaneous tail coiling test might be used in environmental risk assessment.

References

1. Faust M, Backhaus T, Altenburger R, et al. Prioritisation of water pollutants: the EU Project SOLUTIONS proposes a methodological framework for the integration of mixture risk assessments into prioritisation procedures under the European Water Framework Directive. *Environ Sci Eur* 2019; 31: 66.
2. Kortenkamp A, Backhaus T and Faust M. *State of the art report on mixture toxicity.* London: University of London, 2009, 391 pp., https://ec.europa.eu/environment/chemicals/effects/pdf/report_mixture_toxicity.pdf (accessed 18 August 2021).

Comparison of Tools for the Analysis of Viral Quasispecies

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Animals have been used for many years to answer important scientific research questions. However, animal models are costly and, depending on the research question, present scientific limitations; computational models can help to fill in these gaps. To this end, the objective of the present work was the use and comparison of software tools for analysis of the variants that are formed within a viral population that has infected an individual. These take the name of quasispecies and confer great genomic variation on the infecting virus, making it remarkably resistant to medical treatments. This makes the study of quasispecies essential, and as a result of such study it should be possible to develop suitable therapies. In the first phase, the main programs developed for the analysis and reconstruction of viral quasispecies — VICUNA, Virus-VG, aBayesQR, TenSQR and QSdpR — were analysed. All these programs were tested by using a set of real Covid-19 samples and the quality of the results was calculated by using the MEC Score, which is a mathematical tool that performs an analysis between the input readings and the output genomes, calculating for each the smallest value of the Hamming Distance and adding them all together to obtain a precise estimate of the sequencing errors present in the program results. This comparison showed that the most accurate program among those studied is TenSQR, but it has an error rate too high to be considered a reliable tool for the reconstruction of quasispecies. This means that studies still need to be carried out to develop an efficient and reliable reassembly algorithm.

Organs-on-chips and Proteomics as Tools to Identify Key Molecular Players Responsible for Human OA Initiation

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Osteoarthritis (OA) is the most prevalent degenerative joint disorder, but despite its prevalence, no reversing therapies are currently available.[1,2] This is partly due to a gap in knowledge on OA mechanisms, linked to the disease's multifactorial aetiology and to the lack of reliable preclinical models.[3] In this context, organs-on-chips are promising solutions, as they are able to recapitulate a complex environment, while reducing the use of animals in accordance with the Three Rs principles.[4] Here, a human cartilage-on-chip model was exploited to unravel key molecular players responsible for triggering OA.[5] A microscaled platform was used to generate a cartilage model by culturing human articular chondrocytes within a fibrin gel, and to induce a shift toward an OA phenotype by applying hyperphysiological compression. Cell culture supernatant was then analysed, comparing static and mechanically stimulated samples, via liquid chromatography-mass spectrometry. This permits the identification of proteins released during culture under hyperphysiological compression, and the investigation of key molecular players triggered by mechanical damage that in turn trigger other events in the OA cascade (e.g. activation of synovial macrophages). Once identified, the role of these key molecular players will be validated within a more complex model comprising both cartilage and synovium, to investigate whether these molecules, when produced by chondrocytes upon mechanical damage, induce synovitis. This would help to elucidate cause–effect relationships among these molecular players in the early stages of OA, as mounting evidence suggests that synovitis plays a pathological role in OA. However, whether synovitis is causative of OA, or a consequence of joint failure, is still not clear.

References

1. Martel-Pelletier J, Barr AJ, Cicuttini FM, et al. Osteoarthritis. *Nat Rev Dis Primers* 2016; 2: 16072.
2. Bijlsma JWJ, Berenbaum F and Lafeber FPJG. Osteoarthritis: An update with relevance for clinical practice. *Lancet* 2011; 377: 2115–2126.
3. Johnson CI, Argyle DJ and Clements DN. *In vitro* models for the study of osteoarthritis. *Vet J* 2016; 209: 40–49.
4. Ferrari E, Palma C, Vesentini S, et al. Integrating biosensors in organs-on-chip devices: a perspective on current strategies to monitor microphysiological systems. *Biosensors* 2020; 10: 110.
5. Occhetta P, Mainardi A, Votta E, et al. Hyperphysiological compression of articular cartilage induces an osteoarthritic phenotype in a cartilage-on-a-chip model. *Nat Biomed Eng* 2019; 3: 545–557.

Functional Foods, Bioactive Compounds from Natural Sources and Food By-products — Their Effects on Human Health

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A recent research interest of our group focused on investigating the bioactivity and bioavailability of innovative, functional foods. These are foods — either natural or processed — which consist of specific bioactive compounds, and thus could contribute to the achievement of specific functional goals in the human body and consequently to the promotion of general health. Firstly, in the context of an epidemiological study based on a population from various regions of Greece, consumers' perceptions and acceptance of innovative functional foods and their frequency of consumption, were investigated and related to anthropometric indicators.[1] Furthermore, the nutritional parameters of natural functional foods, food industry by-products and created innovative functional foods were also studied.[2] Finally, the effects of the consumption of these novel functional foods on the postprandial metabolic biomarkers of chronic diseases were evaluated in healthy volunteers via a crossover interventional study.[3] The epidemiological study showed correlation between increased specific functional food consumption and improved anthropometric indices, such as BMI. The produced innovative functional foods, such as cheese fortified with mountain tea and orange peel extract, appeared to increase total antioxidant activity, and the total phenolic and carotenoids content. The crossover interventional study showed that the consumption of the innovative cheese increased the plasma total antioxidant capacity after three hours, and may affect triglyceride and glucose postprandial increase. The development of new functional foods from natural sources rich in bioactive compounds could be a basic goal in order to improve the nutritional intake of the population.

References

1. Ntrigiou V, Ntrigios I, Rigopoulos N, et al. Functional foods consumption correlates with anthropometric characteristics and body composition in healthy adults. *Curr Top Nutraceutical Res* 2018; 16: 279–288.
2. Dimou C, Karantonis C, Skalkos D, et al. Valorization of fruits by-products to unconventional sources of additives, oil, biomolecules and innovative functional foods. *Curr Pharm Biotechnol* 2019; 20: 776–786.
3. Papagianni O, Loukas T, Magkoutis A, et al. Postprandial bioactivity of spread cheese, enhanced with mountain tea and orange peel extract, in healthy volunteers. A pilot study. *Proceedings* 2021; 70: 19.

Application of Chemical Methods to Estimate the Dietary Exposure of Bisphenol A from Packed Food

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Bisphenol A (BPA) is defined as an endocrine disruptor and a reproductive toxicant in experimental animals. According to the EFSA,[1] the largest contributor to BPA exposure is the oral route of entry into the human body, mainly through canned food. Metal packaging for canned foods is produced with an inner protective layer of epoxy resin that comes into contact with the food. The main monomer of epoxy resins production is BPA. The aim of the present work was to determine the levels of BPA, which can migrate in the food from different metal packaging with inner epoxy coating, by using a modified standardised HPLC-FL analytical method. Various food simulants imitating a range of food types were used and the effect of temperature on migration levels was studied. The range of established BPA migration levels in canned foods varies between values of below LOD 0.005 mg/kg for deionised water and 0.015 mg/kg for 50% ethanol. The correlation was experimentally established between the migration of BPA and the duration of storage in metal packaging for soft drinks. In other types of metal packaging, the results obtained exceed the limit of specific migration of 0.05 mg/kg (*EU Regulation No. 10/2011*)[2] at different temperatures. The specific migration limit of BPA was also exceeded for aluminium chocolate tubes. Some metal packaging has been found to exhibit significantly higher values of BPA migration and non-compliance with current legislation due to epoxy coatings. The study shows that canned foods may pose a risk of BPA exposure for vulnerable populations. The obtained data on BPA levels in canned foods are suitable for assessing the dietary exposure to BPA, after combining them with the relevant information on the consumption of these foods.

References

1. EFSA. Scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. *EFSA J* 2015; 13(1): 3978.
2. European Commission. *Commission Regulation (EU) No 10/2011* of 14 January 2011 on plastic materials and articles intended to come into contact with food. *Off J Euro Union* 2011; L12, 15.01.2011: 1–89.

Finding Critical Transitions in the Development of Non-alcoholic Fatty Liver Disease

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Non-alcoholic fatty liver disease (NAFLD) is a liver condition that is especially prevalent in the Western world, where its prevalence is 20–30%. It ranges from simple steatosis to more serious liver conditions, such as liver cirrhosis, liver fibrosis and liver failure. An important characteristic of NAFLD is altered fatty acid metabolism, which can be observed as liver cells containing lipid droplets. A lack of knowledge regarding the mechanism of disease development at the cellular level limits *in vivo* investigations in humans, mice and rats. We track the development of NAFLD *in vitro* by using human liver cells. NAFLD is induced chemically and the cells are tracked on topography plates, advancing the lifespan of the cells by several weeks. A lifespan of over three weeks allows us to investigate beyond acute toxicity effects. The same single cells are tracked to obtain single-cell development data, in order to deal with the high level of heterogeneity among these cell populations. We identify critical transitions in the development to NAFLD by using the morphological changes observed with live-cell fluorescence microscopy, visualising the nucleus, the mitochondria and the lipid droplets. The cells in the images are interpreted by calculating over a thousand features per cell that represent the cell's development. The molecular investigation of these critical transitions is performed by using RNA-Seq, meaning that the morphological tracking serves as a guideline for the time points of molecular analysis. The results of the sequencing are used to describe the molecular changes in each stage, leading to a developmental description of NAFLD that can suggest therapy options.

Medical Devices Testing *In Vitro*: Development and Optimisation of the *In Vitro* Protocol for Screening Ocular Irritation and Photo Irritation using 3-D Reconstructed Human Cornea-like Tissue Models

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In recent years, the ISO standard 10993 for the Biocompatibility Testing of Medical Devices implemented a series of *in vitro* methods that help safety assessors in the screening of potential health hazards of medical devices before conducting animal studies. Success has been achieved with the development and validation of *in vitro* protocols for sub-cutaneous irritation testing that led to the release of the new ISO standard 10993-23, published in January 2021.[1–4] 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 with an *in vitro* 3-D reconstructed cornea-like tissue model (3-D RHC). In the initial experiments, we have evaluated the tolerance of the 3-D RHC models toward an increasing dose of UVA and visible light, and selected a dose that is sufficiently high to cause excitation of the tested compounds, while at the same time remaining non-toxic to the 3-D model. In the subsequent experiments, we tested the phototoxic benchmark, 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. This research

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References

1. International Organisation for Standardisation. *ISO 10993-23:2021. Biological evaluation of medical devices — Part 23: Tests for irritation*. Geneva: International Organisation for Standardisation, 2021, 60 pp.
2. De Jong WH, Hoffmann S, Lee M, et al. Round robin study to evaluate the reconstructed human epidermis (RhE) model as an *in vitro* skin irritation test for detection of irritant activity in medical device extracts. *Toxicol In Vitro* 2018; 50: 439–449.
3. Kandarova H, Willoughby JA, De Jong WH, et al. Pre-validation of an *in vitro* skin irritation test for medical devices using the reconstructed human tissue model EpiDerm™. *Toxicol In Vitro* 2018; 50: 407–417.
4. Pellevoisin C, Videau C, Briotet D, et al. SkinEthic™ RHE for *in vitro* evaluation of skin irritation of medical device extracts. *Toxicol In Vitro* 2018; 50: 418–425.

Cholangiocarcinoma-on-a chip: A Platform for 3-D Liver Tumour Model

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Intrahepatic cholangiocarcinoma (iCCA) is a deadly cancer of the biliary epithelium with very limited therapeutic options. This highlights the importance of deciphering iCCA mechanisms for effective therapeutic strategies. Nevertheless, the complexity of *in vivo* cellular interactions has hindered an effective recapitulation of the human milieu through *in vitro* 2-D standard culture systems. We aim to develop an *in vitro* 3-D microfluidic device with patient-specific cocultures of the main cells involved in iCCA. Primary iCCA cells were isolated from patients subjected to surgical resection at the division of Hepatobiliary Surgery, ICH. A microfluidic device was fabricated in PDMS at the Polytechnic of Milan. The chip was composed of a central microchannel fluidically connected to the two lateral channels, but with independent inlet and outlet ports. To recapitulate iCCA microenvironment, the central channel was seeded with primary iCCA cells in a 3-D hydrogel (fibrin/collagen gel). After 72 hours, LIVE/DEAD assay confirmed high cell viability. Following hydrogel protocol standardisation, the lateral channels were filled with cholangiocytes (H69) and endothelial cells (HUVEC) forming tubular vessels. Fluorescence microscopy with specific antibodies corroborated the tube formation. For all cell types, proliferation assay, morphology assessment and RT-PCR were performed to assess the maintenance of their phenotype inside the chip, cultured with an *ad hoc* combination of media. Our results showed that we were able to recreate a more reliable liver iCCA microenvironment in a 3-D microfluidic device. The reconstruction of this device will permit the elucidation of the biological mechanisms involved in iCCA progression and may provide an efficient clinical tool for personalised drug testing.

Investigating the Effects of Chronic Dosing on the Detection of Non-genotoxic Carcinogens and the Mechanisms Utilised

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Non-genotoxic carcinogens (NGCs) make up approximately 15% of all carcinogens and are largely undetected in carcinogenicity testing due to their complex nature. NGCs use alternative mechanisms such as upregulating cell proliferation, inhibiting apoptosis and inducing oxidative stress in order to promote oncogenesis. Chronic dosing, ROS induction and cell cycle analysis are examples of endpoints analysed to investigate the mechanism of action (MOA). Both acute and chronic dosing relative population doubling (RPD) studies were carried out for several NGCs. These treatments were followed by a mononucleate micronucleus assay to determine whether evidence of genotoxicity was present. In addition, multiple mechanistic studies are carried out for nickel chloride, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and two forms of arsenic, in order to understand the MOA utilised by each chemical. However, further mechanistic understanding is required to establish the routes used by a number of NGCs as they are complex chemicals.

Currently, the main assay used to test for NGCs is the two-year rodent bioassay, which uses excessive numbers (> 600) of organisms per chemical tested and is often unsuccessful. A Three Rs approach to detecting NGCs *in vitro* is required, which would reduce the numbers of rodents used if a reliable alternative is found.

The Caco-2 Based Coculture Model as a Tool to Study Nanoparticles Absorption, According to Three Rs Principles

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The Three Rs principles refer to three essential concepts regarding animal use in scientific experimentation: *Replacement*, *Reduction* and *Refinement*. The aim is, whenever possible, to completely replace the use of animals, to reduce the number of animals used, and to improve their welfare and limit their suffering when they are used. This involves, among other strategies, the development of alternative methods to animal testing and encouraging the use of *in vitro* approaches. Although cell cultures are a valid alternative in toxicological studies, cell monocultures present limitations, mainly related to the greater complexity of human physiological structures. Therefore, cell cocultures might represent a useful tool to reproduce human models that are closer, from a morphological/functional point of view, to the *in vivo* situation. The intestinal barrier model, based on Caco-2 cells (derived from a human colon carcinoma), is a well-established *in vitro* model to mimic active and passive absorption of drugs and chemicals. In long-term culture, Caco-2 cells spontaneously differentiate into enterocyte-like monolayer, but a monoculture model does not represent the complexity of the intestinal barrier, since it lacks mucus and absorptive M cells. The use of cocultures has greatly expanded the potential applications of this model, in particular to assess the absorption/uptake of nanomaterials.[1,2] The Italian National Institute of Health, supported by the Ministry of Health, is involved in the implementation of a triple cell coculture model, consisting of Caco-2, HT29-MTX (mucus-secreting) cells, and Raji B lymphocytes, to mimic a more realistic intestinal barrier. This activity is also carried out in the framework of the NanoHarmony EU project.

References

1. Schimpel C, Teubl B, Absenger M, et al. Development of an advanced intestinal *in vitro* triple culture permeability model to study transport of nanoparticles. *Mol Pharm* 2014; 11: 808–818.
2. García-Rodríguez A, Vila L, Cortés C, et al. Exploring the usefulness of the complex *in vitro* intestinal epithelial model Caco-2/HT29/Raji-B in nanotoxicology. *Food Chem Toxicol* 2018; 113: 162–170.

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Comparison of the Franz Diffusion Cell and a Novel Fluido-dynamic System (MIVO®) for the *In Vitro* Evaluation of the Penetration of Caffeine Through Different Skin Models

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Transdermal drug delivery is increasingly gaining interest in the pharmaceutical industry. *In vitro* diffusive models provide important tools for screening multiple drug formulations, evaluating skin permeation-enhancing properties and mechanism of action of the carrier systems, and estimating the rank of skin transport for several drug molecules.[1] The degree of skin penetration of topical drugs is a crucial point in studying their effects and tolerability. Therefore, there is a growing need for reliable *in vitro* skin absorption methods since the EU have stipulated that animal experiments should be avoided, in line with the Three Rs approach.[2] Several *in vitro* assays have thus been developed with the aim of testing drug delivery across the skin barrier. The aim of our study was to compare the Franz diffusion cell with a novel fluido-dynamic platform, named MIVO®, by evaluating caffeine penetration from different vehicle solutions into two skin models (Strat-M® synthetic membrane, kindly provided by Merck, and porcine skin biopsies). The results showed a similar trend of caffeine penetration kinetics for both diffusive systems. As expected, this similarity and reproducibility of data is higher with the Strat-M than

the biopsies, due to the biological heterogeneity among different donors. Moreover, the amount of penetrated caffeine is higher in Strat-M in all experimental conditions, meaning that the artificial membrane-based skin models are more permeable than porcine biopsies. Finally, the percentage of caffeine permeated through porcine skin in MIVO was in line with values observed in literature, confirming MIVO as a reliable alternative candidate to Franz cells.

References

1. van de Sandt JJ, van Burgsteden JA, Cage S, et al. *In vitro* predictions of skin absorption of caffeine, testosterone, and benzoic acid: a multi-centre comparison study. *Regul Toxicol Pharmacol* 2004; 39: 271–281.
2. Vinken M. 3Rs toxicity testing and disease modeling projects in the European Horizon 2020 research and innovation program. *EXCLI J* 2020; 19: 775.

An *In Silico* Tool to Assess the Systemic Toxicity Safety Assessment of a Cosmetic Fragrance Without Animal Data

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Systemic toxicity is an important endpoint to consider, in order to assess the safety of cosmetic ingredients. No alternatives exist to replace the 90-day repeated exposure on animals. Animal experiments are now forbidden in Europe and a new generation of safety assessment is taking place. We developed an innovative *in silico* tool to assess the safety of fragrances in a completely animal-free way. This method is based on three different steps. The first step is to calculate the consumer exposure, or the Systemic Exposure Dose (SED) of the fragrance ingredient. It is the amount expected to enter the bloodstream and therefore be systemically available.[1] The second step is to perform the safety evaluation without animal data. We compare the SED with food consumption, as many fragrances are also flavouring agents. We can also use a model called the Creme Aggregate Exposure Model to determine the fragrance concentration in hydroalcoholics from survey data.[2] Those data reflect the average presence of a compound in cosmetics in Europe. Historical data from RIFM former publications, who advised safe levels in the 70s can also be used. The last step is to check if the consumer exposure would be under the calculated exposure based on food consumption and RIFM data. If this is the case, we consider that the fragrance is safe within the cosmetic product for systemic toxicity. This innovative method will help the new generation of toxicologists to assess the safety of cosmetic ingredients in a relevant and complete cruelty-free way.

References

1. Scientific Committee on Consumer Safety. *Notes of guidance for the testing of cosmetic ingredients and their safety evaluation, 10th revision*. Brussels: European Commission, 2018, 147 pp.
2. Api AM, Belsito D, Bruze M, et al. Criteria for the Research Institute for Fragrance Materials, Inc. (RIFM) safety evaluation process for fragrance ingredients. *Food Chem Toxicol* 2015; 82: S1–S19.

Automated High-content Imaging for Carcinogenicity Testing *In Vitro*

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Carcinogenicity testing is a regulatory requirement for pharmaceutical development and other regulated sectors. However, the current *in vitro* and *in vivo* testing paradigm fails to accurately predict carcinogenic potential in humans, with up to 95% of drugs failing at the clinical trial stage. Thus, there is an expressed need to change the current testing paradigm. This project aims to integrate traditional genotoxicity data with more advanced holistic data on cell phenotype by using image analysis to provide an advanced multi-disciplinary *in vitro* testing platform. This novel testing platform is currently being validated at Swansea University (UK) and compared to a similar robotic Multi-Endpoint Genotoxicity Assessment (MEGA) system under development at AstraZeneca laboratories (Cambridge, UK). The MEGA-Screen based on confocal microscopy and image analysis allows for a multiplexed assessment of both DNA damage and phenotypic markers in a single assay. At Swansea, another multi-endpoint system for carcinogenic mode action looks at morphological analysis, cell cycle and cell signalling perturbations, Micronuclei induction, ROS generation and mitochondrial toxicity separately

measured with parallel platforms. By evaluating the two systems in parallel, we can refine the optimal testing platform for safety assessment in drug development and give a more reliable prediction of carcinogenicity. Cisplatin and temozolomide were two test compounds initially assessed for genotoxicity by using the micronucleus assay, and for cell cycle perturbations by using flow cytometry, in both TK6 and A549 cell lines; the results were compared across both test systems as an initial phase of validation.

Effect of Flame Retardants on Human Health and Immune System: The Importance of Microbiota

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Flame retardants are compounds largely used to prevent flames from spreading. Prolonged exposure to these compounds may cause intestinal alterations, microbiota imbalance and can lead to immune system malfunction.[1–3] Although their toxicity has been studied, the exact molecular mechanisms of action are not yet well understood. The aim of our work is to obtain a deeper understanding of the mechanism of flame retardant toxicity and its effects on human health, with specific focus on microbiota-mediated immune system alterations. We are currently conducting a statistical analysis of microbiota composition on a large range of the population, linking it with their dietary habits and toxic exposure. We will use computational modelling, such as PBPK and GSMN, to investigate how flame retardants might affect the microbiota's metabolic pathways and composition, and then check the impact on immune system regulation and function. Finally, we will perform *in vitro* and/or *in vivo* experiments to validate our results. We expect to generate a model that will give us a better understanding of the mechanism of toxicity induced by flame retardants, and its effects on human health and the immune system.

References

1. Wang X, Wei L, Zhu J, et al. Tetrabromoethylcyclohexane (TBECH) exhibits immunotoxicity in murine macrophages. *Environ Toxicol* 2020; 35: 159–166.
2. Cui H, Chang Y, Jiang X, et al. Triphenyl phosphate exposure induces kidney structural damage and gut microbiota disorders in mice under different diets. *Environ Int* 2020; 144: 106054.
3. Cruz R, Mendes E, Maulvault AL, et al. Bioaccessibility of polybrominated diphenyl ethers and their methoxylated metabolites in cooked seafood after using a multi-compartment *in vitro* digestion model. *Chemosphere* 2020; 252: 126462.

Organotypic Cornea 3-D Model with a Collagen Alternative Scaffold Based on Extracellular Matrix Hydrogel Derived from Bovine Cornea

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To reduce the use of animals to assess toxicity potential, 3-D cell culture models based on scaffolds have been used because they better represent the microarchitecture of the tissues, as well as the matrix–cell interactions compared to 2-D models.[1] As an example, 3-D models are being developed for a better evaluation of ocular toxicity, as an alternative to the Draize test. The objective of this study was to propose a new model of 3-D corneal epithelium for assessing ocular toxicity based on the use of HaCaT cells and a natural biomaterial scaffold derived from a decellularised bovine corneal extracellular matrix (ECMd) to replace commercial collagen I rat tail. The hydrogel was prepared from the decellularisation and digestion of bovine corneas to obtain the pre-gel. The pre-gel was neutralised and went through the gelation process in Transwell inserts, to be later populated with HaCaT cells on its surface, to finally obtain the 3-D corneal epithelium model. The models were characterised by histology and maintained for 21 days to monitor their stability and cell viability. The model proposed by the TOXIN[2] laboratory, based on the use of rat tail collagen, was used as the control. The results showed that the bovine corneal ECMd hydrogel was biocompatible and the cells remained viable for five days. This demonstrates that the model based on this natural biomaterial resembles the model that is based on collagen. It also remained stable for a longer time, thus suggesting that it could be a better and more sustainable alternative to collagen in this context.

References

1. Ramadan Q and Zourob M. Organ-on-a-chip engineering: toward bridging the gap between lab and industry. *Biomicrofluidics* 2020; 14: 041501.
2. da Silva ACG, Chialchia AR, de Castro EG, et al. A new corneal epithelial biomimetic 3D model for *in vitro* eye toxicity assessment: development, characterization and applicability. *Toxicol In Vitro* 2020; 62: 104666.

Development and Application of Physiological Kinetic Modelling to Inform *In Vitro* Testing Relevant to Metabolic Disruption Using Human Biomonitoring Data

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Human safety testing has taken a clear direction toward non-animal based testing strategies, with *in vitro* and *in silico* methodologies being employed to inform decision-making. Notable issues associated with the use of *in vitro* toxicity data to describe human risk are the confident and reliable translation of biomonitoring data to *in vitro* relevant concentrations and, in reverse, the extrapolation of *in vitro* concentration–response curves to *in vivo* dose–response curves to derive safe human exposure levels for humans. The GOLIATH project aims to broaden our understanding on metabolic disruption (MD) induced by chemicals, by focusing on developing a battery of assays to capture MD mode of action.[1] To do that, six case chemicals have been selected with documented MD activity to facilitate assay development based on different types of cell line. Along with the optimisation of *in vitro* protocols, priority is given to capturing exposure levels that could potentially translate to human exposure. The purpose of this work is to create a workflow on how PBK applications can be utilised to tailor *in vitro* testing, using current knowledge on mechanism of action, human biomonitoring data and *in silico* methods. Consideration is also given to the advantages and caveats that need to be addressed to increase confidence of model predictability. By using the case of triphenyl phosphate, the presented work will showcase this workflow and discuss applications.

References

1. Legler J, Zalko D, Jourdan F, et al. The GOLIATH Project: towards an internationally harmonised approach for testing metabolism disrupting compounds. *Int J Mol Sci* 2020; 21: 3480.

Understanding the (Dys)regulation of Human Islet Amyloid Polypeptide (hIAPP) Expression in Type 2 Diabetes Mellitus (T2DM)

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Protein misfolding and aggregation has been associated with many human diseases, including type 2 diabetes mellitus (T2DM). T2DM is a metabolic disorder, which is principally characterised by hyperglycaemia and pancreatic β-cell dysfunction. Human islet amyloid polypeptide (hIAPP), or amylin, is a 37-amino acid long peptide hormone that is co-stored and co-secreted with insulin from the pancreatic β-cells. Under normal physiological conditions, hIAPP remains in solution; however, under hyperglycaemic conditions associated with T2DM, hIAPP is known to misfold and aggregate. Autopsied pancreas from T2DM patients exhibit up to 50–60% reduction in β-cell mass and show presence of hIAPP amyloid deposits. Little information is available on the metabolic re-programming that occurs as a result of hIAPP aggregation in pancreatic beta cells, its effect on insulin secretion and its regulation. Previous studies from our laboratory have shown that a chronic exposure of pancreatic β-cells to hIAPP results in their death.[1] In the present work, we attempted to further gain mechanistic insights into pancreatic β-cell death by exposure to hIAPP. A time-course study revealed that even a 1-hour exposure of INS-1E cells (pancreatic β-cell insulinoma from rat) to hIAPP affected their viability and was associated with increased ROS levels, membrane damage and reduced mitochondrial membrane potential. ^{1}H NMR spectra revealed significant changes in the metabolites extracted at different time points. We aim to investigate the metabolic disturbance that occurs in pancreatic beta cells upon IAPP exposure. We will also attempt to understand the influence of identified metabolite on IAPP expression and aggregation in pancreatic cells, and gain further insights into the pathways involved in the regulation of these processes.

References

1. Dubey R, Minj P, Malik N, et al. Recombinant human islet amyloid polypeptide forms shorter fibrils and mediates β-cell apoptosis via generation of oxidative stress. *Biochem J* 2017; 474: 3915–3934.

The Inflammatory Framework of Respiratory System Cells as an *In Vitro* Tool for the Assessment of Respiratory Sensitisers

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Respiratory sensitisers are chemicals that promote allergic responses in the respiratory tract given sufficient/repeated exposures.[1] In certain cases, they can be considered Substances of Very High Concern (SVHC),[2] which are poorly addressed with the animal-based models available for sensitisation assessment. Moreover, there are no validated methods for the evaluation of pulmonary allergenicity, since the Adverse Outcome Pathway (AOP) for lung sensitisation remains incompletely understood.[3] Aiming to contribute to Mode of Action (MoA) elucidation, we selected seven known respiratory chemical allergens and evaluated their pro-inflammatory effects, after exposure to 80% cell viability concentrations (CV_{80}), on four different cell types: bronchial epithelial cells (BEAS-2B); lung fibroblasts (MRC-5); endothelial cells (EA.hy926); and monocytic cells (THP-1). The results of cytokine quantitation demonstrated that respiratory sensitisers promoted a significant increment in IL-8 production by bronchial epithelial cells and lung fibroblasts, as well as an increase in IL-6 production by endothelial and monocytic cells. Besides that, we demonstrated decreased epithelial mucin production (MUC1), an increase in ICAM-I expression by endothelial cells, and the activation of dendritic cells (THP-1) by augmented expression of CD86/HLA-DR. Furthermore, we integrated the aforementioned cells in a 3-D tetraculture model, to sequentially address the effects of aerosolised sensitiser exposure at the air–liquid interface, and to assess the impact of cell crosstalk on the inflammatory response profile. Taken together, these data suggest that respiratory sensitisers trigger specific alterations in respiratory system cells — knowledge that can contribute to both a better mechanistic understanding and the establishment of assessment tools for respiratory sensitisation for regulatory purposes.

References

1. Basketter DA and Kimber I. Assessing the potency of respiratory allergens: uncertainties and challenges. *Regul Toxicol Pharmacol* 2011; 61: 365–372.
2. European Commission. *Regulation (EC) no. 1907/2006* of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending *Directive 1999/45/EC* and repealing *Council Regulation (EEC) No 793/93* and *Commission Regulation (EC) No 1488/94* as well as *Council Directive 76/769/EEC* and *Commission Directives 91/155/EEC*, *93/67/EEC*, *93/105/EC* and *2000/21/EC*. *Off J Euro Union* 2006; L349: 1–849.
3. Charry A, Hennen J, Klein SG, et al. Respiratory sensitization: toxicological point of view on the available assays. *Arch Toxicol* 2018; 92: 803–822.

In Vitro Evaluation of the Toxicological Impact of the Brominated Flame Retardant TBBPA and Nanoplastics on the Caco-2 Cell Line

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Tetrabromobisphenol A (TBBPA) is classed as an emerging contaminant (EC). These are substances whose presence in the environment is not new, but for which there is no regulation or harmonised information on the levels and effects detected in ecosystems.[1] TBBPA is one of the most commonly used brominated flame retardants,[2] present in a wide variety of consumer goods. As a result, its presence is ubiquitous in the environment.[3] Like TBBPA, plastics are also widespread in the environment,[4] with their degradation resulting in the so-called 'nanoplastics' — i.e. small particles with sizes from 0.1 to 0.001 μm. Here, we tried to determine the cytotoxic effects induced by these substances alone and to assess whether their association potentiates the effects observed separately. To do so, we determined the cytotoxic effects on Caco-2 human intestinal cell line by using different *in vitro* conditions. After an acute exposure of 24 hours, in 2% and 10% serum-supplemented media under a wide range of concentrations, we chose the most relevant conditions to be further tested for

21 days. This chronic exposure timing corresponds to the differentiation period of the cell line. The results suggest that, after 24 hours, high concentrations of TBBPA induce severe damage, especially at the low serum concentration: in the presence of 10% serum, EC_{50} values were almost three times those obtained with 2%. Similar outcomes were seen for the non-toxic chemical concentration (NtC) values. After 21 days, the EC_{50} values were like those obtained after 24 hours. Furthermore, co-exposure of TBBPA with nanoplastics suggested that this association is relevant, given that nanoplastics alone did not induce significant damage, either after the 24-hour or 21-day exposure.

References

1. Darnerud PO, Eriksen GS, Jóhannesson T, et al. Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology. *Environ Health Perspect* 2001; 109, Suppl. 1: 49–68.
2. Sjödin A, Hagmar L, Klasson-Wehler E, et al. Flame retardant exposure: polybrominated diphenyl ethers in blood from Swedish workers. *Environ Health Perspect* 1999; 107: 643–648.
3. Montie EW, Letcher RJ, Reddy CM, et al. Brominated flame retardants and organochlorine contaminants in winter flounder, harp and hooded seals, and North Atlantic right whales from the Northwest Atlantic Ocean. *Mar Pollut Bull* 2010; 60: 1160–1169.
4. Barnes DK, Galgani F, Thompson RC, et al. Accumulation and fragmentation of plastic debris in global environments. *Philos Trans R Soc Lond B Biol Sci* 2009; 364: 1985–1998.

Identifying Markers Indicative of Feminisation and Steroidogenesis Deregulation Caused by Endocrine Disruptors

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Published results from EuroMix[1,2] have revealed that exposure to chemicals with endocrine disruptive properties evokes feminisation effects in male offspring, such as reduced anogenital distance (AGD) index, as well as altered steroidogenesis. Total RNA sequencing was performed in the testis of weaning rat pups, showing prominent feminisation effects following perinatal exposure to endocrine disruptors (flutamide 25 and 50 mg/kg bw/d, mixture of flutamide 25 mg/kg bw/d + dienestrol 3 μg/kg bw/d, mixture of flutamide 25 mg/kg bw/d + linuron 12.5 mg/kg bw/d, $n = 3$ per treatment group, dam exposure during GD06-PND21, testis tissue obtained at PND21). The ultimate aim was to identify markers (genes and/or regulatory RNAs) that could be efficiently assessed in *in vitro* systems (e.g. in testicular or H295R cell lines) and potentially reduce/avoid unnecessary animal testing for assessing robust feminisation parameters *in vivo* (such as AGD index, nipple retention, etc.), and thus largely contribute to the Three Rs principles. The sequencing of total RNA was performed by using Illumina RNA-seq technology. RNA-seq analysis of differentially expressed RNA molecules was performed using the *edgeR* package and custom scripts in the R language environment. The RNA-seq analysis indicated a list of 11 RNA transcripts that are significantly deregulated (FDR < 0.1, $p < 0.001$) in at least two of the treatment groups, and are thus worth considering as markers of feminisation and impaired steroidogenesis. Four of these transcripts were found to be deregulated in three treatment groups, and thus seem the most prominent protein-coding RNAs for further use as markers (*Meox2, Gpc3, Tbx22, Fgf3*). Five additional RNA transcripts were near the significance level (FDR < 0.2, $p < 0.001$). The implication of these molecules in feminisation and impaired steroidogenesis needs to be verified in a larger pool of samples or/and by RT-qPCR. Additionally, selection of the most suitable *in vitro* systems to ensure the human-relevance of the results is deemed necessary. GO pathway enrichment analysis revealed that embryo development and circulatory system development biological pathways were affected by two treatments, indicating perturbations in developmental pathways, among others.

References

1. Katsanou ES, Batakis P, Spyropoulou A, et al. Maternal exposure to mixtures of dienestrol, linuron and flutamide. Part II: Endocrine-related gene expression assessment on male offspring rat testes. *Food Chem Toxicol* 2020; 144: 111603.
2. Schreiber E, Garcia T, González N, et al. Maternal exposure to mixtures of dienestrol, linuron and flutamide. Part I: Feminization effects on male rat offspring. *Food Chem Toxicol* 2020; 139: 111256.

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A Future Perspective to Use Human Biomonitoring (HBM) and Physiologically-based Kinetic (PBK) Modelling for Refined Risk Assessment of Genotoxic Carcinogens Present in Immunomodulator-targeted Jamu

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Jamu is being explored by the Indonesian public as traditional medicine to enhance self-immunity against various infections, including the novel coronavirus (SARS-CoV-2). Our previous research indicates that risk management actions for jamu should be prioritised because genotoxic carcinogens, including alkenylbenzenes (ABs), pyrrolizidine alkaloids (PAs) or aristolochic acid (AAs), were detected in jamu collected on the Indonesian market by a targeted approach.[1–3] The occurrence data can be used for an exposure assessment on these compounds. However, human biomonitoring (HBM) may provide a more accurate exposure assessment. This study presents an overview of the current state-of-the-art on the identification of potential biomarkers for human exposure to ABs, PAs and AAs. Urinary metabolites and/or persistent DNA or protein adducts in blood or liver samples might provide parameters for HBM, to evaluate exposure to ABs, PAs or AAs and related elevated cancer risks. Urinary and blood samples could be collected from jamu consumers, while liver samples could be obtained from patients with liver cancer who had consumed jamu. By using physiologically-based kinetic (PBK) modelling-facilitated reverse dosimetry, the HBM data can be converted into exposure estimates. It is concluded that combining HBM data with PBK modelling could provide insight into exposure to ABs, PAs and AAs via the use of jamu, thereby refining the current risk assessment of some selected genotoxic carcinogens shown previously to be present in jamu. Furthermore, the results would indicate whether this approach could also become of use to tackle the lack of epidemiological data in exposure and safety assessment of Indonesian herbal products.

References

1. Suparmi S, Widiastuti D, Wesseling S, et al. Natural occurrence of genotoxic and carcinogenic alkenylbenzenes in Indonesian jamu and evaluation of consumer risks. *Food Chem Toxicol* 2018; 118: 53–67.
2. Suparmi S, Ginting AJ, Mariyam S, et al. Levels of methyleugenol and eugenol in instant herbal beverages available on the Indonesian market and related risk assessment. *Food Chem Toxicol* 2019; 125: 467–478.
3. Suparmi S, Mulder PPJ, Rietjens IMCM. Detection of pyrrolizidine alkaloids in jamu available on the Indonesian market and accompanying safety assessment for human consumption. *Food Chem Toxicol* 2020; 138: 111230.

Sensitisation Potential of Medical Devices Detected by *In Vitro* and *In Vivo* Methods

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Medical devices have to be tested before marketing, in accordance with ISO EN 10993-10, in order to avoid skin sensitisation. This standard predominantly refers to the *in vivo* test; however, it does not exclude the use of *in vitro* methods that have been sufficiently technically and scientifically validated for the purpose of medical devices testing. It is foreseen that due to the complexity of the sensitisation endpoint, a combination of several methods will be needed, in order to address all key events occurring in the sensitisation process. The objective of this follow-up study was to evaluate the sensitisation potential of real samples of medical devices by using a combination of *in vivo* (LLNA DA, OECD TG 442A), *in chemico* (DPRA, OECD TG 442C) and *in vitro* (LuSens, OECD TG 442D) methods, and to enhance the testing strategy for the safety assessment of medical devices extracts. This limited study aims to optimise the use and preparation of extracts, with reference to our previous study.[1] A good agreement between *in vitro* and *in vivo* results was achieved regarding the absence of skin sensitisation potential. However, discrepancies in positive classifications have been recorded. The mismatch

between *in vitro* and *in vivo* results might be caused by the specific response of the immune system of the living organism.

References

1. Svobodová L, Rucki M, Vlkova A, et al. Sensitization potential of medical devices detected by *in vitro* and *in vivo* methods. *ALTEX* 2021; 38: 419–430.

Low Toxicity of Various Microplastics in Monocultures and Cocultures of Lung and Immune Cells

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The presence of plastic debris in the environment has increased during recent years due to the high demand in various applications,[1] and this represents a threat to biodiversity.[1] Scientific findings indicate that, in the last decade, microplastics (MPs) have started to pose an additional risk to animals.[2] Furthermore, due to the abundance of MPs in the environment, in combination with lack of data on their toxic potential on humans,[3] they have become a global health concern. Some MPs can be inhaled and reach the lungs due to their small size. Few toxicity studies on MPs have been reported and, so far, the focus has mainly been on polystyrene (PS) MPs.[4] In the current study, we evaluated the toxic effects of different types of MPs: PS; polyethylene terephthalate (PET); and polyethylene (PE). Importantly, these MPs were produced in a manner that simulates weather-induced degradation of bigger plastic items (i.e. secondary MPs). As a positive control, amine-coated PS nanoplastics (NPs) were included. Human bronchial epithelial cells (HBECs), monocytes (THP-1) and macrophages (differentiated THP-1), both in monocultures and cocultures, were used and cytotoxicity was evaluated with the Alamar blue assay. The results showed that all MPs were relatively non-toxic and only PE MPs decreased cell viability, but only at very high doses (500, 1000 μg/ml). This effect was more pronounced in the cocultures. In contrast, amine-coated PS NPs were toxic even at very low concentrations (≥ 5 μg/ml). These findings suggest that the surface charge plays a key role in the toxic potential of the respective particles, and that secondary plastic particles in the size range of MPs seem to be relatively inert. Further studies — including additional endpoints, time points and sizes of MPs — are needed to fully evaluate the toxicity of plastic particles.

References

1. Bucci K, Tulio M, Rochman C. What is known and unknown about the effects of plastic pollution: a meta-analysis and systematic review. *Ecol Appl* 2020; 30: e02044.
2. Anbumani S and Kakkar P. Ecotoxicological effects of microplastics on biota: a review. *Environ Sci Pollut Res* 2018; 25: 14,373–14,396.
3. Prata J. Airborne microplastics: consequences to human health? *Environ Pollut* 2018; 234: 115–126.
4. Yong C, Valiyaveetill S and Tang B. Toxicity of microplastics and nanoplastics in mammalian systems. *Int J Environ Res Public Health* 2020; 17: 1509.

In Vitro Evaluation of the Inhalation Toxicity of the Cosmetic Ingredient Aluminium Chlorohydrate (ACH)

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Dermal contact, as the primary exposure route for most cosmetic products, is prioritised in cosmetic safety assessments. However, some cosmetic formulations (e.g. aerosols) might favour inhalation exposure, and potential adverse effects on the respiratory tract should be investigated to provide a more accurate toxicological evaluation.[1,2] Aluminium chlorohydrate (ACH) is an important aerosol component frequently used as the active ingredient in antiperspirants,[3] and *in vivo* studies have raised a concern about its inhalation toxicity (e.g. increase in alveolar macrophages). [4,5] Still, few studies have addressed its effects on the human respiratory tract.[6] Therefore, we evaluated the inhalation toxicity of ACH regarding oxidative stress, immunotoxicity and epigenetic changes. A549 cells exposed to three non-cytotoxic concentrations of ACH (0.25, 0.5 and 1 mg/ml) for 24 hours were used as an *in vitro* model of human alveolar cells. Our data showed that ACH induced reactive oxygen species (ROS) production (H_2DCFDA probe, flow cytometry) with a two-fold increase in median

fluorescence intensity (MFI). However, ACH did not alter the released cytokine profile (Cytometric Bead Array (CBA) human inflammatory cytokine kit). Moreover, no alterations to the global DNA methylation pattern (5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC)) or to the histone modification associated with DNA damage (phospho-histone H2AX (γ-H2AX)) were observed (immunostaining for flow cytometry). Our data suggest that ACH might be safe for the human respiratory tract concerning immunotoxicity and epigenetic changes, but it may induce oxidative stress on alveolar cells. Hence, further research is needed to ensure the inhalation safety of ACH.

References

1. Rothe H, Fautz R, Gerber E, et al. Special aspects of cosmetic spray safety evaluations: principles on inhalation risk assessment. *Toxicol Lett* 2011; 205: 97–104.
2. Steiling W, Bascompta M, Carthew P, et al. Principle considerations for the risk assessment of sprayed consumer products. *Toxicol Lett* 2014; 227: 41–49.
3. Pineau A, Fauconneau B, Sappino AP, et al. If exposure to aluminium in antiperspirants presents health risks, its content should be reduced. *J Trace Elem Med Biol* 2014; 28: 147–150.
4. Stankus RP, Schuyler MR, D'Amato RA, et al. Bronchopulmonary cellular response to aluminum and zirconium salts. *Infect Immun* 1978; 20: 847–852.
5. Drew RT, Gupta BN, Bend JR, et al. Inhalation studies with a glycol complex of aluminum-chloride-hydroxide. *Arch Environ Health* 1974; 28: 321–326.
6. Agency for Toxic Substances and Disease Registry (ATSDR). *Toxicological profile for aluminum*. Atlanta, GA: US Department of Health and Human Services, Public Health Service, 2008, 310 pp.

Evaluation of Advanced Cell Models for Inhalation Risk Assessment

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The exposure-led, hypothesis-driven approach in Next Generation Risk Assessment (NGRA) integrates new approach methodologies (NAMs) to assure human safety without the use of animal data. We are currently evaluating our NGRA approach for inhalation exposure by using hypothetical risk assessment case studies, such as film-forming polymers within personal care products (e.g. antiperspirants) and silanes in cleaning products. From the most common consumer exposure scenarios (e.g. daily antiperspirant use), impairment of mucociliary clearance, lung fibrosis and lung surfactant inhibition were identified as relevant endpoints. To investigate these endpoints, the *in vitro* testing of two cell models was carried out. The first, representing the bronchial region, was the MucilAir™-HF cell model (Epithelix), which consists of ciliated as well as mucus-producing cells. The second was the EpiAlveolar™ cell model (MatTek), which is characteristic of the alveolar tract and is a coculture system of AT1 cells, AT2 cells, fibroblasts and THP1 cells. In addition to the two selected case study chemicals, 16 benchmark substances were selected that are either well-known for their effects within specific areas of the lungs, or have a history of safe use with respect to inhalation exposure. Other benchmarks were also chosen with chemical/physical similarities to the case study chemicals. Cells were exposed daily for up to 12 days *in vitro* and different endpoints were measured. Preliminary results from the alveolar model were found to be more sensitive for some of the pro-inflammatory substances tested. For example, polyhexamethylene guanidine phosphate over the 12-day treatment induced mild inflammatory response in the MucilAir-HF system, whereas significant cytotoxicity was induced in EpiAlveolar system after four days of exposure.

Development of a Database of Physiologically-based Kinetic Models

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Systematic reviews make use of all available data by identifying, evaluating and summarising the information from all relevant studies in the literature. Systematic reviews can help with the Three Rs (*Reduction*, *Refinement* and *Replacement*) through making the available data more readily accessible. Read-across is one of the main alternatives used in regulatory submissions for chemical

safety assessment, based on information from data-rich chemicals to make predictions for similar, data-poor chemicals. Information on toxicokinetics (TK) is important for reliable read-across. TK data may be derived from physiologically-based kinetic (PBK) models; these determine concentration–time profiles at the organ level. However, PBK models are time consuming to develop with demanding data requirements. Therefore, being able to inform the development of new PBK models (for chemicals lacking data) using existing data for similar chemicals would be a valuable asset for read-across, with applications in industrial and regulatory sectors. Herein, a systematic review is described that has been undertaken to create a readily available resource for identifying chemicals for which PBK models are publicly available. This includes models for multiple species (e.g. rat, human, mouse, cow, aquatic species, etc.) and various routes of administration (oral, dermal, intravenous, etc.). The development of the systematic review protocol, data extraction methodology and a summary of the resulting PBK model data resource is presented.

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Towards a Better Trial of Occupational Exposures: *In Vitro* Macrophage Systems for Nanomaterial Exposure Study

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Synthetic amorphous silica (SAS) is one of the most commonly used nanomaterials (NMs), with various applications, such as a food additive or cosmetic ingredient, or in tyres to reduce rolling resistance. Considering these uses encountered in daily life, a single high-dose exposure mode is not the most relevant mode to assess the relevant biological effects of NM exposure. Therefore, we have developed an *in vitro* system of NM exposure to evaluate the influence of the dosing rate (bolus *versus* repeated fractionated dose) and the persistence of effects via a recovery period. The effects of repeated exposure of macrophages (RAW 264.7) to SAS and colloidal silica,[1] as well as the persistence of the effects after pyrogenic SAS exposure,[2] have been investigated. The repeated exposure protocol has shown a higher accumulation of SAS particles in the macrophages and different inflammatory responses (TNF secretion and phagocytic function), as compared to the single bolus protocol. The recovery study reported a significant persistence of effects after pyrogenic SAS exposure, as illustrated by an increase in the pro-inflammatory response (TNF and IL-6) and changes in protein abundance (cell adhesion, carbon metabolism or mitochondrion), even after a 72-hour recovery period. Our systems allow the testing of important determinants of toxicity by investigating the general cell functions and specialised functions of macrophages following exposure to NMs. Furthermore, our *in vitro* protocols yield results coherent with those from *in vivo* mouse models.[3,4]

References

1. Torres, Dalzon B, Collin-Faure V, et al. Repeated vs. acute exposure of RAW264.7 mouse macrophages to silica nanoparticles: a bioaccumulation and functional change study. *Nanomaterials* 2020; 10: 215.
2. Torres, Dalzon B, Collin-Faure V, et al. How reversible are the effects of fumed silica on macrophages? A proteomics-informed view. *Nanomaterials* 2020; 10: 1939.
3. Toybou D, Celle C, Aude-Garcia C, et al. A toxicology-informed, safer by design approach for the fabrication of transparent electrodes based on silver nanowires. *Environ Sci: Nano* 2019; 6: 684–694.
4. Dalzon B, Aude-Garcia C, Diemer H, et al. The longer the worse: a combined proteomic and targeted study of the long-term versus short-term effects of silver nanoparticles on macrophages. *Environ Sci: Nano* 2020; 7: 2032–2046.

Different Models to Disentangle Hormone Receptors Signalling Pathways in Breast Cancer

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About 75% of breast cancers are defined as hormone receptor positive (HR^+). These tumours are characterised by the expression of oestrogen receptor (ER), progesterone receptor (PR) and androgen receptor (AR). Activation of these hormone receptor signalling pathways is known to affect breast cancer, but to what extent and by which mechanisms is still debated. The study of hormone action in the human breast has been hampered by a lack of adequate model systems. In order to uncover the roles of the hormone receptors, we have used innovative models: i) the hormone receptor positive breast cancer cell line MCF-7, in which the expression levels of hormone receptors was analysed following treatment with oestrogen and fulvestrant; ii) human microstructures, which were stimulated with hRANKL[1] and their transcriptomic profiles analysed by using microarray methodology. Human microstructures are prepared from fresh human breast tissue specimens, from material discarded following surgery such as reduction mammoplasty, which are mecanically and enzymatically digested to obtain tissue fragments containing ducts and lobules. Both oestrogen and fulvestrant treatments of the MCF-7 cells resulted in an increase in mRNA and protein levels of the AR, whereas fulvestrant decreased PR mRNA levels. Across all the RANKL-stimulated human microstructures, an effective response was achieved only in the tissues of those patients whose blood showed appreciable levels of P4. This result highlights the importance of P4 levels for the sensitivity of the mammary epithelium to RANKL. Pathway analysis detected the enrichment of TNF-α signalling pathway among the RANKL upregulated genes, confirming the successful outcome of hRANKL treatment.

References

1. Tanos T. Progesterone/RANKL is a major regulatory axis in the human breast. *Sci Transl Med* 2013; 5: 182ra55.

Sunscreens and Human Safety Evaluation from the Industrial Point of View

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Sunscreen products fall under different regulations across different countries, and they are still among the most debated products and the most difficult formulas to develop. Key to the sunscreen formula, UV filters protect the skin by absorbing, scattering or reflecting UV radiations. In the EU, sunscreen formulas are regulated as cosmetics. A list of authorised filters is defined after assessment from European scientific experts and their conditions of use are detailed in Annex VI of the Cosmetics Regulation. However, the evaluation of many endpoints is challenging, and data gaps remain for many authorised filters, allowing for recurring controversies on their possible hazards. Moreover, concerns are rising, especially since recent FDA clinical studies revealed systemic exposure of commercially available sunscreens under maximal use conditions. These new findings highlight once more the difficulty of evaluating sunscreen filters with alternative methods only. Indeed, the evaluation strategy of the FDA, waiving some non-clinical toxicology studies for sunscreen development based on an exposure threshold, is questioned. In this context, companies aiming to develop new filters must adapt their safety evaluation strategy and also anticipate regulatory changes. Therefore, we propose an integrated approach, evaluating hazards with New Approach Methodologies and risk with New Generation Risk Assessment tools. Thus, *in silico* models and *in vitro* tests can be used to fill specific data gaps, like sensitisation, carcinogenicity, endocrine disruption, reproductive toxicity, etc. Finally, the robustness of this approach is evaluated. Such approaches are critical from the industry's point of view to prevent innovation attrition and develop new efficient UV filters safely.

3-D Bioprinting of a Microfluidic Renal Corpuscle Model

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The renal corpuscle plays a crucial role in the kidney, being responsible for blood filtration. It has also been pointed out as one of the components responsible for drug failure during clinical trials due to unexpected nephrotoxicity.[1] Despite its importance, few *in vitro* models have aimed to mimic its functionalities.[2] Here, a new application of digital light-assisted (DLP) bioprinting technique is proposed for building 3-D models of the renal corpuscle. To do so, a top-down DLP 3-D bioprinter is assembled and a bioink is proposed. The bioprinter was specifically designed to have excellent accuracy that matches the small feature size required for miniaturistic biomimetic structures, such as the renal corpuscle. The bioink was specifically designed with the same purpose based on gelatin methacryloyl (GelMA) and combined with the visible light crosslinking system formed by ruthenium and sodium persulphate. Through the addition of a biocompatible photoabsorber (red food dye), the crucial fine tuning was achieved. The successful manufacturing of the convoluted shapes that resemble the structure of the renal corpuscle, proves the suitability of the DLP 3-D bioprinting approach. Nevertheless, the cell-friendliness of the model remains to be assessed. This new method aims to join the advantages between microfluidic devices (organ-on-a-chip) and 3-D bioprinting, expanding the available methods for building renal corpuscle models. Bridging the gap between the two approaches could be beneficial not only for *in vitro* kidney models used in disease modelling and drug development, but it also has the potential to be applied to other tissues.

References

1. Saran R, Robinson B, Abbott KC, et al. US Renal Data System 2018 Annual Data Report: epidemiology of kidney disease in the United States. *Am J Kidney Dis* 2019; 73: A7–A8.
2. Sánchez-Romero N, Schophuizen CMS, Giménez I, et al. *In vitro* systems to study nephropharmacology: 2D *versus* 3D models. *Euro J Pharmacol* 2016; 790: 36–45.

Comparison of Cobalt Skin Absorption in Different Skin Models

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Cobalt (Co) is a known toxic metal and skin exposure may lead to contact allergy. The process of skin absorption and skin retention of cobalt is poorly understood. Skin absorption studies are usually performed with human *ex vivo* skin or pig skin because of its similarities to human skin. However, the ethical issues and regulatory restrictions surrounding the use of animals have resulted in the development of alternative skin models, such as Reconstructed Human Epidermis (RhE) skin models. Of the commercially available skin models, EpiSkin™ has shown to be an appropriate alternative to human and pig skin for *in vitro* absorption studies.[1] The overall aims of this study were to compare whether absorption factors of Co change depending on the form of cobalt at exposure (in solution as $CoCl_2$ or in particle form as CoNP), and to determine whether a RhE model could be used as a substitute for stillborn piglet skin. To identify a possible RhE model, a literature review was performed to gather information about RhE models that have been used for absorption studies. We identified EpiSkin as a suitable model for our project. Franz diffusion cell experiments with stillborn piglet skin were done according to OECD guidelines for absorption studies,[2,3] and for the RhE model the experiments were performed without mounting the model in a diffusion cell.[4]

References

1. Schafer-Korting M, Bock U, Diembeck W, et al. The use of reconstructed human epidermis for skin absorption testing: results of the validation study. *Altern Lab Anim* 2008; 36: 161–187.
2. Organisation for Economic Co-operation and Development. *Test no. 428: skin absorption:* in vitro *method*. OECD Guidelines for the Testing of Chemicals, Section 4. Paris: OECD Publications Office, 2004, 8 pp.
3. Organisation for Economic Co-operation and Development. *Guidance document for the conduct of skin absorption studies. OECD Series on Testing and Assessment, No. 28*. Paris: OECD Publications Office, 2004, 31 pp.

4. Eilstein J, Léreaux G, Budimir N, et al. Comparison of xenobiotic metabolizing enzyme activities in *ex vivo* human skin and reconstructed human skin models from SkinEthic. *Arch Toxicol* 2014; 88: 1681–1694.

Human Exposure-based Mixture of Organochlorines and its Endocrine-disrupting Effects on Male Fertility: An Integrative *In Vitro* Approach

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The effects of exposure to individual chemicals are well-reported in the literature, but the consequences of exposure to mixtures is overlooked. Exposure to organochlorines and their mixtures poses a risk to male reproductive health. Therefore, this study aims to develop a complex, rapid and (semi)-versatile automatic battery of *in vitro* assays, mainly in high-content analysis (HCA) mode, for monitoring endocrine-disrupting potential and reproductive toxicity of human-relevant defined mixtures. This approach was applied to assess a human exposure-based mixture of organochlorines with already established *in vivo* reproductive toxicity.[1] First, we evaluated the toxicity profile of the mixture by using a battery of transfected reporter gene assays to study interactions with a variety of nuclear receptors. Strong anti-androgenic effects and weak oestrogenic effects of the mixture were detected. Secondly, we focused on the direct effects of the mixture on testicular somatic cells in a single cell type culture model of pre-pubertal Leydig or Sertoli cells. The mixture affected both testicular cell types and their functions in a dose- and time-dependent manner. Finally, we carried out a hypothesis-driven mechanistic approach to study the mechanisms involved. We identified that the mixture of organochlorines rapidly dysregulated the gap junctional intercellular communication (GJIC) in both testicular cell types by using a simple, cost-effective and (semi)high-throughput assay, based on the scrape-loading/dye transfer technique.[2] In conclusion, organochlorine mixtures could be a potential aetiological agent contributing to reproductive dysfunction in males through impairment of testicular GJIC and endocrine disruption.

References

1. Anas M-KI, Guillemette C, Ayotte P, et al. In utero and lactational exposure to an environmentally relevant organochlorine mixture disrupts reproductive development and function in male rats. *Biol Reprod* 2005; 73: 414–426.
2. Dydowiczová A, Brózman O, Babica P, et al. Improved multiparametric scrape loading-dye transfer assay for a simultaneous high-throughput analysis of gap junctional intercellular communication, cell density and viability. *Sci Rep* 2020; 10: 730.

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Latent Infection of Resting CD4$^+$ T-cell Transmigrating Through Microvascular and Lymphatic Endothelial Cells in Response to Homeostatic or Inflammatory Chemokines

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A small pool of naïve and memory CD4$^+$ T-cells harbouring latent HIV and persisting indefinitely despite anti-retroviral therapy represents the major barrier toward a cure for HIV-1 infection.[1] It is, therefore, important to investigate the pathways involved in the establishment of latent infection in these cells. Although resting CD4$^+$ T-cells are not susceptible to HIV-1 infection *in vitro*, recent studies have shown that chemokines can render these cells permissive to latent infection by inducing subcortical filamentous actin depolymerisation, thus favouring HIV-1 translocation to the cell nucleus. We therefore hypothesise that HIV-1 latency can be established in resting CD4$^+$ T-cells during their constitutive homing to tissues in response to homeostatic chemokines, or upon their recruitment to inflamed tissue by inflammatory chemokines produced at sites of HIV-1 infection. Furthermore, recent data indicate that the HIV-1 Tat protein released by HIV-1 productively infected CD4$^+$ T-cells renders activated endothelial cells permissive to HIV.[2] In addition, HIV-1 internalised by endothelial cells can be stored in non-degradative compartments and transferred to CD4$^+$ T-cells. We therefore further hypothesise that the infected endothelium may transfer HIV-1 to CD4$^+$ T-cells during endothelial cell

transmigration by a direct cell-to-cell transmission pathway. The HIV-1 Tat protein may be important in these processes. Organs-on-chips and organoids are key for these studies, as they permit $CD4^+$ T-cell migration and transendothelial migration in a context that is more similar to the *in vivo* microenvironment.

References

1. Palermo E, Acchioni C, Di Carlo D, et al. Activation of latent HIV-1 T cell reservoirs with a combination of innate immune and epigenetic regulators. *J Virol* 2019; 93: e01194-19.
2. Cafaro A, Barillari G, Moretti S, et al. HIV-1 Tat protein enters dysfunctional endothelial cells via integrins and renders them permissive to virus replication. *Int J Mol Sci* 2021; 22: 317.

New Approaches for the Safety Assessment of Novel Proteins and Their Capacity to Trigger Celiac Disease

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The world population is expected to grow by more than 10 billion by 2100[1] and, with it, the demand for new food proteins. The dietary use of novel proteins has to be carefully assessed regarding their potential adverse effects on the human body, such as celiac disease (CD). CD, estimated to affect 1 in 100 people worldwide,[2] is a non-IgE immunoreaction provoked by gluten proteins.[3,4] The only treatment so far is the elimination of gluten from the diet.[2] The elicitation of the CD pathogenic pathway in the human body mainly involves three key elements that can be used in risk assessment:[3,4] the enzymatic degradation during digestion, affecting the immunogenic properties of gluten; the epitope binding to the HLA-DQ receptor of the antigen-presenting cell; and the activation of pro-inflammatory $CD4^+$ T-cell, which recognises the HLA-DQ–epitope complex and initiates the inflammatory response. Data originating from humans and animal models[5,6] have allowed the scientific community to develop different studies that have been used for the development of predictive models, such as *in vitro* assays (e.g. digestion and proliferation assays) or *in silico* models (e.g. enzymatic cleavage and affinity models).[7,8] Initial approaches in this direction for the risk assessment of novel proteins have already been pursued.[4] Additional work on how best to use *in silico* and *in vitro* studies to predict the potential of novel proteins to trigger CD, replacing human and/or animal models data, is ongoing, with great potential for improvement in the near future.

References

1. United Nations. *World population prospects 2019: highlights*, https://population.un.org/wpp/ (accessed 18 August 2021).
2. Celiac Disease Foundation. *What is celiac disease?* https://celiac.org/about-celiac-disease/what-is-celiac-disease/ (accessed 22 February 2020).
3. The University of Manchester. *Literature review: 'non-IgE-mediated immune adverse reactions to foods'. EFSA supporting publication 2013*: EN-527, 40 pp.
4. EFSA panel on genetically modified organisms. Guidance on allergenicity assessment of genetically modified plants. *EFSA J* 2017; 15: 4862.
5. Sollid LM, Tye-Din JA, Qiao SW, et al. Update 2020: nomenclature and listing of celiac disease-relevant gluten epitopes recognized by $CD4^+$ T cells. *Immunogenetics* 2020; 72: 85–88.
6. Marietta EV, David CS and Murray JA. Important lessons derived from animal models of celiac disease. *Int Rev Immunol* 2011; 30: 197–206.
7. Stoven S, Murray JA and Marietta EV. Latest *in vitro* and *in vivo* models of celiac disease. *Expert Opin Drug Discov* 2013; 8: 445–457.
8. De Re V, Magris R and Cannizzaro R. New Insights into the pathogenesis of celiac disease. *Front Med* 2017; 4: 137.

Assessing the Endocrine Disruption Potential of REACH Chemicals: A Case Study of Two Plasticisers

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In the course of the European Commission's Green Deal Agenda toward a non-toxic environment,[1] endocrine disruptors (EDs) have been highlighted as substances of high health concern. Here, two plasticisers — a phthalate and a non-phthalate — were scrutinised for ED potential by assessing both the available registration data and all other scientific data retrieved by systematic review methodology from the open literature. It was of interest to find out whether the current standard information requirements for chemicals regulated under REACH are efficient in identifying EDs. To aid the assessment process, the guidance document on the identification of EDs, developed by the European Chemicals Agency and the European Food Safety Authority and designed for biocidal and plant protection products,[2] was used. Applying Weight-of-Evidence methodology, the following conclusions were drawn: a) Regarding the non-phthalate plasticiser, ED potential is unlikely, but terminal tests on mechanistic data are needed to draw a clear conclusion. b) The phthalate plasticiser formally met the ED criteria, but data gaps and uncertainties remain regarding its mechanism of action. c) In a broader perspective, the required data for identifying an ED according to the EU's criteria are not completely covered by the standard information requirements. Thus, for an ED assessment of REACH chemicals according to the guidance document, it may be necessary to request additional *in vivo* data, raising ethical concerns about animal welfare. d) Better collaboration between registrants, i.e. data sharing of full studies, is indispensable for a sound ED assessment and avoidance of further animal testing.

References

1. European Commission. Communication from the Commission to the European Parliament, the European Council, the Council, the European Economic and Social Committee and the Committee of the Regions. The European Green Deal. COM/2019/640 final, https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=COM%3A2019%3A640%3AFIN (accessed 18 August 2021).
2. European Chemical Agency (ECHA) and European Food Safety Authority (EFSA) with the technical support of the Joint Research Centre (JRC), Andersson N, Arena M, Auteri D, et al. Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009. *EFSA J* 2018; 16: 5311.

Evaluating Further Refinement Methods and a Newly Developed Sustained-release Buprenorphine to Relieve Pain in Mouse Osteotomy Models

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Untreated or insufficiently treated pain impacts animal welfare and can have effects on the organism such as impaired healing, disrupted regeneration or immunosuppression; therefore, it potentially affects the scientific merit of the experimental results. Thus, adequate pain management in animal experiments is essential both for ethical and scientific reasons. Our collaborative project aims to refine commonly used pain management protocols in animal experiments which currently cannot yet be replaced in many areas of (bio)medical research, and therefore we hope to improve animal welfare in science. Buprenorphine is one of the most commonly used analgesics in rodents. The standard application route is i.p. or s.c. injection every 6–12 hours, which requires frequent additional handling. This translates into additional stress for the animals, thereby possibly increasing the overall burden. However, many studies indicate that application every 12 hours is not enough to provide continuous pain relief. Thus, the animals suffer periods of inadequate or no pain relief, although a 12-hour interval is already stressful for the animals and the experimenter. Other protocols rely on the voluntary intake of medication such as tramadol via the drinking water, but this intake might be decreased in animals in pain. The availability in Europe of sustained-release buprenorphine would solve several problems; however, the import of commercially available preparations from the USA is not possible. Hence, in our current *refinement* study, we are focusing on the testing of a newly developed sustained-release formulation of buprenorphine[1] in two mouse osteotomy models, as a proof-of-concept for a wide range of rodent models. It is hoped that this sustained-release treatment can be effectively used as a potential alternative to the application of tramadol via the drinking water.[2]

References

1. Schreiner V, Durst M, Arras M, et al. Design and *in vivo* evaluation of a microparticulate depot formulation of buprenorphine for veterinary use. *Sci Rep* 2020; 10: 17295.
2. Jirkof P, Durst M, Klopfleisch R, et al. Administration of tramadol or buprenorphine via the drinking water for post-operative analgesia in a mouse-osteotomy model. *Sci Rep* 2019; 9: 10749.

HepG2 Cell Culture Confluence Measurement in Phase-contrast Micrographs: A User-friendly, Open-source Software-based Approach

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Phase-contrast micrographs are often used for the confirmation of results in proliferation and viability assays. However, they are usually only a qualitative tool and fail to exclude with certainty the presence of assay interference by test substances. The complexity of the image analysis workflow hinders life scientists from routinely utilising micrograph data. Here, we present an open-source software-based, combined ilastik[1] segmentation/ImageJ[2] measurement of area (ISIMA) approach, for cell monolayer segmentation and confluence percentage measurement of phase-contrast micrographs of HepG2 cells. The aim of this study was to test whether the proposed approach is suitable for quantitative confirmation of proliferation data, acquired by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Our results showed that ISIMA is user-friendly and provides reproducible data that strongly correlate with the results of the MTT assay. In conclusion, ISIMA is an affordable, simple and fast approach for confluence quantification by researchers without a background in image analysis.[3]

References

1. Berg S, Kutra D, Kroeger T, et al. ilastik: interactive machine learning for (bio)image analysis. *Nat Methods* 2019; 16: 1226–1232.
2. Schneider CA, Rasband WS and Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012; 9: 671–675.
3. Yordanov, Y. Hep G2 cell culture confluence measurement in phase-contrast micrographs — a user-friendly, open-source software-based approach. *Toxicol Mech Methods* 2020; 30: 146–152.

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An Effect-based Comparison of Common Drinking Water Treatments in a Full-scale *versus* Pilot-scale System: The Use of *In Vitro* Bioassays in Water Quality Evaluations

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Drinking water treatment plants (DWTPs) often employ different combinations of coagulation, sedimentation, filtration and disinfection methods to provide clean drinking water safe from harmful pathogens. However, such methods might not completely eliminate micropollutants present in the raw water. As such, there is a need for vital research into the effectiveness of existing water purification methods and a push toward the development of new technologies. In the current study, bioassays were used to assess a pilot-scale treatment process (ozonation and granular activated carbon (GAC) filtration) *versus* a full-scale DWTP (coagulation/sedimentation/rapid sand filtration, biologically activated carbon (BAC) filtration, UV disinfection, monochloramine dosing). The effectiveness of the treatments was assessed by *in vitro* bioassays for the following endpoints: oxidative stress (Nrf2 activity), genotoxicity (micronuclei formation), aryl hydrocarbon receptor (AhR) activation, and hormone-mediated (oestrogen receptor (ER), androgen receptor (AR) receptor activation). Compared to most of the full-scale treated samples, lower Nrf2, AhR, ER bioactivities and genotoxicity were observed in the pilot-scale samples following ozonation or GAC filtration across two sampling events. These findings highlight the effectiveness of ozonation or GAC filtration in reducing bioactive compounds during drinking water treatment. In

comparison, higher bioactivities were detected in most of the full-scale samples and variability was observed between sampling events. No AR activities were observed in any of the samples. The conclusions made from this study, regarding both the pilot-scale and full-scale drinking water treatment methods, provide important insights into the optimisation of existing drinking water treatment designs and utilisation of alternative treatment technologies. Also, *in vitro* bioassays were proven to be promising tools in the improvement of drinking water quality from a regulatory perspective.

Effects of Endocrine Disrupting Chemicals (EDCs) on Molecular Endpoints in Human Mesenchymal Stem Cells

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Intricate and highly regulated hormone signalling is essential for correct metabolic programming that, in turn, is central for postnatal health.[1–5] Exposure to endocrine disrupting chemicals (EDCs) during critical periods of development has been associated with a variety of metabolic diseases that transpire later in life.[6–8] Human mesenchymal stem cells (hMSCs) are multipotent progenitors with the ability to differentiate into a range of cell lineages, including adipose.[9] Thus, they represent a relevant model to study key events during early human metabolism and growth development. Prenatal EDC exposure can affect differentiation of hMSCs through the alteration of their DNA methylation patterns, leading to abnormal development.[10] Current hazard and risk assessments of EDCs do not accurately reflect the effects of chemical mixtures during metabolic programming. Thus, there is an urgent need to expand the testing and screening methodology for developmental exposure effects of EDC mixtures on metabolic programming. In this context, experimental data on novel, more sensitive endpoints for mixture effects are central to the development of new testing and risk assessment methodology. This study aims to validate a panel of molecular candidate endpoints that could predict effects of EDCs, either individually or in mixtures, on hMSC differentiation. This study is based on an EDC mixture identified in EDC-MixRisk that was associated with adverse outcomes in humans at common exposure levels. Exposure of hMSCs to this mixture resulted in increased adipogenesis. Additionally, genome-wide DNA methylation analysis using Illumina EPIC bead array identified a panel of DNA methylation changes induced by the mixture that could be involved in changed metabolism. Therefore, in this project this panel of endpoints was validated in DNA samples collected from the mixture-exposed hMSCs. DNA methylation patterns at regions of interest were assessed by using bisulphite-pyrosequencing.

References

1. Litwack G. Chapter 1: Hormones and perinatal development. In: *Hormonal signaling in biology and medicine*: comprehensive modern endocrinology. Cambridge, MA: Academic Press, 2020, pp. 1–11.
2. Christoforou E and Sferruzzi-Perri A. Molecular mechanisms governing offspring metabolic programming in rodent models of in utero stress. *Cell Mol Life Sci* 2020; 77: 4861–4898.
3. Brenseke B, Prater MR, Bahamonde J, et al. Current thoughts on maternal nutrition and fetal programming of the metabolic syndrome. *J Pregnancy* 2013; 2013: 368461.
4. Fowden A, Giussani D and Forhead A. Endocrine and metabolic programming during intrauterine development. *Early Hum Dev* 2005; 81: 723–734.
5. Howard S. Developmental exposure to endocrine disrupting chemicals and type 1 diabetes mellitus. *Front Endocrinol* 2018; 9: 513–513.
6. Filardi T, Panimolle F, Lenzi A, et al. Bisphenol A and phthalates in diet: an emerging link with pregnancy complications. *Nutrients* 2020; 12: 525.
7. Chen J, Brown T and Russo J. Regulation of energy metabolism pathways by estrogens and estrogenic chemicals and potential implications in obesity associated with increased exposure to endocrine disruptors. *Biochim Biophys Acta* 2009; 1793: 1128–1143.
8. Alonso-Magdalena P, Quesada I and Nadal A. Endocrine disruptors in the etiology of type 2 diabetes mellitus. *Nat Rev Endocrinol* 2011; 7: 346–353.
9. Ksiazek K (2009). A comprehensive review on mesenchymal stem cell growth and senescence. *Rejuvenation Res* 2009; 12: 105–116.
10. Schug T, Janesick A, Blumberg B, et al. Endocrine disrupting chemicals and disease susceptibility. *J Steroid Biochem Mol Biol* 2011; 127: 204–215.

Deciphering the Molecular Mechanisms of Resveratrol as a Neuroprotective Agent in Multiple Sclerosis Induced by Environmental Factors: An *In Silico* Approach

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Multiple sclerosis (MS) is a chronic autoimmune inflammatory disease of the central nervous system (CNS), characterised by demyelination, neuronal injury, and breaching of the blood–brain barrier.[1] Epidemiological studies have shown that immunological, genetic, and especially environmental factors contribute to the progression and development of MS.[2] Although improvements have been made in disease therapy, effective treatment of progression remains an unmet need because current therapies confer only partial protection against the neurodegenerative component of MS.[3] Therefore, intervention approaches targeting phytochemicals have been recommended as an alternative form of treatment.[4] The aim of this study was to elucidate the molecular mechanisms of resveratrol as a potential neuroprotective agent in MS therapy by using an *in silico* approach. The Comparative Toxicogenomics Database (CTD), Cytoscape software package version 3.8.0 (plug-ins: GeneMANIA, CytoHubba) ToppGene Suite portal and Metascape were used as the main data-mining tools. Resveratrol targeted 34 genes linked to MS development (shown by the CTD). GeneMANIA revealed that the majority of the selected genes were co-expressed (72.81%). The CytoHubba plug-in identified 10 hub genes: CXCL8; IL6; TNF; RELB; TRAF1; IL1B; NFKBIA; NFKB2; TNFAIP3; and ICAM1. The subgroup Process Enrichment Analysis (Metascape) showed that NF-kappa B signalling and TNF signalling were most significant pathways, which was confirmed by ToppGene analysis along with Cytokine Signaling in the Immune System. Positive regulation of the neuroinflammatory response and immunoglobulin-regulated adaptive immune response were highlighted as important biological processes. Since MS is predominantly driven by a systemic immune response, adjuvant therapies targeting neuroinflammation should be investigated further.[5]

References

1. Lassmann H. Multiple sclerosis pathology. *Cold Spring Harb Perspect Med* 2018; 8: 1–15.
2. Waubant E, Lucas R, Mowry E, et al. Environmental and genetic risk factors for MS: an integrated review. *Ann Clin Transl Neurol* 2019; 6: 1905–1922.
3. Hauser SL and Cree BAC. Treatment of multiple sclerosis: a review. *Am J Med* 2020; 133: 1380–1390.
4. Yu S, Liu M and Hu K. Natural products: potential therapeutic agents in multiple sclerosis. *Int Immunopharmacol* 2019; 67: 87–97.
5. Dendrou CA, Fugger L and Friese MA. Immunopathology of multiple sclerosis. *Nat Rev Immunol* 2015; 15: 545–558.