EMERGING FIELDS IN 3Rs

RE-Place
ALTERNATIVE METHODS TO ANIMAL TESTING

IC-3Rs
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JOINT 3R SYMPOSIUM
19-20-21 SEPT 2023
BRUSSELS · BELGIUM

The Innovation Centre 3Rs (IC-3Rs), together with RE-Place and the EU-TWINALT consortium invite you to attend their first Joint Symposium where (inter)national scientists will provide an insight into their research using the most advanced 3R-alternative models.

The Joint 3R Symposium runs during 3 consecutive days and will start with a closed pre-meeting of the TWINALT consortium in the morning of the 19th September 2023. TWINALT is a European Horizon 2020 funded project between the Nofer Institute of Occupational Medicine (NIOM), the Vrije Universiteit Brussel (VUB), the Norwegian Institute for Air Research (NILU) and the University of Milan (UMIL). The aim of TWINALT is to increase the capacity of the institute NIOM, based in the Widening Country Poland, towards available *in vitro* methods for toxicity testing by knowledge exchange and trainings. The afternoon session is open to all and will be devoted to keynote lectures on TWINALT relevant topics.

RE-Place is organizing the morning session on Wednesday the 20th of September. RE-Place is a scientific collaboration between the VUB and Sciensano, the Belgian institute of public health. RE-Place collects different types of replacement methods in one central database for which experience and expertise exists in Belgium. Six experts from the RE-Place database will be invited to present their work. During the afternoon session, young researchers will have the opportunity to competitively present their 3R research.

The Annual IC-3Rs Symposium will be organized on the last day, Thursday the 21st of September. Until recently, most research in complex fields such as neurology, reproductive biology, neurotoxicology and reproductive toxicology, was carried out using experimental animals. However, studies of the brain and reproductive system in rodents rarely yield results applicable to humans. Therefore, new 3R-alternative methods for research on the brain and reproductive organs are emerging and will receive special attention during that day.

Poster sessions will be organized during coffee and lunch breaks over the full three days to trigger informal discussions among all participating researchers. Two prizes (250€ each) will be awarded: the best oral presentation by a young researcher and the most interesting poster.

We are happy to welcome you at this exciting event!

Prof. Tamara Vanhaecke
VUB, TWINALT, IC-3Rs

Dr. Birgit Mertens
Sciensano, RE-Place

Em. Prof. Vera Rogiers
VUB, TWINALT, RE-Place, IC-3Rs

Prof. Edyta Reszka
NIOM, TWINALT

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Programme

TUE 19.09 • TWINALT

CLOSED SESSION

Moderation Prof. E. Reszka (NIOM,PL)

09.30 – 10.00 Morning coffee

10.00 – 10.05 **Welcome** Prof. Tamara Vanhaecke (VUB, BE) & Prof. Edyta Reszka (NIOM, PL)

10.05 – 10.20 **TWINALT pillars/objectives 1) scientific development; 2) dialogue with industry; 3) raising awareness; 4) approachable knowledge** Prof. Edyta Reszka (NIOM, PL)

10.25 – 10.40 **WP 1: Elaboration of a common RTD and innovation strategy on alternative methods for toxicity assessment** Dr. Elise Rundén-Pran (NILU, NO)

10.45 – 11.00 **WP 2: Exchange of know-how with partners through training mobility** Dr. Joanna Roszak (NIOM, PL)

11.05 – 11.20 **WP 3: Communication, exploitation and dissemination** Prof. Tamara Vanhaecke (VUB, BE)

11.25 – 12.00 **Return mechanism workshop** Dr. Joanna Roszak (NIOM, PL)

S&T 7b - Course on GLP for *in vitro* nano and genotoxicology, best practices with nanomaterial handling and preparation for testing Dr. Gloria Melzi (UMIL, IT)

S&T 8 – New tools in the assessment of neurotoxicity and developmental neurotoxicity Dr. Tanima Sengupta (NILU, NO) & Dr. Anna Wolniakowska (NIOM, PL)

S&T 9 – Imaging Course @ NILU
Marta Biesiekierska (NIOM, PL) & Marth Stinckens (VUB, BE)

S&T 10 – Course on *in silico* methods in safety assessment of chemicals Kateryna Tarhonska (NIOM, PL)

12.00 – 12.15 **Results obtained by NIOM for each of the 4 pillars, lessons learned and next steps to be taken** Prof. Edyta Reszka (NIOM, PL)

12.15 – 12.30 **General Discussion**

TUE 19.09 • TWINALT

OPEN SESSION

Welcome & moderation Prof. T. Vanhaecke (VUB, BE)

12.30 – 14.00 Lunch & Poster sessions

14.00 – 14.30 **Use of Adverse Outcome Pathways (AOPs) for hazard identification**
Prof. Barbara Viviani (UMIL, IT) • Page 20

14.40 – 15.10 **An AOP-based IATA to pave the way towards animal-free genotoxicity testing**
Dr. Birgit Mertens (Sciensano, BE) • Page 22

15.20 – 15.40 **Influence of the experimental approach in the assessment of nanomaterial hazard *in vitro* – engineered and incidental nanoparticles relevant to the ceramic industry as case study**
Prof. Sónia Fraga (INSA, PT) • Page 24

15.50 – 16.10 **Metabolic disruption in the liver: assays to detect hepatic triglyceride accumulation in human liver cells *in vitro***
Prof. Albert Braeuning (BfR, DE) • Page 26

16.10 – 17.30 Networking reception

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Programme

WED 20.09 • RE-PLACE & YSC

Knowledge Sharing on NAMs

Moderation Dr. B. Mertens (Sciensano, BE)

09.00 – 09.30 Morning coffee

09.30 – 09.40 **Welcome** Dr. Birgit Mertens (Sciensano, BE)

European Databases on NAMs and education of the next generation scientists

09.40 – 10.10 **Fostering critical thinking about science, its methods and the 3Rs: resources from EURL-ECVAM for established researchers and the scientists of tomorrow** Dr. Annalisa Gastaldello (Project officer at the European Commission, EU) • Page 28

Belgian Database on NAMs and initiatives to promote its use

10.10 – 10.35 **RE-Place: a bottom-up approach to collect the existing expertise on NAMs in Belgium** Mieke Van Mulders & Maude Everaert (VUB/Sciensano, BE) • Page 30

Expertise from the RE-Place database: Modelling tools in practice

10.35 – 10.55 **Mathematics as an alternative for animal experiments in burn injuries** Prof. Fred Vermolen (UHasselt, BE) • Page 34

10.55 – 11.15 **Towards a Hippocampal Formation Model of Temporal Lobe Epilepsy** Prof. Emmeric Tanghe (UGent, BE) • Page 36

11.15 – 11.35 Coffee break & Poster sessions

Expertise from the RE-Place database: Progress in 3D tissue models

11.35 – 12.00 **Using lung organoids to study the role of the chitinase-like protein Ym2 in allergic asthma** Dr. Ursula Smole (UGent, BE) • Page 38

12.00 – 12.20 **Optimized High-Throughput Screening of 3D Precision-Cut Tissue Slices** Dr. Bella B. Manshian (KU Leuven, BE) • Page 40

12.20 – 12.40 **MISpherID: A knowledgebase and transparency tool for Minimum Information in Spheroid IDentity** Eva Blondeel (UGent, BE) • Page 42

WED 20.09 • RE-PLACE & YSC

Young Scientists Competition

Moderation Prof. J. De Kock (VUB, BE)

12.40 – 14.00 Lunch & Poster sessions

14.00 – 14.15 **The GENOMARK transcriptomic biomarker: a new approach methodology for qualitative and quantitative genotoxicity assessment of chemicals** Anouck Thienpont (VUB, BE) • Page 62

14.15 – 14.30 **A human bronchial epithelial spheroid model to study respiratory toxicity *in vitro*** Thomas Celis (KU Leuven, BE) • Page 63

14.30 – 14.45 **Receptor ontology and the importance of glutamatergic subunit composition in the assessment of developmental neurotoxicity: focus on the GluN2B/GluN2A switch in primary versus hIPSCs-derived neurons** Melania Serafini (Università di Milano, IT) • Page 64

14.45 – 15.00 **Perioperative care for complex abdominal surgery in a novel mouse model** Louis Onghena (Ugent, BE) • Page 65

15.00 – 15.30 Coffee break & Poster sessions

15.30 – 15.45 **Production of a reusable micromolded microcavity insert to standardize spheroid generation for drug screening** Helia Fernandes (Politecnico di Milano, IT) • Page 67

15.45 – 16.00 **Comparison of advanced and traditional cell culture models for lung and liver – cyto- and genotoxicity of NM-300K silver nano-particles** Elisabeth Elje (NILU, NO) • Page 68

16.00 – 16.15 **Assessment of cytotoxic effect mechanisms of circadian REV-ERB agonist in urothelial cells** Marta Biesiekierska (NIOM, PL) • Page 69

16.15 – 16.30 **Planarians as an *in vivo* model for a high-throughput assessment of developmental, neuro- and genotoxicity** Karolien Bijnens (UHasselt, BE)• Page 70

16.30 End of the day

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Programme

THU 21.09 • IC-3Rs

The lifecycle in neurotoxicity

Moderation Dr. B. Garthoff (BIO.NRW, DE)

08.30 – 09.00 Morning coffee

09.00 – 09.15 **Welcome** Prof. Vera Rogiers (IC-3Rs, BE)

09.15 – 09.30 **Some Words** from Bernard Clerfayt (Min. Brussels Region, Animal Welfare, BE)

09.30 – 09.55 **Introduction to life stage-dependent neurotoxicity, including current status of AOPs**
Dr. Oddvar Myhre (Norwegian Institute of Public Health, NO) • Page 44

10.05 – 10.30 **The *in vitro* test battery for developmental neurotoxicity assessment towards regulatory acceptance (DNT IVB)**
Prof. Ellen Fritsche (University of Düsseldorf, DE) • Page 46

10.40 – 11.20 Coffee break & Poster sessions

11.20 – 11.45 **Neurodevelopmental toxicity in the European H2020 ONTOX project**
Dr. Eliška Kuchovska (University of Düsseldorf, DE) • Page 48

11.55 – 12.20 **Brain organoids for personal medicine modelling in neurodegenerative disease**
Prof. Ira Espuny Camacho (ULiège/GIGA, BE) • Page 50

THU 21.09 • IC-3Rs

Reproductive health: *in vitro* modelling

Moderation Prof. J. Castell (Uni Valencia, ES)

12.30 – 14.00 Lunch & Poster sessions

14.00 – 14.30 **Endocrine disruptive effects on the male reproductive system: animal versus non-animal data?**
Prof. Terje Svingen (Technical University Denmark, DK) • Page 52

14.40 – 15.10 **Development of a representative, reliable and reproducible model for (human) testis**
Prof. Yoni Baert (Vrije Universiteit Brussel, BE) • Page 54

15.20 – 15.35 Coffee break

15.40 – 16.10 **Implantation (female) models including endometrial organoids and assembloids**
Dr. Thomas Rawlings (University of Warwick, UK) • Page 56

16.20 – 16.50 **Stem cells and embryo development**
Prof. Karen Sermon (Vrije Universiteit Brussel, BE) • Page 58

17.00 – 17.15 Award ceremony

17.15 Networking reception

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Symposium Moderators

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Vera Rogiers

Emeritus Professor
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After many years of leading the department of *In Vitro* Toxicology and Dermato-Cosmetology at the VUB in a successful way, Emeritus professor in Toxicology Vera Rogiers is actually still teaching dermato-cosmetics at the VUB and the University of Ghent. She also gives a limited number of lessons to the University of Namur. She yearly organizes international courses on Cosmetics and Risk Assessment. She is the Director of the Innovation Centre-3Rs (IC-3Rs) at the VUB, supported by the scientific Chair Mireille Aerens, both with focus on replacing experimental animals by novel technologies. At the EU level, she is Co-chair of the Scientific Committee on Consumer Safety (SCCS) and member of the Mirror group of the European Partnership on Alternative Approaches to Animal Testing (EPAA). Her main research activity was many years situated in the development of *in vitro* models as an alternative to the use of experimental animals. Actual focus is on the differentiation of human skin-derived stem cells to functional hepatic cells and their application for drug discovery and the detection of drug-induced liver injury. She has been promoter of 33 doctoral theses, is author or co-author of > 450 publications in international peer reviewed scientific journals and is editor of several scientific books. She is an often-invited speaker (>400) and participated in the organization of more than 60 international congresses. She has coordinated 2 EU research projects and was partner in several FP6, FP7 EU and Horizon 2020 research projects concerned with *in vitro* methodology development. Of the obtained scientific results, several patents have been filed. Throughout her career she received several international scientific awards for her pioneering role in *in vitro* Experimental Toxicology.

Tamara Vanhaecke

Professor
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Tamara Vanhaecke is Full Professor Toxicology and Head of the Department *In Vitro* Toxicology and Dermato-Cosmetology (IVTD) at the faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel (VUB), that is striving for excellence in the domain of 3R alternative methods, referring to the Reduction, Replacement, Refinement of animal use in research and testing. She has a background in cell and gene biotechnology, holds a doctoral degree in experimental *in vitro* toxicology, is a European registered toxicologist and a trained chemical risk assessor. Her research is situated in the field of *in vitro* experimental toxicology with focus on the development of human stem cell-derived hepatocyte-based culture models for the detection of drug-induced liver injury and in the area of *in vitro* liver disease modelling. She also has several research collaborations with Sciensano (Belgian public institution for public and animal health) that address toxicity concerns related to e-cigarettes, food contact materials, cosmetics and feminine intimate products. She has more than 150 scientific publications in peer-reviewed journals and books, and is reviewer for several scientific journals in the field of pharmaco-toxicology, (*in vitro*) toxicology and hepatology. She has several times been invited speaker on international congresses. She is at the VUB co-holder of 3 patents and obtained 2 international research awards in the field of 3R alternative methods. She has acted as promoter of 10 doctoral theses in pharmaceutical sciences and is promoter of 11 additional PhD theses that are still ongoing. She was the VUB promotor of the European FP7 project START-UP and was involved in the FP7 SEURAT-1 projects DETECTIVE and HeMiBio, and the FP6 projects ESNATS, carcinoGenomics, Predictomics and Liintop. At the EU level, she is Member of the Scientific Committee on Consumer Safety (SCCS) and former Member of the ECVAM Validation Management Group concerning toxicokinetics and metabolism.

Joery De Kock

Professor
***In Vitro* Toxicology and Dermato-Cosmetology (IVTD)**
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- Pharmacist (Vrije Universiteit Brussel, 2006)
- Doctor in Pharmaceutical Sciences (Vrije Universiteit Brussel, 2012)

Joery De Kock graduated in 2006 as Pharmacist from the Vrije Universiteit Brussel (VUB) and obtained his PhD in Pharmaceutical Sciences in May 2012 under the mentorship of Prof. Vera Rogiers. During his PhD, he managed for the first time to differentiate so-called human skin-derived precursor cells (hSKP) into hepatic cells. These hSKP-derived hepatic cells (hSKP-HPC) have provided a solid basis for multiple successful follow-up PhD projects over the last years. He is since 2017 a full-time professor affiliated to the Faculty of Medicine and Pharmacy at the research group of *In Vitro* Toxicology and Dermato-Cosmetology (IVTD) and was previously a postdoctoral research fellow of the Research Foundation Flanders (FWO) from 2012 until 2017. From 2016 to 2018, he was a visiting researcher at the Institute of Biotechnology of the RWTH Aachen University in Germany. During this period he acquired exper- tise in state-of-the-art directed protein evolution technology. His ongoing re- search uniquely combines gene and stem cell therapy with directed protein evolution technology in order to develop next generation medicines to cure inborn errors of liver metabolism.

Bernward Garthoff

CEO
BIO Clustermanagement NRW GmbH, Germany
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- Veterinary Medicine (Hannover University, 1974)
- Doctor in Veterinary Medicine (1975)
- Board certified pharmacologist and toxicologist (1984)
- Lectureship for pharmacology (Ruhr-University of Bochum)
- Variety of major positions in research and development at Bayer AG (1976 - 2009)

Dr. Bernward Garthoff is the CEO of the BIO Clustermanagement NRW GmbH. Before, he was the Biotechnology Representative for the Federal State of North Rhine-Westphalia, Germany, and chair of the Biotech Cluster BIO.NRW. Prior to that, he held several positions in the pharmaceutical and plant protection business of Bayer in Germany, the USA and Japan. In 1994, Dr. Garthoff had joined the top management of Bayer's Crop Protection Business, managed the integration process associated with the acquisition of Aventis CropScience and was member of the Board of Management of Bayer CropScience AG. He is member of the Boards of several foundations and until recently Supervisory Board member of Rottendorf Pharmaceuticals GmbH. He has also been chairman of the German Association of Biotechnology Industries (DIB), a member of the Private Sector Committee of CGIAR (World Bank) and of the EuropaBio Board. He has been a member of ESAC, of ecopa, co-chair of epaa and of the German foundation/ platform for alternatives "set". He serves in diverse committees of the Federal Ministry of Agriculture of Germany dealing with animal welfare.

José V. Castell

Emeritus Professor
Department of Biochemistry and Molecular Biology, University of Valencia, Spain
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José V. Castell. PhD in Biochemistry and MD in Medical Biochemistry, Graduate in Pharmacy. Postdoctoral at the ETH-Zürich and Max-Planck Institut, Göttingen. Invited professor at the Biochemisches Institut de Univesität Freiburg. Emeritus Professor of the Chair of Medical Biochemistry and Molecular Biology at the Faculty of Medicine of Valencia where he also reads a postgraduate course on Molecular Toxicology and Clinical Immunology. Head of the Unit of Experimental Hepatology at the Research Institute of the University Hospital La Fe (Valencia). Ex-Director of the Medical Research Center of the University Hospital La Fe (Valencia).

In 1990, Professor Castell was given the International Award of the European Federation of Pharmaceutical Industries (Heidelberg 1990) for the development of cultured human hepatocytes to investigate the hepatotoxic risk of new drugs to man.

His major research interests include: *in vitro* drug metabolism and hepatotoxicity, drug induced liver injury in clinics, cell reprogramming to hepatocytes, cell therapy with human hepatocytes and PSC-derived hepatocytes. He has authored 410 papers, including scientific articles published in relevant international journals, book chapters and several reviews.

His current research interest involve the use of metabolomics to assess drug hepatotoxicity in the clinics and *in vitro* (drug development, metabolism and toxicity).

Former member of the Scientific Advisory Committee of the European Center for Validation of Alternative Methods (ECVAM), Scientific Committee of INVITOX, and Scientific Committee ILSI.

Prof. Castell has been actively involved in over 25 EU research projects; and has served as coordinator in 3 of them, and is presently involved in “ONTOX”.

Speakers

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Barbara Viviani

Associate Professor
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Barbara Viviani is Associate Professor of Pharmacology at the Department of Pharmacological and Biomolecular Sciences (DiSFeB), Università degli Studi di Milano. Barbara Viviani graduated in Biological Sciences and obtained a PhD in Experimental medicine, Environmental and Food toxicology.

Her research activity focuses on hazard characterisation, qualitative and/or quantitative assessment of the nature of adverse effects associated with biological and chemical agents, with a particular focus on neurotoxicity.

Barbara Viviani is tenure of courses in the field of Toxicology at the Faculty of Pharmacy, University of Milan. She collaborates with the European Food Safety Authority (i) as an external scientific expert for the execution of Scientific Opinions of the PPR and CONTAM Panels, (ii) for scientific and technical support and (iii) as project/team coordinator. She is involved in Horizon 2020 and Horizon Europe consortia including the Partnership for the Assessment of Risks from Chemicals (PARC).

Since 2020 BV is the Italian delegate to the OECD in the Advisory Group on Molecular Screening and Toxicogenomics (EAGMST, now ESCA, serving as a contribu-ting author of Developers' Handbook 2.5, contributor to the initiative on Systematic Methods in AOP development and AOP coach). BV is the promoter and coordinator of the Centre for Research on Novel Approach Methodologies (NAMs) in Chemical Risk Assessment, Università degli Studi di Milano.

Use of Adverse Outcome Pathways (AOPs) for hazard identification

Barbara Viviani

An Adverse Outcome Pathway (AOP) is a theoretical framework that describes a sequence of causally related events (Key Events, KE) leading to a toxic effect, synthesising existing data available in the literature and databases. AOPs are intended to provide a mechanistic basis to support animal and epidemiological studies and to facilitate the development of Integrated Approaches to Testing and Assessment (IATA). To be of potential predictive value for regulatory use, an AOP's constitutive event (i.e. Molecular Initiating event, MIE; KE; Adverse Outcome, AO) must focus on critical steps leading to adversity, be measurable, and be identified in biological models representative of a pathway relevant to a human disease. A key requirement for AOPs to support regulatory application is the confidence and precision with which they facilitate the extrapolation of data measured at lower levels of biological organisation to predicted outcomes at higher levels of organisation.

The AOP "Activation of uterine estrogen receptor-alfa leading to endometrial adenocarcinoma, via epigenetic modulation" is discussed as an example of a transparent, structured and reproducible approach that supports the development of an evidence-based AOP. The approach is articulated in different phases: 1. a priori definition of the problem and method to be adopted (protocol), 2. application of machine learning technique (Topic modelling) that automatically analyzes text data to identify biologically plausible MIEs/KERs independently from prototypical stressors, 3. systematic literature review and critical appraisal of prioritized evidence, taking into account human, *in vivo* and *in vitro* studies, 4. Integration of the appraised evidence by means of the AOP conceptual network according to OECD guidelines. The advantages and disadvantages of this approach are discussed, together with a recommendation for the adoption of a fit-for-purpose process for AOPs. ■

Birgit Mertens

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Birgit Mertens obtained her Master's in Pharmaceutical Sciences and PhD in Neuropharmacology from the Vrije Universiteit Brussel in Belgium. She joined Sciensano in 2010 and is currently a senior toxicologist and team leader of the Risk and Health Impact Assessment service. She coordinates and participates in multiple (inter)national research projects and activities on the genotoxicity of physical and chemical agents, focusing on food contaminants and developing and applying new approach methodologies. Within the European Partnership for the Assessment of Risks from Chemicals (PARC), she is co-leading the project on the development of an integrated approach to testing and assessment (IATA) for genotoxicity. She has published over 45 peer-reviewed scientific papers and contributed to multiple (inter)national scientific advices. She is the National Coordinator of the Test Methods Programme both at the European and OECD level and the Belgian contact point for the Preliminary Assessment of the Regulatory Relevance of Alternative Methods (PARERE) network. She is also a member of the OECD genotoxicity expert group. She participates in different (inter)national working groups and scientific committees on hazard and risk assessment and is the current president of the Belgian Environmental Mutagenesis Society. She is also a guest lecturer in the toxicology courses of different Belgian universities.

An AOP-based IATA to pave the way toward animal-free genotoxicity testing

Birgit Mertens

Genotoxicity is an important toxicological endpoint that drives the safety assessment of chemicals. Current genotoxicity assessment approaches face several limitations, including the high number of misleading positive results triggering unnecessary animal testing, the limited mechanistic information provided, and the need for more integration of new approach methodologies (NAMs) developed over the last years. One strategy to improve genotoxicity testing and include NAMs involves developing 'integrated approaches to testing and assessment (IATA)'. The selection of NAMs to be included in the IATA for genotoxicity should be structured and science-driven, a process in which adverse outcome pathways (AOPs) could play an important role.

Within the framework of the European Partnership for the Assessment of Risks from Chemicals (PARC 101057014), we will develop an AOP-based IATA to support the transition to animal-free genotoxicity assessment. Based on the AOPs found in the AOP-Wiki and literature, a first AOP network for genotoxicity has been drafted. Several Key Events (KEs) and Key Event Relationships (KERs) in this network are now being further characterized and, where possible, quantified. Afterward, suitable *in vitro* assays for measuring Molecular Initiating Events (MIEs) and KEs within the AOP network will be identified in accordance with available OECD guidance to design an IATA. NAMs detecting mechanisms not directly supported by AOPs may also be integrated into the IATA. Case studies will be performed to evaluate and further optimize the IATA. The uncertainties of the IATA will be analyzed and compared to those associated with the current genotoxicity approaches. Kinetic and Quantitative *In Vivo* to *In Vitro* Extrapolation (QIVIVE) aspects will also be considered. The final IATA will help to harmonize NAM-based genotoxicity testing and fill mechanistic data gaps in genotoxicity allowing more-informed regulatory decision-making. ■

Sónia Fraga

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Sónia Fraga is Researcher of the Department of Environmental Health at the Portuguese National Institute of Health Dr. Ricardo Jorge (INSA) and coordinates the Nanomaterials - Safety and Health group at the Laboratory for Integrative and Translational Research in Population Health (ITR), Porto, Portugal. She is also Professor of Pharmacology at the Faculty of Medicine of the University of Porto, Portugal. Her main research interests include environmental genotoxicity, chemicals' hazard assessment, and nanotoxicology. She has been involved in several national and international projects dealing with different aspects of the health and safety hazards of nanomaterials, including the occupational risks associated with manufacture of nanomaterials, establishment of nanomaterials' grouping approaches to support risk assessment and nano-bio interactions at biological barrier level.

Influence of the experimental approach in the assessment of nanomaterial hazard *in vitro* – engineered and incidental nanoparticles relevant to the ceramic industry as case study

Sónia Fraga

In the recent years, manufacture and application of nanomaterials has developed quickly and global production of new entities is expected to increase exponentially in the coming years. The broad spectrum of nanomaterials applications, and consequently the increased risk of environmental and human exposure to these materials have raised concerns about their safety and potential adverse health effects. Nanomaterials specific properties such as smaller size and larger surface area, high catalytic reactivity and distinctive physicochemical characteristics compared to their respective bulk forms offer a challenge for a reliable safety assessment using the conventional testing approaches and techniques.

Advanced ceramic technologies have a strong potential for airborne (nano)particle formation and emission, meaning that workers of those industries are at great risk of exposure to these particles. Taking as a starting point the nanoparticles intentionally used or incidentally released into the workplace air during advanced ceramic processes, practical examples will be presented to illustrate how different aspects of the experimental design (e.g. dose metrics, exposure conditions, cell type) can greatly impact the findings and conclusions of *in vitro* toxicity studies. ■

Albert Braeuning

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Prof. Dr. Albert Braeuning is head of the unit "Effect-based analytics and toxicogenomics" at the German Federal Institute for Risk Assessment since 2014, and professor for toxicology at the Charité University Hospital in Berlin. He is a biochemist and toxicologist with a strong research focus on omics technologies to study the molecular mechanisms of toxicity of food constituents and contaminants. He is active in different international research projects including different EU-funded projects (EDCMET, BoostCrop, ITN Perforce 3, SCENARIOS). He has authored >180 peer-reviewed publications with an H-index of 30.

Metabolic disruption in the liver: assays to detect hepatic triglyceride accumulation in human liver cells *in vitro*

Albert Braeuning

The focus of endocrine disruptor research has been on sex hormone-related effects for a long time. More recently, the ability of certain chemicals to alter endogenous metabolic pathways, for example related to fat or energy metabolism, has gained increasing attention. However, there is still a lack of mechanistic knowledge on metabolic endocrine disruption, as well as a lack of validated test methods to identify metabolic endocrine disruptors. Liver steatosis is a major health concern especially in Western countries, while fatty acid-related liver changes are also a frequent histological finding in rodent studies, for example caused by certain pesticidal active compounds.

Following an adverse outcome pathway (AOP)-based approach, a toolbox of *in vitro* assays covering different molecular initiating events and key events of the AOP for liver steatosis was established in HepaRG human liver cells. This toolbox includes nuclear receptor activation assays, analyses of gene and protein expression, as well as testing methods to determine cellular triglyceride accumulation as an *in vitro* surrogate for liver steatosis. The work done demonstrates the important role especially of the nuclear receptor PXR (pregnane-X-receptor) in HepaRG triglyceride accumulation and highlights the need to further improve the detailedness of existing AOPs. A novel transcriptomic signature to predict triglyceride accumulation using mRNA expression data was established.

As the most promising assay of the test battery, the AdipoRed triglyceride accumulation assay selected for further development. The experimental protocol was extensively optimized within the course of the EU-funded project EDCMET (Metabolic effects of Endocrine Disrupting Chemicals: novel testing METhods and adverse outcome pathways). Using a large panel of test compounds, a good concordance of *in vitro* triglyceride accumulation results and rodent *in vivo* data was recognized. Following preparation of a detailed standard operating procedure for the test system, it was selected by the PEPPER initiative for formal validation. ■

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Annalisa Gastaldello joined the Joint Research Centre (JRC) of the European Commission in Ispra (Italy) in September 2022 as Project Officer working on non-animal based-approaches in biomedical research. Prior to this position, she spent 13 years in the UK where she gained a PhD and Masters in Cardiovascular Science from the University of Edinburgh, and worked as a researcher in several fields of biomedicine (endocrinology, cardiovascular diseases, immuno-oncology). She has extensive lab experience with both animal and non-animal models. She also has expertise in Covid testing, organic farming and a Masters in Nutritional Science.

Fostering critical thinking about science, its methods and the 3Rs: resources from EURL-ECVAM for established researchers and the scientists of tomorrow

Annalisa Gastaldello

EURL-ECVAM at the JRC of the European Commission provides an array of resources on the 3Rs for both already established scientists and the scientists of tomorrow.

For the former, a series of studies were performed to investigate human relevant approaches used in biomedical research in seven disease areas.
In total, several thousand of articles were screened and analysed, and 3049 non-animal methods were selected to create highly curated and publicly available databases that can be exploited to explore the strengths and limitations of both animal and non-animal models used in biomedical research.

For the scientists of tomorrow, EURL ECVAM has undertaken initiatives aimed at strengthening 3Rs education for school-goers. Emphasis has been placed on the creation, testing and dissemination of teaching materials to teachers to allow them to deliver 3Rs lessons in the classroom.
The resources provided include learning scenarios for primary and secondary schools, references lists, slide decks, games and podcasts, as well as examples of career paths centred on the 3Rs in the form of podcasts and videos. Additionally, a report informing key education decision-makers on how to facilitate the incorporation of the 3Rs into their syllabus and curricula is included.

EURL ECVAM is also developing an open access virtual reality teaching resource to support 3Rs education for students aged 14-19, with the goal to educate on important technologies not requiring animals used in science.

During this talk, an overview of the abovementioned resources will be given, with the hope to create awareness and increase interest in the 3Rs in both the older and younger generations of scientists. ■

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Mieke Van Mulders obtained her Master's degree in Biomedical Sciences, with a major in Nutrition and Metabolism, from the University of Ghent (2014). Mieke started her career as a consultant in the pharmaceutical industry where she was in charge of several projects in the Departments of Regulatory Affairs and Quality Assurance.

Since 2017, Mieke is fully dedicated to the RE-Place project, a joint research collaboration between the Vrije Universiteit Brussel and Sciensano. She has been responsible for the development of the RE-Place platform and currently works on its further optimization. The RE-Place project focuses on increasing knowledge sharing on alternative methods to animal testing, also known as 'New Approach Methodologies' (NAMs) and fosters collaborations across life sciences sectors/ stakeholders.

Mieke is also involved in the activities of the Belgian 'Network for Preliminary Assessment of Regulatory Relevance (PARERE)', which provides EURL ECVAM with upstream input and preliminary views on potential regulatory relevance of methods or approaches submitted for validation and/or peer review.

Furthermore, Mieke is a steering committee member of the Belgian Society of Toxicology and Ecotoxicology (BelTox), where she improves the communications strategy with fellow steering committee members. She is also an active member of the Flemish and Brussels animal testing committees in Belgium.

Maude Everaert

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Maude Everaert studied in biomedical sciences at the University of Mons and obtained her master's degree in 2021. After graduation she worked for 6 months in clinical trials and phage therapy at the military hospital in Brussels. In May 2022, Maude started to work as a scientist on the RE-Place project. This project aims to collect all available Belgian expertise on alternative to animal testing in one central database. Her main tasks include contacting researchers specifically in the Brussels region, taking care of the social media and supporting the RE-Place team in updating the website and maintaining the database.

RE-Place: a bottom-up approach to collect the existing expertise on NAMs in Belgium

Mieke Van Mulders, Maude Everaert, Birgit Mertens, Vera Rogiers

Animal experimentation is tracked in Europe through the publication of non-technical summaries and annual statistics. In contrast, there is no clear overview on the use of alternative methods, also known as 'New Approach Methodologies' (NAMs). Several knowledge resources are available to search for NAMs, including the 'Inventory of 3Rs Knowledge Sources' and the 'DataBase service on ALternative Methods' from the European Commission. Still, these do not provide insights into the actual use of NAMs.

In Belgium, the RE-Place project was launched as a bottom-up strategy to map all available expertise on the use of NAMs in one central, open-access database. This database, available via www.re-place.be, provides an overview of the existing ▶

NAMs in Belgium and links them with the names of experts and institutes where the methods were developed and/or are applied. By providing a direct point of contact, networking opportunities are greatly facilitated between stakeholders (scientists, ethical committees, regulators, competent authorities …). This unique approach is an important added value compared to other databases.

Since its launch in 2017, 243 methods have been collected in the database covering a wide array of topics in biomedical research, regulatory testing and education. These methods have been submitted by 166 experts from 29 institutes. Although many efforts have been taken to expand the content of the RE-Place database, convincing scientists to submit their methods remains the major challenge in this project. Several incentives have been created to stimulate contribution including, amongst others, awards, publication in an international journal, active dissemination of information on NAMs from the RE-Place database via the website, social media channels and newsletter, and the set-up of opportunities to present ongoing research during study days, symposia or webinars.

By centralizing the existing knowledge on NAMs and providing the names of experts, the RE-Place database can be used as a practical tool by all stakeholders. Moreover, it helps to facilitate the implementation of new methods, models and technologies, thereby supporting the replacement of animal testing in the long term. ■

Notes

Fred Vermolen

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Prof.dr.ir. F.J. (Fred) Vermolen is a Full Professor in Computational Mathematics at the University of Hasselt in Belgium. He obtained his PhD degree from the TU Delft in 1998. Thereafter he worked at CWI and from 2000 he joined the TU Delft as an assistant professor in the section Numerical Analysis. His research is related to analysis, numerical methods and uncertainty quantification for partial differential equations. He has given courses in numerical analysis for more than 10 years.

Mathematics as an alternative for animal experiments in burn injuries

Fred Vermolen

Burn injuries can have a devastating impact on the lives of patients, since burns may cause hypertrophic scars or problematic contraction of skin. This contraction may even lead to disability of the patient, who is no longer able to move his or her joints. In order to be able to optimize therapies that are administered to patients, it is crucially important to understand the underlying biological mechanisms. For this reason, animal experiments are carried out in many groups all over the globe. In all these studies, it is evident that animals are used as a model for the behaviour of human skin, and that it is questionable whether animals provide an accurate model for humans. In this talk, some mathematical frameworks in terms of partial differential equations and random processes will be presented in an accessible manner. These partial differential equations are based on physical concepts like conservation of mass, momentum and evolution of strain. The random (stochastic) processes are motivated via cellular processes like cell differentiation, death and proliferation (division). This generates an *in silico* (computational) model for the behaviour of post-burn human skin. During this talk, merely the implications of the model, rather than the underlying mathematics, will be discussed and their use in the clinic and as an alternative for animal experiments. Furthermore, the use of machine learning solutions will be discussed as a means to make it possible to use mathematical modelling in clinical practice. ■

Emmeric Tanghe

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Emmeric Tanghe received the M. Sc. degree in Electrical Engineering from Ghent University (Ghent, Belgium) in 2005 and the Ph. D. degree in electrical engineering from the same institution in 2011. From September 2005 until May 2011, he was a research assistant with the Department of Information Technology at Ghent University (imec-UGent/INTEC). His scientific work focused on the modeling of indoor and outdoor radio propagation through field measurements. Since May 2011, he has been a postdoctoral researcher at the same institution and continues his work in radio propagation modeling. Since October 2015, he is a part-time professor in medical applications of electromagnetic fields in and around the human body. In this function, he focuses on computational biophysical modeling of brain networks. These models are used for *in silico* optimization of (non-invasive) neurostimulation therapies for neurological diseases.

Towards a Hippocampal Formation Model of Temporal Lobe Epilepsy

Emmeric Tanghe

Temporal lobe epilepsy (TLE) is the most common epilepsy in adults and the most difficult to treat with antiepileptic drugs. In TLE, seizures are generated by the cortico-hippocampal circuit. Neurostimulation techniques are widely investigated as alternative to hippocampal resection. Our project adopts the computational neuroscience new approach methodology. A conductance-based hippocampal formation model of TLE is developed. This *in silico* model is to be used as a tool to gain more insights in the mechanisms of various stimulation techniques, e.g., optogenetics or deep brain stimulation, which will contribute to the design of better treatments.

The model of the hippocampal formation is constructed in NetPyNE, i.e., an open-source Python package to facilitate neuronal network simulations using the NEURON simulator. The model of Cutsuridis et al. (2015) provides the basis. It is a network of three hippocampal subregions, i.e., the dentate gyrus (DG), CA3 and CA1, that are biophysically represented by 100 multicompartmental neurons of each region's principal cell type (granule cell, and CA3 and CA1 pyramidal cell, respectively). It includes a total 14 interneurons of 6 different types: 2x mossy cell (DG), hilar perforant path associated cell (DG), 2x basket cell (DG, CA3 and CA1), axo-axonic cell (CA3, CA1), bistratified cell (CA1), oriens lacunosum-moleculare cell (CA3, CA1). Simplified morphologies are used to reduce the number of compartments and thus the computational burden. The CA1 pyramidal cell is substituted by a model created by Tomko et al. (2021). The cells are placed according to the hippocampal formation's anatomy. The axon is not modeled. Therefore, synaptic delays are imposed which are made distance dependent. Their gains are drawn from normal distributions. The extracellular potentials are calculated with the line source approximation in an Ohmic medium with conductivity = 0.3 mS/mm. The connections between subregions are validated by comparing I/O curves with experimental data of paired pulse simulations. The pathological changes observed in mesial TLE are included to make the model epileptic. Mossy fiber sprouting is modeled by adding recurrent connections between the DG granule cells, resulting in hyperactivity. This spreads to the other regions when hippocampal sclerosis is included, modeled by reducing GABAergic gains. ■

Ursula Smole

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Ursula Smole is a postdoctoral researcher based at the Inflammation Research Centre of the Vlaams Institute of Biotechnology in Gent. She is currently working together with Prof. Dr. Bart Lambrecht on the establishment of murine and human lung organoids. She received her PhD from the University in Vienna and has been training as a postdoctoral fellow at the Johns Hopkins Bloomberg School of Public Health and the Medical University of Vienna.

Using lung organoids to study the role of the chitinase-like protein Ym2 in allergic asthma

Ursula Smole, Hamida Hammad, Bart Lambrecht

Allergic asthma is a chronic inflammatory disorder of the airways in response to inhaled allergens. The airway epithelium plays a critical role in the development, progression, and exacerbation of the disease. We here employ airway epithelial cell (AEC)-derived organoids in functional assays to assess their role on development of Th2-high pathologies. AEC organoids can recapitulate lung structure and function ex vivo while being amenable to experimental manipulation and are hence a new and exciting model system to investigate lung biology. ■

Bella B. Manshian

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The group of Bella Manshian (PhD), part of the Translational Cell and Tissue Research Unit, works on translational nanomedicine using advanced 3D precision cut tissue models and engineered nanoformulations for drug delivery. They also collaborate closely with the NanoHealth and Optical Imaging Group of Stefaan J. Soenen (PhD), focusing on non-invasive monitoring of nanoparticle distribution in preclinical model systems.

Optimized High-Throughput Screening of 3D Precision-Cut Tissue Slicesa

Ara Sargsian, Gianluca Matteoli, Bo-Jun Ke, Saeed Abdurahiman, Bart Ghesquière, Stefaan J. Soenen, Bella B. Manshian

Development of sound disease models has been a continuous effort with, till date, often suboptimal outcomes due to imperfect mimicking of the complex human body environment. Additionally, increasing efforts have been made to reduce the number of laboratory animals, in spirit of the 3Rs principle (Refinement, Reduction and Replacement). Therefore, Precision-Cut Tissue Slices (PCTS) have gained particular interest as three-dimensional (3D) organotypic ex vivo models. PCTSs are clearly defined tissue sections of uniform thickness, generated from a single organ, either human or animal. PCTSs retain the anatomical architecture of the organ, in addition to organ-specific features such as metabolic activity and tissue homeostasis. In contrast to some other 3D systems, cell types are present at the same ratios and with the same cell-cell and cell-matrix interactions as *in vivo*.

Despite the successful generation of these models, an in-depth comparison with the *in vivo* equivalent is currently lacking. Therefore, we have generated PCTS from an orthotopic lung cancer model in mice and validated the ex vivo model with the equivalent *in vivo* model. Comparison was performed using high throughput screening methods. Furthermore, in our work we were able to culture various organ specific PCTSs for an extended amount of time, thereby offering a long-term model, closely mimicking the organ which they are derived from. These were confirmed through PCR screening and histological analysis of the tissue slices. Culture conditions were further optimised following metabolomics studies. Given the high-level retainment of tissue properties, we were able to use some patient derived PCTS to confirm the therapeutic effect of two drug formulations. Therefore, we trust that PCTSs can strongly support the 3Rs principles and bridge the gap in translation of preclinical research into the clinic. ■

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Eva Blondeel graduated in 2019 as a Biomedical scientist at Ghent University. She continued as a doctoral fellow in the Laboratory of Experimental Cancer Research Ghent under supervision of Prof. Dr. Olivier De Wever. In 2020 she obtained a Research Foundation Flanders (FWO) aspirant fellowship. During her PhD she focusses on three-dimensional tumour modelling. More particularly, in her first two years she worked on MISpheroID, which is a knowledgebase and transparency tool for minimum information in spheroid research (https://mispheroid.org/). The MISpheroID paper was published in Nature Methods in November 2021. Currently, she is working on multiple projects around standardization, assay optimization, resource development and tumour biology *in spheroid* and *ex vivo* patient-derived tissue fragment models.

MISpherID: A knowledgebase and transparency tool for Minimum Information in Spheroid IDentity

Eva Blondeel

Spheroids are three-dimensional (3D) cellular models that show widespread application across academic and industrial research groups. These 3D cultures provide more physically relevant and predictive data that overcome the correlation mismatch between the preclinical and clinical situation. The MISpheroID consortium (Blondeel, E.; Peirsman, A.; MISpheroID consortium; De Wever, O. (2021) MISpheroID: a knowledgebase and transparency tool for minimum information in spheroid identity. Nat. Methods 18, 1294–1303) recently presented a crowdsourcing knowledgebase (mispheroid.org) that assembles experimental parameters of more than 3,000 published spheroid-related experiments. Interrogation of the knowledgebase revealed heterogeneity and lack of transparency in the methodological setup of spheroids. The consortium identified 4 parameters to embody minimum information about a spheroid which we defined as the MISpheroID string; cell line, culture medium, formation method and size. Empirical evaluation and interlaboratory validation of variations in each of these methodological parameters identified a striking impact on a diverse set of spheroid metrics: RNA fingerprints, presence of cell death, ATP content, ratio of lactate secretion to glucose uptake, secreted protein signatures, circularity, size and cancer therapy response. These results strongly suggest that reporting of these 4 essential experimental parameters is an absolute prerequisite to interpret and reproduce a spheroid experiment, with a likely beneficial use for other emerging 3D model systems as well. In conclusion, we aim to advance 3D biology in both an academic and industry environment by removing barriers of inconsistency and unawareness. ■

Link of methodology: https://www.re-place.be/method/mispheroid-knowledgebase-and-transparency-tool-minimum-information-spheroid-identity

Oddvar Myhre

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Dr Oddvar Myhre is a neurotoxicologist with 25 yrs of experience within *in vitro* and *in vivo* toxicology and as a Study Director from pharmaceutical industry with special interest in developmental neurotoxicology, AOP development and over the recent years *in vitro* DNT NAMs development. He is also scientific advisor for scientific bodies like the Norwegian Environment Agency and the European Chemical Agency. He also has active roles in several ongoing EU funded projects within developmental neurotoxicology like ONTOX and PARC, and in EFSA projects.

Introduction to life stage-dependent neurotoxicity, including current status of AOPs

Oddvar Myhre, Eliska Kuchovska, Tia Heikkinen, Malene Lislien, Nicola M. Smith, Marcin W. Wojewodzic, Birgitte Lindeman, Nur Duale, Ellen Fritsche, Antonios K. Stratidakis, Dimosthenis A. Sarigiannis, Barbara Viviani, Karine Audouze, Hubert Dirven

Experimental and epidemiological studies suggest possible long-term neurodevelopmental and neurotoxic effects in adults arising from repeated low-level exposure to environmental chemicals. Because neurodevelopmental disorders, such as attention-deficit hyperactivity or autism spectrum disorders cannot be fully explained by genetic risk factors alone, understanding the possible hazard of chemical exposures is critical. Animal experiments with rats are currently the gold standard in developmental neurotoxicity (DNT) testing. These are specified in the OECD test guidelines 426 or 443 with the DNT cohort included. However, the current regulatory DNT animal guidelines are not sensitive and specific enough to identify hazards. Adult neurotoxicity hazard is of particular interest for the regulatory risk assessment of pesticides being part of the EU legislation data requirement. Meta-analyses suggest an association between pesticide exposure and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis. Epidemiological studies, while not proving causality, raise concerns and questions about the adequacy of *in vivo* regulatory studies to provide information on complex human health outcomes. Human-based *in vitro* New Approach Methods (NAMs), combined with exposure assessment models and the Adverse Outcome Pathway (AOP) framework, represent the strategy to date to address these limitations and develop Next Generation Risk Assessment (NGRA). A vision of the ONTOX and PARC projects is to develop NGRA of chemicals to protect human health. Experimental mechanistic data from tailored NAMs data using human iPSC-derived cell lines will be presented, together with data from PARC summarizing the AOPwiki inventory of AOPs for DNT and ANT. We will suggest classification of an AOP network created by mapping AOPs related to (D)NT adverse outcomes using the 11th International Classification Diseases system (ICD-11) provided by the World Health Organization. Altogether, this will increase mechanistic understanding of human neurological and neurodevelopmental disorders and advance NGRA without the use of animals. ■

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Ellen Fritsche is PI at the IUF – Leibniz Research Institute for Environmental Medicine and appointed University Professor for Environmental Toxicology at the Heinrich-Heine University in Duesseldorf, Germany. She did a postdoc at the National Institute for Environmental Health Sciences at Research Triangle Park in North Carolina, USA and habilitated in 2008 on the role of the aryl hydrocarbon receptor in skin. She has been collaborating with international agencies like the European Food Safety Authority and the US-Environmental Protection Agency for many years with the goal of advancing alternative methods for developmental neurotoxicity testing for regulatory application.

The *in vitro* test battery for developmental neurotoxicity assessment towards regulatory acceptance (DNT IVB)

Ellen Fritsche

For developmental neurotoxicity (DNT) evaluation, *in vitro* methods allow a more efficient testing for hazard identification compared to traditional animal experiments. Recently, a DNT-*in vitro* battery (DNT IVB) has been compiled under international regulatory guidance, which is the sum of its single assays and represents a large variety of neurodevelopmental key events like human neural progenitor cell (hNPC) proliferation, their neural differentiation, migration and maturation. An OECD DNT IVB guidance document is being composed that should facilitate IVB interpretation.

To gain confidence in the DNT IVB, we pursued four strategies with the EU part of the DNT IVB. First, within the European H2020 project ONTOX we are generating physiological maps for brain development. These maps are then compared to the current DTN IVB. Secondly, we employed molecules altering signalling pathways known to be crucial in brain development/human neurodevelopmental diseases. Results from both efforts inform on IVB gaps. Third, we calculated battery performance based on chemical testing of a reference set of 45 positive and negative DNT compounds. Last, we strengthen the relevance of IVB hits by assembling transcriptome-supported DNT adverse outcome pathways (AOPs).

In summary, an international effort led to the first generation DNT IVB. Gap and strength analyses reveal areas where more scientific input is needed. The collective work of the scientists involved will increase confidence in the battery and help decrease uncertainty for its regulatory application. ■

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Eliska Kuchovska graduated with a double Ph.D. degree from the University of Bordeaux (France, EPOC) and Masaryk University (Czech Republic, RECETOX) in environmental health sciences focusing on the developmental toxicity towards aquatic non-target organisms. She received the Vice-Chancellor's Award for Excellence in Doctoral Studies. After her studies she specialized in developmental neurotoxicology (DNT) at the Leibniz Research Institute for Environmental Medicine in Düsseldorf (Germany), where she is currently employed as a postdoctoral researcher. She is striving to develop and promote new approach methodologies for DNT in the framework of the European H2020 ONTOX project. She is an enthusiastic early-career researcher often engaging in science communication activities to raise public awareness about toxicology and risk assessment.

Neurodevelopmental toxicity in the European H2020 ONTOX project

Eliška Kuchovská, Kristina Bartmann, Luiz Carlos Maia Ladeira, Arif Dönmez, Lynn-Christin Saborowski, Nicolai Goerts, Denis Polozij, Farina Bendt, Mats Schade, Georgea Raad, Raphaëlle Lesage, Alessio Gamba, Bernard Staumont, Job H. Berkhout, Aldert Piersma, Juliette Legler, Harm J. Heusinkveld, Malene Lislien, Graciela Lopez Soop, Tim Hofer, Oddvar Myhre, Hubert Dirven, Liesbet Geris, Ellen Fritsche

The current regulatory developmental neurotoxicology (DNT) guidelines are not fit-for-purpose for the hazard assessment of the immense chemical universe. They require using *in vivo* tests that are burdened with ethical, practical (time & cost), and scientific (limited predictivity for human health) issues. Thus, more reliable and efficient human-based new approach methodologies (NAMs) are needed.

The ONTOX project aims to develop such NAMs, combining *in vitro* and *in silico* approaches, to predict repeated-dose DNT effects of chemicals. The core of the NAM is an AI-driven ontology, a framework qualitatively and quantitatively integrating state-of-the-art DNT knowledge. As the first pillar of the ontology, we created a physiological map (PM) of the developing human brain. The PM web interface interactively displays the neurodevelopmental processes and physiological mechanisms necessary for healthy brain development. Moreover, a plugin was designed to show relevant neurodevelopmental disorders on the map. The PM also serves to identify gaps in existing DNT *in vitro* methods and enables the derivation of new adverse outcome pathways (AOPs). Furthermore, the PM is used as a scaffold for the generation of an *in silico* model of the neural tube closure to be used for toxicological predictions. The second pillar of the DNT ontology is an AOP network, created by mapping twelve AOPs related to decreased cognition in children after prenatal exposure to DNT chemicals. The ontology is linked with tailored *in vitro* battery (IVB) using human primary and iPSC-derived cell lines (2D and 3D). This IVB is being intensively characterized to support the regulatory acceptance of the DNT *in vitro* NAMs by increasing its understanding and demonstrating the similarity between the physiology of the test system and human biology.

This approach will, together with exposure assessment, advance human risk assessment in line with the next-generation risk assessment principles and without the use of animals. ■

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Dr. Ira Espuny-Camacho has a long standing track record in the fields of human stem cells, human brain development and neurodegeneration, and particularly in the field of Alzheimer's disease. I. Espuny- Camacho obtained her Ph.D in Biomedical Sciences from the KU-Leuven, Belgium in 2007. During her doctoral work, in the laboratory of Bart De Strooper, she focused on the search of new therapeutic targets to treat Alzheimer's disease. In 2008, she moved to the lab of Pierre Vanderhaeghen, at ULB, Brussels. During this postdoctoral scientific experience she pioneered the differentiation of human cortical neurons from pluripotent stem cells and its application to study brain disease and repair. As a second major achievement she generated a novel *in vivo* human chimeric model to study Alzheimer's disease. In 2017 she was awarded a Marie Curie MSCA-IF to focus on the generation of hPSC-derived brain organoids to study Huntington's disease in the University of Milan, Italy. In September 2019 she returned to Belgium to start her own group as Associate Professor at the University of Liège working on hPSC-derived brain organoid models to understand brain disease. She has been awarded twice the SAO- Standard fellowship and the Young-Researcher Prize from the Alzheimer Association, Belgium to unravel early AD mechanisms of disease and to study the role of microglia cells in the first steps leading to Sporadic Alzheimer's disease. Further, she has recently been awarded a consortium based grant ARC, from the Federation Wallonie-Bruxelles region (FWB) to study in close contact with the clinics the effects of hypothalamus-cortex regulation on circadian rhythms and susceptibility to disease. Currently her team is composed of several Ph.D students, a postdoc and Master thesis students working on various projects aiming at understanding human brain maturation and degenerative diseases.

Decoding intrinsic features of human brain maturation and disease using human brain organoids

Ira Espuny-Camacho

The maturation of the human brain shows species-specific differences of neoteny when compared to lower mammals. This process encompass a time window that expands from late embryonic stages to early adolescence. Major features of brain maturation are the acquisition of phenotypic complex traits such as axonal and dendritic trees and the presence of dendritic spines which correlate with higher functionality and connectivity of the neurons. Interestingly, human transcriptomic data has shown a narrow time window from the birth of the individual to the first two years of life where major transcriptomic changes occur. Among those changes, there is an striking accumulation of alternative splicing events such as the splicing of the MAPT gene (TAU), whose role is essential in several neurodegenerative conditions such as Alzheimer's disease.

Here, we address human-specific species features of brain maturation using a multicellular human *in vitro* brain organoid model composed of neurons and glia cell types. Long-term human brain organoids are analysed functionally for the acquisition of mature neuronal phenotypes to understand the time line of maturation compared to the *in vivo* situation. Further, we show that long-term human brain organoids can recapitulate hallmarks of AD such as high levels of Tau phosphorylation and degeneration *in vitro*. ■

Terje Svingen

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Terje Svingen is Head of Research group for Molecular & Reproductive Toxicology at the National Food Institute, Technical University of Denmark (DTU Food). His research focuses on how early-life exposure to chemical substances can disrupt normal development and cause disease later in life. With early training as a developmental biologist focussing on gonadal sex determination and sexual differentiation, Dr Svingen now applies this background to elucidate the molecular and cellular underpinnings on toxic effects in mammals, with special focus on reproductive toxicity. His research groups applies a broad pallet of methods and approaches in their research, from *in vitro* assays to animal toxicity studies. His group also provides expert advice to national and international agencies on endocrine disrupting chemicals.

Effects of endocrine disruption on the developing male reproductive system: animal versus non-animal data

Terje Svingen

Male reproductive development is dependent on spatiotemporally regulated hormone signalling. Gonadal sex determination, which specifies the differentiation of functional testicles is largely driven by genetic cues, whereas development of other male reproductive organs and general sex characteristics hinges largely on a surge in androgens during the masculinization programming window, albeit with several other signalling pathways also playing important roles. With this hormonal dependence, male reproductive development is sensitive to endocrine disruption at critical life stages, not least to chemical substances exerting anti-androgenic or estrogenic effects. Although we have good knowledge about how male sexual development is regulated by, for instance, endocrine signalling, it remains challenging to predict *in vivo* effect outcomes from non-animal experimental data. Some of these challenges are difficult to overcome due to the share complexity of the biological system we try to recapitulate in artificial or reductionists testing systems, whilst other challenges may be more to poor design of test methods relative to effect outcomes. With renewed focus on new approach methodologies (NAMs) and increased knowledge about interactions between man-made endocrine disrupting chemicals and biomolecules, or biological systems, there are real opportunities to improve on current testing regimens and gradually reduce our dependence on *in vivo* toxicity studies for chemical testing and hazard identification/risk assessment. ■

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Dr. Yoni Baert graduated in 2016 with a PhD dissertation on fertility preservation in male cancer patients. He then specialised further in bioengineered systems for *in vitro* spermatogenesis and performed research at the internationally renowned Karolinska Institute as well as the Monash University. Dr. Baert is the former president of the Network of Young Researchers in Andrology and works currently as an assistant professor and postdoctoral researcher at the Vrije Universiteit Brussel with affiliation to the *In Vitro* Toxicology & Dermato-Cosmetology and Biology of the Testis laboratories.

Development of a representative, reliable and reproducible *in vitro* model for (human) testis

Yoni Baert

Diseases, chemicals, and genetic alterations that effectuate male infertility remain largely understudied. A comprehensive physiomimetic *in vitro* testis model is long overdue and highly needed to better understand the causes of male infertility and find effective treatments.

Nowadays, the prevailing method for *in vitro* spermatogenesis in mammals typically involves the use of animal organ culture systems. Despite its successes, an important shortcoming of this approach is the entrapment of cells within the boundaries of the tissue fragment, making them inaccessible for specific manipulations and turning organ cultures inefficient for exploratory studies. Moreover, the translation of the system to humans is hampered by the limited access to human donor tissue. In contrast, the testicular organoid (TO) culture system is tailorable and scalable, and it requires low cell input. TOs are therefore an excellent male fertility R&D tool.

Although the TO research area is still relatively new, it evolves at a fast pace. Given the recent achievements in TO cultures related to recapitulation of testicular architecture, synthesis of testicular hormones, and spermatogenesis, advanced (human) testis physiomimicry *in vitro* seems to be only a matter of time. ■

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Dr. Thomas Rawlings is a National Centre for the 3Rs (NC3Rs) funded Post Doctoral Research Fellow at Warwick Medical school, University of Warwick, in the Professor Andrew Blanks Lab. Previously I was awarded a Warwick-Wellcome Translational Research Fellowship under the supervision of Professor Jan Brosens. Previously, I completed a PhD in Interdisciplinary Biomedical Research, funded by the Warwick Medical School Medical Research Council Doctoral Training Partnership. My current research aims to develop complex 3D cell culture models of the endometrium and myometrium, such as the endometrial assembloids, to study implantation, and to investigate pharmacological strategies to rebalance the aberrant subpopulations seen in recurrent miscarriage endometrium.

Implantation (female) models including endometrial organoids and assembloids

Thomas Rawlings

The mid-secretory phase endometrium undergoes dynamic changes known as decidual remodelling. This process involves a brief optimal period for embryo implantation, followed by either breakdown and menstruation or transformation into a strong and stable matrix capable of supporting the fetus throughout pregnancy. To investigate the underlying mechanisms of these processes, we developed the endometrial assembloids. These assembloids consisted of primary stromal cells and gland organoids. Through single-cell transcriptomics, we observed that the decidualized assembloids closely resemble the mid-secretory phase of the endometrium. They contain different subpopulations of both differentiated and senescent cells in the glands and stromal cells. Our study revealed that acute senescence in the glandular epithelium triggers the secretion of various factors crucial for implantation. It also influences the emergence of anti-inflammatory decidual stromal cells and pro-inflammatory senescent decidual cells. By inhibiting stress responses in pre-decidual cells using pharmacological methods, we prevented the emergence of senescent decidual cells. In co-culture experiments, the absence of senescent decidual cells resulted in the entrapment of collapsed human blastocysts in a sturdy, non-dynamic decidual matrix. Conversely, in the presence of senescent decidual cells, a dynamic environment conducive to implantation was created, allowing space for embryo expansion and attachment. However, if senescent decidual cells persisted, the structural integrity of the assembloid gradually declined. Our findings highlight the significant role of decidual senescence in controlling the fate of the endometrium during implantation. Furthermore, they emphasize the potential of endometrial assembloids for accelerating the discovery of novel treatments to prevent reproductive failure. ■

Karen Sermon

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Karen Sermon trained as an MD and received her PhD in medical sciences at the VUB. As an FWO postdoctoral fellow her main interest was on preimplantation genetic diagnosis for monogenic diseases, which she helped develop at the UZ Brussel. Later her interests have veered towards human embryonic stem cells and especially those stem cells that carry a monogenic disease and can be used as disease models. Her group have contributed to the stem cell field with several papers in high impact factor journals. Recently, she also researched the origin of chromosomal abnormalities in IVF embryos, resulting in several papers on the topic. She was also appointed coordinator of the ESTEEM study by ESHRE. She is currently the chair-elect of ESHRE.

Stem cells and embryo development

Karen Sermon

Since the advent of *in vitro* fertilisation (IVF) and the birth of the first IVF baby in 1978, researchers have been able to study the first days of development *in vitro*. This is not without difficulties however, since using human embryos for research – and a fortiori creating them for research – has always been ethically burdened. Moreover, the study material available has always been determined by what remains after the patients complete their treatment, and therefore is often insufficient in both quantity and quality.

Around the turn of the century, embryo research made it possible to obtain human embryonic stem cells, which are derived from the inner cell mass of 5-day old preimplantation embryos, which will give rise to the embryo proper. These stem cell lines remain in an undifferentiated state indeterminately, but retain the potential to differentiate into derivatives of the three embryonic germ layers. They therefore represent great hopes for research into developmental biology and regenerative medicine, as well as drug discovery and drug testing.

More recently, we have come full circle with the differentiation of hESC into embryo-like spherical structures that recapitulate preimplantation development at day 7. This has led to a race between a handful of international groups to push the limit of synthetic embryo culture even further, to day 14, which includes implantation, gastrulation and emergence of primordial germ cells. While these are important breakthroughs, there are still no other models available for the first 5 days of development besides animal models.

I will discuss these developments, and how they will help to model a window of development that has remained a black box, leading to a better understanding of developmental disorders, early miscarriage and infertility. I will also touch upon the importance of a legal framework such as in Belgium to allow these important developments. ■

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The GENOMARK transcriptomic biomarker: a new approach methodology for qualitative and quantitative genotoxicity assessment of chemicals

A. Thienpont, E. Cho, A. Williams, M. J. Meier, C. L. Yauk, S. Verhulst, V. Rogiers, T. Vanhaecke and B. Mertens

Genotoxicity assessment is a critical endpoint in the development and safety evaluation of chemicals. The traditional *in vitro* genotoxicity tests demonstrate high sensitivity for hazard identification. However, they are relatively low throughput, show a low specificity and provide limited mechanistic information. New approach methodologies (NAMs) that generate quantitative data are needed to modernize genotoxicity assessment beyond hazard identification and reduce reliance on experimental animals. An interesting group of NAMs are transcriptomic signatures, or biomarkers, consisting of a subset of genes that robustly and consistently respond to chemicals with specific mode of actions. Previously, we developed a transcriptomic biomarker, GENOMARK, consisting of 84 genes to identify genotoxic substances in metabolically competent human HepaRG™ cells. The biomarker shows a very high prediction performance to classify genotoxicants based on gene expression data collected with microarray or RT-qPCR. Here, we demonstrate how GENOMARK gene expression data can be used quantitatively, i.e. for potency ranking of genotoxic chemicals. First, the cross-platform applicability of GENOMARK was investigated using TempO-Seq®, a high-throughput technique to generate gene expression data. To this extent, HepaRGTM cells were exposed to 10 chemicals, including 8 known *in vivo* genotoxic and 2 *in vivo* non-genotoxic chemicals, in an adequate concentration range and gene expression data for the 84 biomarker genes were extracted from the transcriptomics dataset. Hazard classification for genotoxicity were obtained with the GENOMARK prediction models. Potency ranking of the chemicals predicted as genotoxic was performed using benchmark dose (BMD) modeling in BMDExpress with a benchmark response value of 1 standard deviation. All 10 chemicals were correctly classified, confirming the applicability of GENOMARK on data generated with the high-throughput platform TempO-Seq®. Quantitative BMD modelling allowed to rank the 8 genotoxic chemicals according to their potency. Overall, this work highlights the potential of GENOMARK to facilitate a rapid and efficient human-relevant identification of genotoxicants while also providing information on potency. ■

A human bronchial epithelial spheroid model to study respiratory toxicity *in vitro*

T. Celis, D. M.A. Bullens, P. H.M. Hoet, M. Ghosh

Over the years, the use of laboratory animals in scientific research has garnered ethical, economic and scientific concerns. As a result, several fields of research have aimed to gradually shift towards the use of validated alternatives such as *in silico* or high throughput cell-based methods. 2D monolayer cell cultures are the most used cell-based method as they are simple in use and can be utilized to assess numerous endpoints, via well documented and optimized assays. However, it is known that the 2D culturing of cells influences the expression of genes and cell-cell interactions. Therefore, translatability of the obtained results to the human response is questionable, especially when assessing the exposure to a drug or toxicant. Alternatively, 3D cultures can better recapitulate the physiological and morphological features present *in vivo*, and therefore, obtained results are believed to be more predictable for human outcomes. Nonetheless, the use of 3D cultures for toxicity assessment is limited and no guidelines or standard operating procedures exist for use in regulatory toxicity assessment. To this extent, we generated a 3D spheroid model comprised of human bronchial epithelial cells, and optimized the use of this model to assess several key endpoints, commonly assessed in the field of inhalation toxicology. Stable spheroids were generated by culturing 16HBE cells as a hanging-drop for 72 hours. Characterization of the spheroids, showed an increased expression of CLDN1 as well as signs of G0/G1 arrest, when compared to 2D monolayer cultured cells. Furthermore, spheroids were successfully utilized to assess genotoxicity, cytotoxicity, cell-cycle analysis, mRNA expression, the apoptotic/necrotic cell populations, and oxidative stress after exposure to known cytotoxic and genotoxic compounds. Eventually, these bronchial

epithelial spheroids can aid in bridging the gap between *in vivo* and *in vitro*, and accelerate the future use of 3D cell cultures for regulatory toxicity assessment. ■

Receptor ontology and the importance of glutamatergic subunit composition in the assessment of developmental neurotoxicity: focus on the GluN2B/GluN2A switch in primary versus hIPSCs-derived neurons

M. Serafini

Neurotoxicology requires methods to assess the function of neuronal receptors and to evaluate compounds that have the potential to interfere with receptor signaling. Receptor composition varies during normal brain development according to specific patterns. For example, GABAAR subunits α2, α3, α5, and β3 are dominant during embryonic development of the rat central nervous system (CNS), whereas subunits α1, β2, and δ increase after birth. Moreover, differences in subunit combinations may affect GABAergic synapses (Kim et al., 2015). Concerning glutamatergic ionotropic NMDARs, GluN2A expression increases while GluN2B decreases during postnatal development, resulting in a reversal ratio between the two subunits called the GluN2B/GluN2A switch (Colantuoni et al., 2011; Kang et al., 2011). NMDARs are critical for neuronal communication, maturation of neuronal circuits, and each receptor subtype has distinct biophysical and pharmacological properties (Vicini et al., 1998; Paoletti et al., 2013). New non-animal approaches are gaining ground, taking advantage of recent discoveries in the field of stem cell reprogramming. Several human-based systems derived from human induced pluripotent stem cells (hIPSCs) have been developed (Logan et al., 2019) and combined in an *in vitro* battery (IVB) of assays that allow the assessment of different outcomes, i.e., neuronal differentiation, neurite outgrowth (Masjosthusmann et al., 2020). However, primary rodent neurons, are still considered the gold standard for studies at the synaptic level, i.e., synaptogenesis, and neuronal network formation, enough to be included in the IVB despite being animal-derived (Masjosthusmann et al., 2020). Our data obtained in rat primary neurons shows the ability of different molecules (i.e., IL-1β, lead, ethinylestradiol) to alter NMDAR expression by acting on specific subunits and affecting the GluN2B/GluN2A switch. Characterization of hIPSCs-derived models in terms of receptor subunit expression using specific rather than panel antibodies is thus of primary importance given the fine effects that neurotoxicants can exert. In keeping with the 3Rs, we also characterized the glutamatergic receptor profile in hIPSCs-derived neurons along different differentiation stages. ■

Acknoledgement

This work was supported by "Twinning towards excellence in alternative methods for toxicity assessment (TWINALT)" EU project (H2020/952404/2020).

Perioperative care for complex abdominal surgery in a novel mouse model

L. Onghena, A. Heldens, L. Devisscher, A. Geerts, Y. van Nieuwenhove, S. Lefere

Background

Bariatric surgery (BS) is an effective therapeutic option involving drastic changes in the gastrointestinal anatomy and gut hormone signalling. While BS mouse models can offer crucial pathophysiological insights, there is a paucity of technical information regarding protocols, outcomes, and perioperative care. This study aims to provide consistency in technique and perioperative care for diet-induced obesity (DIO) mice, in order to minimalize mortality.

Methods

We designed a mouse model care protocol with novel BS at the University Hospital in Ghent, and performed vertical sleeve gastrectomy (VSG), vertical sleeve plication (VSP), one-anastomosis gastric bypass (OAGB) and Roux-en-Y gastric bypass

(RYGB) in C57BL/6 male mice. DIO mice were fed Western diet (rich in saturated fat, sucrose and cholesterol, Teklad® custom diet) from 8 to 20 weeks of age, consequently receiving surgery.

Results

Mice received liquid diet (Nutrison® Protein Plus Multi Fibre 1.28 kcal/ml) pre- and postoperative, after adapted acclimatization (Table 1). A medication stock solution was designed for pre-, peri- and postoperative care with buprenorphine hydrochloride 0.1 mg/kg, meloxicam 1 mg/kg, enrofloxacin 10 mg/kg and metoclopramide 1 mg/kg. Peri-operative mice were kept on a heating pad and the peritoneum was wetted with physiological saline (heated to 38°C) every 15 minutes. Post-surgery mice were kept in cages of max 4 mice, on wired platforms for five days. Post-operative day (POD) 1 they received water supplemented with electrolytes, with gradual induction of fluid diet on POD2. Solid food was reintroduced POD3, wetted or crushed. Weekly vitamin substitution (Vitamin B1, B2, B3, B5, B6, B12, B8, C, magnesium, and iron) was added to their food, as well as biweekly injection of iron dextran 5mg/kg (Table 2). Relative weight loss up to 40 – 50% is seen in these procedures, indicating the necessity of daily control and weighing of the animals. Survival in our model is acceptable, i.e., 90.0% VSP, 90.0% VSG, 72.8% OAGB and 62.5% RYGB survival on POD14. Residual cause of death included intestinal obstruction (bezoar), anastomotic leak (15 - 20%) and biliary obstruction due to anatomical kinking.

Conclusions

Our designed protocol offers a broad insight in care for obese mice following complex, novel BS procedures. The model is multi-applicable in basic science and provides maximal support and care for laboratory mice. ■

Production of a reusable micromolded microcavity insert to standardize spheroid generation for drug screening

H. Fernandes, V. Valeri, C. Degrassi, M. Rasponi and C. Mota

Introduction

Drug screening relies mainly on simple *in vitro* and *in vivo* models that even when combined, do not provide a perfect representation of human physiology. Likewise, due to the ethical concerns surrounding the wide use of animals for research, the improvement of *in vitro* models to support the 3R's policy is crucial. Considering the liver's pivotal role in drug metabolism and its high susceptibility to toxicity over time, better 3-dimensional (3D) liver models are needed for efficient drug screening. We developed an approach based on micromolding to generate a reusable microcavity insert in Polydimethylsiloxane (PDMS), to facilitate spheroid generation.

Experimental

An acrylic mold with small microcavities was designed and after double-casting, both a negative and a microcavity insert in PDMS were produced. The inserts were attached to the bottom of a 48-well plate, sterilized and coated to prevent cell adherence. HepG2 cells were seeded and after 72h, treatment was initiated. The efficacy of Sorafenib was evaluated by assessing the drug's impact on the spheroid size, shape and viability.

Results and discussion

The PDMS negative and microcavity insert were successfully developed, presenting the desired topography. The insert was used to seed HepG2 cells and after 48h, 1500 compact spheroids were observed per insert, similar in shape and size. Upon treatment, the spheroids showed reduced viability, loss of shape and size reduction with increasing concentration of the compound.

Conclusion

We were able to develop a reusable micromolded microcavity insert, in which we can generate thousands of homogeneous spheroids, in a simple and fast manner. Compact spheroids are obtained and easily retrieved. Sorafenib treatment was

performed, and a dose-dependent effect was observed. The developed microcavity insert is an encouraging platform to screen drugs *in vitro*, on more reliable and physiologically relevant models, reducing the need for animal research. ■

Comparison of advanced and traditional cell culture models for lung and liver – cyto- and genotoxicity of NM-300K silver nanoparticles

E. Elje, E. Mariussen, N. El Yamani, E. M. Longhin, L. M.A. Camassa, S. Zienolddiny-Narui, M. Dusinska, E. Rundén-Pran

For the next-generation risk assessment (NGRA) of chemicals and nanomaterials, new approach methodologies (NAMs) are needed for hazard assessment in compliance with the 3R's to reduce, replace and refine animal experiments. As part of this, the development of advanced *in vitro* models is needed for genotoxicity assessment of chemicals and nanomaterials. Inhaled compounds will reach the liver after entering systemic circulation, and thus both respiratory and liver models are of importance for hazard assessment.

The aim of this study was to establish and characterize two different types of advanced *in vitro* cell models representing lung and liver, by cultivating commonly used human cell lines in 3D arrangements. For the advanced respiratory model, the cells were cultivated at the air-liquid interface (ALI) in mono- or cocultures, and for the liver model in hanging drop spheroids. Hazard identification in the advanced models were compared to results from traditional 2D models with submerged exposure. All the models were exposed to nanosilver (NM-300K) for 24 hours before further processing to measure cellular viability (alamarBlue assay), inflammatory response (enzyme-linked immunosorbent assay), DNA damage (enzyme-modified comet assay), and chromosomal damage (cytokinesis-block micronucleus assay).
We demonstrated that both the advanced lung and liver models are applicable for genotoxicity assessment of nanomaterials. Cytotoxicity and genotoxicity induced by NM-300K were dependent on both the cell types and model. This study brings important knowledge for the further development of advanced 3D *in vitro* models for the most reliable human hazard assessment based on NAMs. ■

Assessment of cytotoxic effect mechanisms of circadian REV-ERB agonist in urothelial cells

M. Biesiekierska, A. Wolniakowska, K. Mierczak, J. Roszak, E. Reszka

The circadian rhythm is a natural process that organizes the functioning of the organism in response to external stimuli. This adaptation process is observed at a physiological level, affecting various molecular pathways such as metabolism, cell proliferation, inflammation, and DNA damage repair On the other hand, circadian rhythm disruption is considered a hallmark of urinary bladder cancer (UBC) development. Although the chronotherapy concept has rapidly evolved, novel therapeutics modulating circadian components are still in search. One of the proposed ones is SR9009, a REV-ERB agonist.

The aim of the research was to assess the cytotoxic effect mechanisms upon the treatment of UBC cells with SR9009. This was performed by the viability analysis of UBC cell lines T-24, 5637 and RT-4 using MTT assay. Cells were treated with SR9009 in a given concentration range and incubated for 24 and 72 hours. Next, the migration test using the Boyden chamber and the colony-forming assay were conducted on T-24 cell line after SR9009 treatment.

The results obtained demonstrate the cell line and time dependency of the drug effect. SR9009 inhibited the proliferation of UBC cells, especially T-24 cell line, suggesting that it may be a potential therapeutic in UBC treatment. Nonetheless, further studies are needed to fill the remaining knowledge gaps in the development and treatment of UBC. ■

Planarians as an *in vivo* model for a high-throughput assessment of developmental, neuro- and genotoxicity

K. Bijnens, M. Heleven, C. Segnana, J. Tytgat, J-P. Ploem, K. Smeets

The use of *in vitro* methods in toxicity testing often fails to include complex toxicological endpoints and is limited in its ability to link observed alterations to adverse outcomes. Here we demonstrate the added value of planarians within the 3R principle for *in vivo* toxicity assessments, with a specific focus on developmental toxicity, neurotoxicity and genotoxicity. The freshwater planarian Schmidtea mediterranea is characterized by a high regenerative capacity, which is attributed to a large pool of pluripotent stem cells (neoblasts). Based on planarian behavior, regenerative success and underlying stem cell responses, we investigate complex toxicological outcomes at different levels of biological organization and aim to identify specific adverse outcome pathways.

To assess developmental toxicity, we exploit the regenerative capacities of S. mediterranea by artificially amputating the worm and subsequently study its regenerative success after exposure to toxicological compounds. Phenotypic abnormalities, aberrant eye development and reduced tissue outgrowth are combined with a well established set of cellular markers to assess underlying stem cell dynamics. Behavioral abnormalities are used to evaluate neurotoxicity, which we further investigate by scoring the development of the planarian brain and central nervous system. To specify neurotoxicity, specific neuronal populations can be visualized. Additionally, based on specific planarian stem cell responses we developed a high-throughput screening alternative in the field of carcinogenicity testing. Our assay quantifies the number of proliferating stem cells and, based on the resulting patterns, enables the prediction of genotoxicity and the classification of carcinogenic compounds as genotoxic or non-genotoxic.

In conclusion, planarians allow for a high-throughput, inexpensive and reliable screening of toxicological compounds, here demonstrated in the field of developmental, neuro- and genotoxicity. Future studies regarding the underlying mechanisms will allow us to identify adverse outcome pathways. ■

Poster competition

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Posters

The posters are available at the expo area from Tuesday 19 through Thursday 21.09. The winner will be announced during the award ceremony on Thursday 21.09 at 17.00.

By following the QR code, you can vote for your favourite poster and give more exposure to your preferred research topic. Voting ends on Thursday 21.09 at 14.00 (after lunch).
* included in the Poster Competition

#	Presenting author	Affiliation presenting author	Abstract title
1*	Sien Lequeue	*In Vitro* Toxicology and Dermato-cosmetology, Vrije Universiteit Brussel	Identification of pharmacological chaperones in the context of alkaptonuria: robust high-throughput screening of an FDA approved compound library
2*	Gloria Melzi	Department of Pharmacological and Biomolecular Science (DiSFeB), Università degli Studi di Milano	Redox-activity: correlation between PM1 oxidative potential and toxicological effect on BEAS-2B
3*	Gloria Melzi	Department of Pharmacological and Biomolecular Science (DiSFeB), Università degli Studi di Milano	Lack of genotoxicity for Rheum palmatum L., Rhamnus purshiana DC, Rhamnus frangula L., Cassia senna L. extracts in OECD 487 micronucleus assay *in vitro*
4*	Constant Gillot	Namur Research Insitute for Life Sciences, Université de Namur	Methods for Assessing Resistance to Non-Integrating Virus Vectors
5*	Marth Stinckens	*In Vitro* Toxicology and Dermato-cosmetology, Vrije Universiteit Brussel	Genetically engineering human skin-derived precursor cells into functional hepatocyte-like cells as a novel liver-based *in vitro* model
6*	Grégory de Bodt	PEDI laboratory, Université Catholique de Louvain	Hepatocellular carcinoma cell lines growth inhibition by liver-derived mesenchymal stem cells in direct 3D co-culture
7*	Ingrid Vernemmen	Equine Cardioteam Ghent, Ghent University	Ultrasound-compatible 3D model of the equine heart to develop advanced intra-cardiac treatment strategies
8*	Engi Ahmed	Laboratory of Mucosal Immunology, Ghent University	Modeling severe asthma using human induced Pluripotent Stem Cells (hiPSC) and combining 3D dimensional organoids and air liquid interface methods
9*	Sybren De Boever	*In Vitro* Toxicology and Dermato-cosmetology, Vrije Universiteit Brussel	Development of human liver cancer 3D spheroid cultures for assessment of nanoplastic toxicity
10*	Carolina Rocha	Marine and Environmental Sciences Centre / Aquatic Research Network, University of Coimbra	Moving towards the refinement of fish ecotoxicological studies: metabolomic effects of microplastic and zinc exposure using meagre liver and macrophage cultures
11*	Nidda Saeed	Laboratory of Experimental Surgery, Ugent	Intraperitoneal Electromotive Nanoparticle Administration: Proof of concept.
12*	Martijn Heleven	Centre for Environmental Sciences, UHasselt	Dynamic *in vivo* processes in planarians as an alternative tool to assess toxicity
13*	Sara Sepehri	*In Vitro* Toxicology and Dermato-cosmetology, Vrije Universiteit Brussel	Development of TOXIN Knowledge Graph, advancing animal-free risk assessment of cosmetics

#	Presenting author	Affiliation presenting author	Abstract title
14	Payal Shaw	*In Vitro* Toxicology and Dermato-cosmetology, Vrije Universiteit Brussel	Investigation of the role of adipocyte-derived exosomes in development of NASH using human stem cell-based *in vitro* models
15	Morgane Leprovost	Institut de Recherche Interdisciplinaire en Biologie Humaine et moléculaire, Université Libre de Bruxelles	Duodenal-derived organoids to investigate gut epithelium in Nonalcoholic steatohepatitis (NASH) patients
16*	Melissa Valussi	Department of Biology/Animal Physiology, Philipps-Universität Marburg	3R-SMART: Information and training platform for methods to replace and supplement animal experiments
17	Sivakumar Murugadoss	SD Chemical and Physical Health Risks, Sciensano	Innovative non-animal based testing platform to detect chemical-induced cardiotoxicity - A roadmap for regulatory uptake
18*	Sven De Windt	Andrology Lab, Pôle de recherche en Physiopathologie de la Reproduction, Université Catholique de Louvain	Comparing enzymatic digestion for the highest recovery of neonatal porcine germ cells to generate testicular organoids
19*	Ine Nulmans	*In Vitro* Toxicology and Dermato-cosmetology, Vrije Universiteit Brussel	Development of a robust high-throughput screenings assay for the evaluation of bacterial tyrosine ammonia lyases in the context of tyrosine-inherited metabolic disorders
20*	Samuel Silva	Biology of the Testis, Vrije Universiteit Brussel	Human testicular organoids for high throughput applications: which is the ideal tissue source?
21*	Julie Sanders	*In Vitro* Toxicology and Dermato-cosmetology, Vrije Universiteit Brussel & SD Chemical and Physical Health Risks, Sciensano	A benchmark dose based strategy for evaluating the combined effects of genotoxicants
22*	Ami Toulehohoun	Laboratory of Hepato-Gastroenterology, Université Catholique de Louvain	The role of intestinal epithelial cells in gut barrier dysfunction in Alcohol Use Disorder (AUD)
23*	Elisabeth Knetemann	Liver Cell Biology, Vrije Universiteit Brussel	Unraveling Tumour Microenvironments: 3D Models of Liver Cancer and Metastasis
24	Flore Laurent	Quality of Vaccines and Blood Products, Sciensano	Validation of an animal-free analytical method for the detection of bacterial endotoxins in human vaccines at Sciensano
25	Matteo Piumatti	Altertox	The Right Tool for the Job: Why and How to Adapt Your Science Communication for In-person and Virtual Events
26	Laure-Alix Clerbaux	Laboratory of GastroEnteroHepatology, Université Catholique de Louvain & European Commission, Joint Research Centre	Beyond chemicals: the Adverse Outcome Pathway (AOP) framework embracing diverse stressors to bridge knowledge on mechanisms of adversity

* included in the Poster Competition

1* Identification of pharmacological chaperones in the context of alkaptonuria: robust high-throughput screening of an FDA approved compound library

S. Lequeue, H. Allach, T. Vanhaecke and J. De Kock

Alkaptonuria (AKU) is a rare genetic disorder caused by a homozygous or compound heterozygous mutation in the homogentisate 1,2-dioxygenase (HGD) gene with a total of 250 human mutations identified so far according to the HGD mutation database. The HGD single gene codes for the HGD protein protomer of 445 amino acids. The active HGD form is organized as a hexamer, associated as a dimer of trimers. Previously, it was described that this highly complex quaternary structure can be easily disrupted by missense mutations, i.e. 68% from all AKU-causing mutations. In this study, we explored pharmacological chaperones (PCs), small molecules that can help misfolded proteins fold correctly and translocate within cells. We established and, subsequently, conducted a high-throughput screening (HTS) using E. coli bacteria expressing the HGD G161R missense variant to identify PCs that could enhance the missense variant's catalytic activity. First, we established an HTS assay that quantifies the increase in maleylacetoacetate over time to calculate enzyme velocities and optimized different enzyme and substrate concentrations. Next, the assay was evaluated for its robustness by assessing plate and spatial uniformity as well as signal variability resulting in optimal HTS assay quality parameters ($Z' > 0.4$, $SW > 2$). Finally, the HTS assay was used to identify PCs by screening a compound library consisting of 2320 FDA approved drugs which identified 14 compounds that increased enzyme activity. Finally, we selected five compounds for a dose response study based on their effectiveness, availability and valorisation potential. Compound 98I showed a remarkable average increase of 1.74-fold and 2.27-fold in enzyme velocities compared to the untreated variant ($p < 0.0001$) at a concentration of 50 µM and 100 µM, respectively. To conclude, we successfully applied our newly developed HTS assay to identify and evaluate PCs that can increase the enzyme activity of the G161R missense variant, opening the path to restore HGD enzyme activity in AKU patients. ■

2* Redox-activity: correlation between PM1 oxidative potential and toxicological effect on BEAS-2B

G. Melzi, L. Massimi, M. Rinaldi, M. Paglione, N. Tarallo, F. Crova, M.A. Frezzini, G. Valli, S. Canepari, S. Decesari, R. Vecchi, M. Marinovich, E. Corsini

Particulate Matter (PM) is a complex and heterogeneous mixture of atmospheric particles recognized as a threat to human health. Oxidative Potential (OP) measurement is a promising and integrative method for estimating PM-induced health impacts since it is increasingly recognized as closely associated with adverse health effects than ordinarily used PM mass concentrations [1]. OP measurements could be introduced in the air quality monitoring, along with the parameters currently evaluated. PM deposition in lungs induces oxidative stress, inflammation, and DNA damage.

The OP measured on PM samples was compared to toxicological effect on BEAS-2B of winter and summer PM1 collected in the Po valley (Italy) during 2021. PM1 was extracted in deionized water by mechanical agitation and tested for OP or used to treat BEAS-2B (diluted 1:10 in complete medium). Cytotoxicity, genotoxicity, oxidative stress, and inflammatory response were assessed with different methods: MTT test, DCFH-DA assay, micronucleus, γ-H2AX formation, comet assay modified with endonucleases, and Real-Time PCR The OP evaluation was performed applying three different assays: dithiothreitol (OPDTT), ascorbic acid (OPAA), and 2', 7'-dichlorofluorescein (OPDCFH).

Most of the samples showed a slight increase of ROS formation, with a similar trend in winter and summer samples. The amount of DNA damage detected with the comet assay highlights the presence of oxidative damage both in winter and in summer samples. On the contrary, DNA damage and genes regulation were mainly detected in winter samples. Positive correlation (Spearman's analysis, $p<0.05$) were detected for IL-8 secretion, γ-H2AX formation, and some genes expression (ATM, HMOX, and NQO1) the OPDCFH results.

These results suggest that acellular OPDCFH analysis implementation in air quality monitoring could be a useful tool to estimate the cellular oxidative stress induced by PM exposure, more than the use OPDTT, OPAA, and mass concentration. ■

3* Lack of genotoxicity for Rheum palmatum L., Rhamnus purshiana DC, Rhamnus frangula L., Cassia senna L. extracts in OECD 487 micronucleus assay *in vitro*

G. Melzi, C.L. Galli, M. Marinovich

Botanical extracts from Rheum palmatum L., Rhamnus purshiana DC, Rhamnus frangula L., and Cassia senna L. are commonly used as nutritional supplements or in traditional medicines, mainly for their laxative properties. These botanicals contain anthraquinones in different amount. Hydroxyanthracenes as aloe-emodin and emodin are a class of anthraquinones, that were considered as genotoxic by the 2018 EFSA-ANS Panel due to the related possible risk factor for colon-rectal cancer development.

The aim of this research is to evaluate the genotoxic potential with the micronucleus assay (OECD487) *in vitro* of the extract of Rheum palmatum L., Rhamnus purshiana DC, Rhamnus frangula L. (bark) and Cassia senna L. (fruits and leaves) obtained following the European Pharmacopeia.

Tests were conducted on human lymphocytes obtained from donors' whole blood (upon informed consent). Treatments were performed 48-hours following Phytohaemagglutinin (PHA) stimulation, during the cell proliferation phase. Two different treatments were applied:

- in the first type of treatment, cytochalasin B was substituted to the extracts after 3-hours and cells were analyzed after 25-hours.
- while in the second type of treatment, cytochalasin B was added in the medium at the same time of the extracts.

S9 mix was used to test genotoxicity of the metabolites only in the 3-hours treatment samples. Cytokinesis-block proliferation index (CBPI) was calculated to obtain information about the possible cytotoxicity induced by the treatments. Hydroxyanthracenes' content varies between 0.06% and 0.23% for aloe-emodin, and between 0.07% and 0.16% for emodin and rhein. No cytotoxicity was detected at the tested concentrations. The results obtained with the micronucleus assay showed lack of genotoxicity for all the extracts at all the concentrations.

These results demonstrate that the Rheum palmatum L., Rhamnus purshiana DC, Rhamnus frangula L., and Cassia senna L. extracts do not induce micronuclei's formation in human lymphocytes *in vitro* following the tested concentrations. ■

4* Methods for assessing resistance to non-integrating virus vectors

C. Gillot, J. Favresse, V. Mathieux, J-M. Dogné, F. Mullier and J. Douxfils

We describe the case of an 83-year-old woman vaccinated with ChadOx1 nCoV-19 who developed a vaccine-induced thrombosis with thrombocytopenia syndrome and who did not develop any antibodies against the spike protein of SARS-CoV-2 at 30 days following the administration of her first dose of ChadOx1 nCoV-19. To investigate the lack of response to the vaccination, a cell model was developed. This model permits to evaluate the interaction between responsive cells (A549) possessing the Coxsackievirus and Adenovirus Receptor (CAR), a defined concentration of ChadOx1 nCoV-19 and serial dilution of the patient or the control serum. The aim was to assess the impact of these sera on the production of the spike (S) protein induced by the transfection of the genetic material of ChadOx1 nCoV-19 into the A549 cells. The S protein is measured in the supernatant. The serum from the patient who developed the vaccine-induced thrombosis with thrombocytopenia

syndrome impaired the production of S protein by the A549 cells transfected with ChadOx1 nCoV-19. This was not observed with the controls since the S protein is retrieved in the supernatant fraction. Based on the data coming from the clinical and the cell model information, we found a possible explanation on the absence of antibody response in our patient. She has or developed characteristic that prevents the production of the S protein in contrast to control subjects. The entire mechanism behind this resistance which deserve further investigations. Based on this analyze, we decide to apply for a patent for this cellular model (patent application number: EP22150499.6). This cell-based model is therefore a test to enable the evaluation of the cellular response of therapies aimed at modifying the expression of proteins at the intra- or extracellular level in the presence of patient plasma or other substances that could potentially alter the functioning of the said therapy. ■

5* Genetically engineering human skin-derived precursor cells into functional hepatocyte-like cells as a novel liver-based *in vitro* model

M. Stinckens, M. Rombaut, R. Marcelino Rodrigues, T. Vanhaecke, J. De Kock

Introduction

Primary human hepatocytes (PHHs) are the current gold standard for *in vitro* liver research, but their use is hindered by scarce availability and dedifferentiation in culture. To address these limitations, surrogate human hepatocytes, particularly those derived from human skin-derived precursor cells (hSKP-HPCs), have been explored by the host lab. Indeed, when exposed to hepatogenic growth factors and cytokines, hSKP-HPCs acquire, to some extent, hepatic properties. Although they serve as a valuable model for studying drug-induced liver steatosis and non-alcoholic steatohepatitis *in vitro*, their hepatic phenotype remains immature when compared to PHHs. Therefore, this study aims to improve the hepatic phenotype of hSKP-HPCs, in particular their biotransformation capacity, by overexpressing hepatogenic programming factors.

Materials and methods

Hepatogenic programming of undifferentiated hSKPs into human skin- derived hepatocyte-like cells (hSKP-HEPs) is achieved by co-overexpression of three liver-enriched transcription factors (LETFs), namely forkhead box A3, hepatocyte nuclear factor-1α and hepatocyte nuclear factor-4α using an all-in-one hepatic programming cassette. The induction of these LETFs was achieved using a CRE/LoxP-based flip excision (FLEx) system. After "switching on" the all-in-one hepatic programming cassette in hSKPs, a previously established 24-day differentiation protocol was used. At day 24, the transcriptomic profile of the obtained hSKP-HEPs was evaluated using microarray and compared to the unswitched controls (hSKP-HPCs) and PHHs.

Results

Microarray analysis showed a significant upregulation of several hepatic markers when compared to the unswitched control, including serum (ALB, FGA/FGB/FGG, SERPINA1, TTR, ...), biotransformation (CYP2C9, UGT1A family, GSTA1, SULT1C2, BAAT, SLC2A2 ...) and lipid metabolism markers (RBP4, APOB, APOH, ...). Furthermore, expression levels of the aforementioned genes were similar to the ones found in PHHs.

Conclusion

An improved hepatic phenotype was successfully achieved after overexpression of the all- in-one hepatic programming cassette in hSKPs. Although their further characterization at the protein level and hepatic functionality is still required, our findings provide promising prospects for generating hSKP-HEPs with a superior hepatic phenotype compared to hSKP-HPCs which would present as a valuable new tool for *in vitro* liver toxicity studies and disease modelling. ■

6* Hepatocellular carcinoma cell lines growth inhibition by liver-derived mesenchymal stem cells in direct 3D co-culture

G. de Bodt, M. Najimi and E. Sokal

Background and Aim

Hepatocellular Carcinoma (HCC) is the third highest cause of cancer-related death. Recently, cell therapy using mesenchymal stem cells (MSCs) is being explored as a potential new therapeutic strategy in oncology. MSCs display innate anti-tumoral properties *in vivo* and *in vitro* but their exact role in tumor emergence and progression is still debated, as other studies also highlighted pro-tumoral effects. Human Adult Liver Progenitor Cells (HALPCs), a liver-derived population of mesenchymal stem cells, are being developed as allogeneic products. The aim of this work is to investigate the therapeutic properties of HALPCs towards HCC through the study of their interactions with three human HCC cell lines (HepG2, Hep3B and HuH7) in a 3D co-culture model.

Method

Spheroids were formed in ultra-low attachment plates. In co-culture spheroids, HALPCs and either HepG2, Hep3B or HuH7 cells were seeded at different cell-to-cell ratios to explore spheroid growth by daily brightfield micrography. Spheroid area measurement was automated using an ImageJ Macro. Cell proliferation was also assessed through luminometry-based measurement of spheroids' ATP content and Ki67 immunostaining on paraffin-embedded spheroid slices.

Results:

The spheroids' size follow-up revealed a statistically significant decrease in spheroid growth velocity when HALPCs were co-cultured with the different cell lines. This effect was dependent on the HALPC ratio in the spheroid. Biochemical and histological analyses supported those results, with a significant decrease of the total ATP content and the number of Ki67 + cells in bi-cellular spheroids when compared to their respective controls.

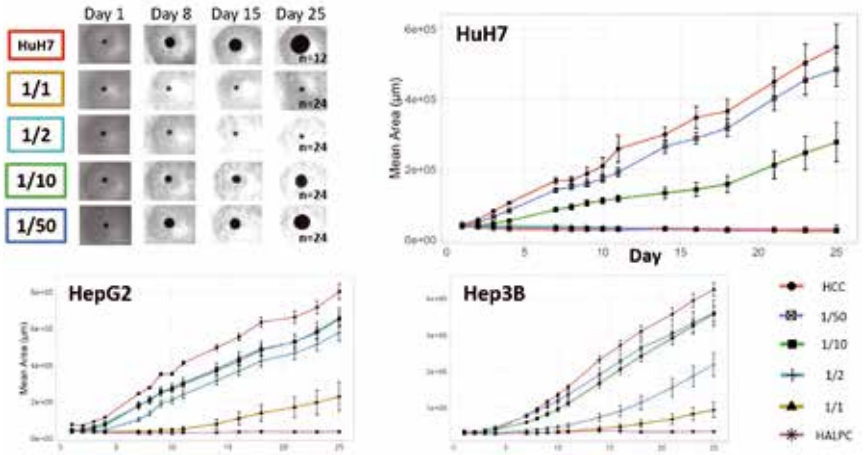


Figure 1: HepG2/HuH7/Hep3B and HALPC coculture spheroids growth follow-up

Method: Coculture spheroids were formed in u-bottom ultra-low attachment plates by seeding a total of 1000 cells/well at varying ratios of both cells types (i.e. 1/1 consists of 500 HALPC cells and 500 HepG2/HuH7/Hep3B cells, 1/2 consists of 333 HALPC cells and 666 HepG2/HuH7/Hep3B cells, 1/10 consists of 100 HALPC cells and 900 HepG2/HuH7/Hep3B cells and 1/50 consists of 20 HALPC cells and 980 HepG2/HuH7/Hep3B cells). Mono-cellular spheroids containing only one of the cell types were used as controls. Representative micrographs of each type of spheroid taken at different days after their formation are displayed to visually illustrate the difference in spheroid growth. Graphs show the mean area of each subtype of spheroid, measured using an automated process with an ImageJ macro each day after their formation, and their individual confidence interval.

Results: A statistically significant decrease in the size and the growth of the spheroids can be seen with each of the three cancer cell line when in the presence of HALPC. In HuH7, a complete stop of spheroid growth and even a decrease of the initial spheroid size can be seen at the 1/1 and 1/2 ratio. The intensity of the spheroid growth inhibition gradually fades in lower ratios, indicating the presence of a dose-response relationship.

Conclusion

In a 3D *in vitro* direct co-culture model, HALPCs are able to reduce the proliferation of three different human HCC cell lines (HepG2, Hep3B and HuH7). This effect was proven morphologically, biochemically and histologically. The deciphering of the mechanisms of action of these effects is ongoing while *in vivo* validation of these results is mandatory. ■

7* Ultrasound-compatible 3D model of the equine heart to develop advanced intra-cardiac treatment strategies

I. Vernemmen, S. Hauspie, G. Van Steenkiste, E. Buschmann, K. Vanderperren, G. van Loon

Background

Arrhythmias are common in horses and can pose a risk to horse and rider. Transthoracic 2D echocardiography (2DTTE) is pivotal in the structural and functional cardiac assessment. In addition, 2DTTE is the standard guiding modality in horses for minimally-invasive intracardiac procedures to diagnose and treat these arrhythmias, because fluoroscopy, CT and MRI cannot be used due to the size of the animal. However, visualisation of catheter manipulation using 2DTTE can be challenging and therefore a 3D ultrasound- compatible equine heart model would be useful in the development of these procedures.

Objective

To develop an ultrasound-compatible 3D model of the equine heart allowing the development of standardised ultrasound-based guidance of intracardiac interventions in horses.

Methods

A contrast-enhanced CT was performed to image the heart of a pony small enough to fit into a CT gantry. Based on these images, a 3D computer model was created and scaled to adult horse size, allowing the production of a 3D heart phantom made of ultrasound-compatible silicone. Placed in a water tank with silicone windows, 2DTTE can be mimicked (Figure 1). Through insertion pieces, catheters can be introduced to simulate intracardiac catheterizations. Images were compared with *in vivo* images.

Results

Echocardiography of the model is feasible. Images acquired on the phantom resemble *in vivo* echocardiographic images of the pony on which the model was based (Figure 2).

Discussion

Procedures can be simulated before performing them in living animals and

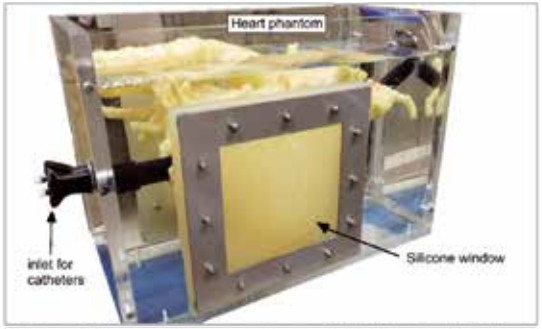


Figure 1. Set-up of the ultrasound-compatible 3D heart model in the water tank. Silicone windows allow echocardiographic imaging.

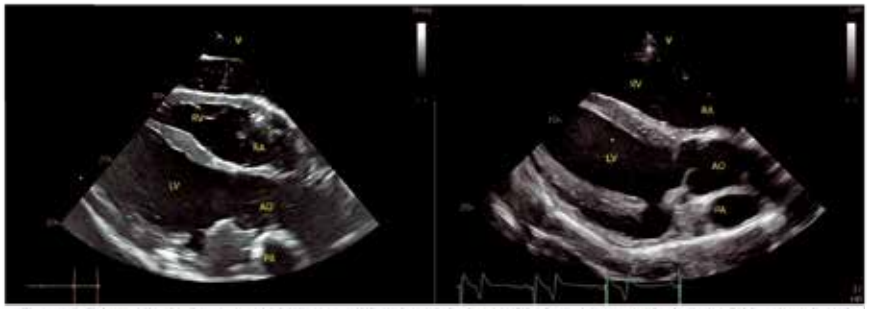


Figure 2. Echocardiographic image of the heart model (left) and the heart of the living pony on which the model has been based (right). The anatomy of the model corresponds well with the in vivo image. AO, aorta; LV, left ventricle; PA, pulmonary artery; RA, right atrium; RV, right ventricle.

echocardiography can be trained, improving experimental animal safety and reducing animal number needed for intervention development. Limitations of the model include the absence of cardiac contractions, flow and heart valves, which might have an influence on catheter manipulations.

Conclusion

An ultrasound-compatible 3D equine heart model has been developed to reduce the number of animals needed to develop and train intracardiac interventions guided by echocardiography. ■

8* Modeling severe asthma using human induced Pluripotent Stem Cells (hiPSC) and combining 3D dimensional organoids and air liquid interface methods

E. Ahmed, F. Foisset, C. Bourdais, S. Assou, A. S. Gamez, J. de Vos, H. Hammad, A. Bourdin and B. Lambrecht

Rationale

Asthma is an heterogenous disease and is the most common chronic inflammatory disorder of the airways. Severe asthma patients with persistent airflow obstruction are characterized by airway obstruction due to mucus plugs containing mucins, fibrin, and eosinophil derived-Charcot-Leyden crystals. The molecular mechanisms underlying this endotype are not clearly understood. Developing new models is mandatory for respiratory research as critical differences exist between human and rodent airway epithelium. 3-dimensional (3D) organoid cultures have led to new physiologically complex *in vitro* models to study human development and disease. We and other teams showed that it is possible to reconstitute *in vitro* a complex and functional airway epithelium displaying all the characteristic features described *in vivo* from hiPSC. Our aim is to establish a human *in vitro* model of severe asthma that will recapitulate epithelium cell remodeling, mucus hyperproduction, and to set up a co-culture with innate immune cells.

Methods

We will engineer a human *in vitro* asthma model system through the directed differentiation of induced pluripotent stem cells (iPSC) from two severe asthmatic patients and two controls. Human manipulation of developmental signaling pathways such as canonical TGF-b pathway, Wnt and BMP signaling allows the generation of NKX2.1 bronchial progenitors. Efficiency will be evaluated at each step by flow cytometry and immunofluorescence. FACS analysis of CXCR4 expressing cell rates as a surrogate marker of definitive endoderm (DE), and NKX2.1 for iPS derived bronchial progenitors. Mucus rheological proprieties and production, ciliary beating, airway epithelium differentiation, epithelium integrity will be assessed.

Results

We successfully generated two human iPS cell lines from highly characterized severe asthma patients (RECHMPL22_0384 – MOSAIC, NCT05616338, Montpellier University Hospital, France). Peripheral blood mononuclear cells (PBMCs) were reprogrammed using integration-free Sendai Virus. The cell lines had normal karyotype, expressed pluripotency hallmarks at mRNA and protein level (i.e. OCT-4, NANOG, SOX2), and differentiated into the three primary germ layers. Cells lines are maintained in feeder free condition (i.e., no use mouse embryonic fibroblasts) and using serum free animal media. Definitive endoderm differentiation efficiency of these two patients was comparable to a healthy control iPSC cell line, UHOMi002-A, cells expressing more than 80% of CXCR4 marker. ALI cultures derived from healthy donors are composed of basal, secretory and multiciliated cells and demonstrate epithelial barrier integrity, motile cilia, and mucus secretion. Differentiation of iPS cell lines from asthmatic patients are ongoing.

Conclusions

3D human bronchial organoids and ALI cultures of human iPSC represent a great tool that recapitulate the human development, bronchial differentiation, and

disease *in vitro*. Human iPSC is also a powerful tool to identify novel pharmaceuticals and represent an opportunity to decrease our reliance upon traditional animal research models of human lung disease and injury. ■

9* Development of human liver cancer 3D spheroid cultures for assessment of nanoplastic toxicity

S. De Boever, K. Leroy, L. Devisscher, M. Vinken

3D spheroid cell cultures are an appropriate alternative to conventional 2D monolayer cell systems because of a higher *in vivo* similarity. However, their use is currently underrepresented in hazard identification, in casu in the field of particle toxicity. In the present study, we focus on the development of 3D spheroid cultures of 7 human liver cancer cell lines and test their interaction with micro- and nanoplastics. Data regarding spheroid growth and viability indicated that 3 out of the 7 cell lines, namely C3A, SK-HEP-1 and Hep3B cells, formed maintainable 3D spheroids over time in non-scaffold culture conditions. A superior sensitivity towards the hepatotoxic compounds menadione and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone was demonstrated for the spheroid-forming cell lines compared to their 2D counterparts. Fluorescent polystyrene particles with a diameter of 0.5 µm interacted with the 3D spheroid cultures in a dose-, time- and cell type-dependent manner. However, larger particles with a diameter of 5 µm were incompatible with the scaffold-free set-up because of excessive particle precipitation, thereby preventing particle-spheroid interaction. The successful development of maintainable spheroid cultures and their potential to interact with nanosized particles advocates their use in the area of particle toxicity. ■

10* Moving towards the refinement of fish ecotoxicological studies: metabolomic effects of microplastic and zinc exposure using meagre liver and macrophage cultures

C. Rocha

Fish ecotoxicological studies remain mostly focused on the use of *in vivo* models. Besides the main ethical issue of, inevitably, causing distress to living organisms, experiments with live organisms face expectable constraints that limit the true reproducibility of the studies. For instance, regarding marine organisms, most studies still use juvenile fish, which, usually, present high heterogeneity and uncertain behaviour when confined. This, together with easily fluctuating environmental conditions, pose constraints to the maintenance of stable system conditions and, consequently, to fish well-being, tampering with any metabolomic parameters aimed to be assessed. Contrastingly, resorting to alternative models, such as organ culture models, enables an easier control of experimental conditions, resulting in the obtention of more trustworthy conclusions and a significant reduction of sacrificed animals, while maintaining the studies' ecological relevance. Micro(nano)plastic research remains a topic of high relevance, due to the knowledge gaps concerning smaller size fractions and the particles' interaction with other contaminants. The present study evaluates single and combined metabolomic effects in gilthead seabream (Sparus aurata) liver and macrophage cultures exposed to zinc chloride and micro- sized (round shapes, ~1µm diameter) plastics. Fish liver was obtained via organ culture methods and macrophages isolated from the head-kidney. Analyses are ongoing, through the assessment of the metabolomic pathways activated and structural effects potentially caused by the single and combined exposures. As environmentally relevant concentrations were used for exposures, we expect to find mostly inflammatory and immunological responses, as well as histological alterations in the used models. Understanding these effects is of utmost importance, both from the perspective of providing further insight in the often-overlooked sublethal consequences of contaminants to marine biota and to further sustain the relevance of applying the 3Rs principles in ecotoxicological studies. ■

11* Intraperitoneal electromotive nanoparticle administration: Proof of concept

N. Saeed, J. Wong Si Min, K. Remaut, A. Coene, and W. Ceelen

Introduction

Intraperitoneal drug delivery is increasingly used to treat peritoneal metastases (PM). However, the low hydraulic permeability of tumor tissue severely limits the depth of drug penetration after intraperitoneal instillation. The addition of an electrical force may enhance nanoparticle (NP) penetration in PM. We studied the effect of electromotive drug administration (EMDA) on NP penetration in porcine peritoneal tissue.

Methods

A double-walled cylindrical device was constructed to expose peritoneal tissue fragments to a temperature- regulated fluid column. Using an immersed spiral electrode, an pulsed DC electrical current (30 mA) was applied during 20 minutes (Fig.1A). Porcine peritoneal tissue fragments (1cm2) were exposed to a solution of positively charged amine-modified polystyrene fluorescent NPs (100 and 200 nm diameter). After exposure, tissue samples were cryo-sectioned into 20 µm slices, and tissue penetration and spatial distribution were measured using confocal microscopy. Mean fluorescence intensity (MFI, dimensionless) was measured in three regions of interest (ROI) with increasing depth drawn in triplicate on each cryosection. We studied the effects of the following parameters on NP penetration: EMDA (versus passive diffusion), NPs size, and liquid temperature. Results are given as mean (SD).

Results

Overall, EMDA resulted in a 4-fold increase in NP penetration and a 2-fold improvement in spatial distribution compared to passive diffusion (PD). Compared to passive diffusion (PD), the use of EMDA resulted in significantly higher NP tissue penetration with a size of 100 nm (MFI 250.9 (44.6) vs 79.6 (29.9), p=0.0001) and 200 nm (MFI 417.7 (70.8) vs 137.5 (47), p = 0.0001). The smaller NPs showed deeper tissue penetration (Fig. 2A). Increasing temperature resulted in a significantly higher tissue penetration of the 200 nm NPs: MFI 491.4 (276), 1261.5 (315), and 1383.1 (329), p=0.006 after exposure to 20°C, 37°C, and 42°C, respectively (Fig.2B).

Conclusion

The use of electromotive drug administration results in significantly increased tissue penetration of positively charged NPs in peritoneal tissue. Increasing temperature further enhances tissue penetration. These results suggest that EMDA may hold promise for intraperitoneal drug delivery for peritoneal metastases. ■

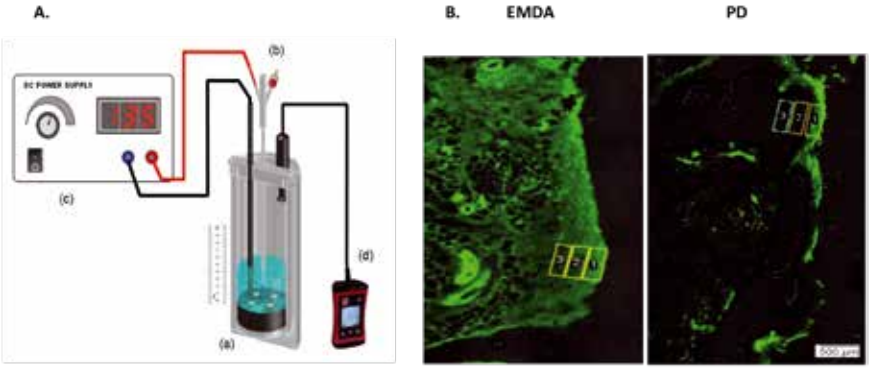
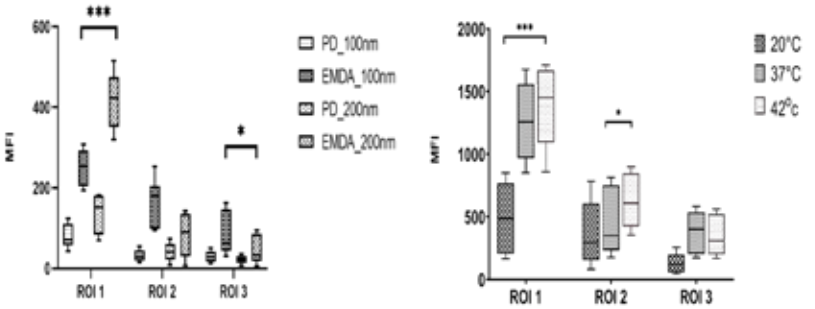


Figure 1A: EMDA Experimental setup containing (a) a partially insulated conductive apparatus comprised of a silver base plate and (b) a spiral electrode connected to (c) an electric generator, and (d) a temperature probe. **1B.** Fluorescence microscopy images of 100nm NPs uptake after EMDA and PD. The scale bar is 500µm.



12* Dynamic *in vivo* processes in planarians as an alternative tool to assess toxicity

M. Heleven, K. Bijnens and K. Smeets

Toxicity tests often rely on *in vitro* methods, which may not fully capture the complexity of the *in vivo* multicellular environment. Furthermore, these methods often fail to capture the immediate responses that occur shortly after exposure or make it impossible to link these short-term alterations to the induced adverse outcomes. Here, we demonstrate how planarians can be used to measure toxicity outcomes *in vivo* using live imaging techniques. This allows us to capture dynamic processes such as redox changes and alterations in neuronal signaling. Planarians possess a rudimentary brain with many features in common with vertebrate brains. They have a sensory nervous system that can be visualized via the autofluorescent properties of riboflavin in neurons. By exploiting these autofluorescent characteristics, alterations in neuronal signaling following exposure to toxic compounds can be monitored and differences in movement, speed, and signal intensity of the autofluorescent signals can be determined.

In addition, the regenerative abilities of the organism allow us to assess the effect of hazardous compounds and materials on developing processes. Planarians are able to fully restore and regenerate their nervous system, and neurodevelopmental processes can be monitored to assess the impact of toxicants on these processes.

Our findings show that the planarian Schmidtea mediterranea is a powerful toolbox for fast and convenient screening to study the effects of toxicants on dynamic processes during development. Live imaging allows the monitoring of dynamic processes such as redox and nerve signaling *in vivo*, which is a promising tool for toxicity testing in the future. Conclusively, this alternative way to determine toxic effects will aid in gaining in-depth knowledge regarding the underlying processes and adverse outcomes induced by toxicants. ■

13* Development of TOXIN Knowledge Graph, advancing animal-free risk assessment of cosmetics

S. Sepehri, J. Maushagen, G. Vrijens, C. Debruyne, R. Marcelino Rodrigues, A. Sanctorum, O. De Troyer, T. Vanhaecke

Background and Aim

Assessing repeated dose toxicity of novel chemical compounds without validated animal-free methods is challenging, especially in the EU's cosmetics industry with a ban on animal testing (Regulation EC N°1223/2009). To address this, OECD (Organisation for Economic Co-operation and Development) introduced Integrated Approaches to Testing and Assessment (IATA) for chemical safety evaluation, utilizing non-testing and alternative methods (e.g., *in vitro* and *in silico*). To support this, the aim of our research is to create a Knowledge Graph (KG) (TOXIN KG), based on collecting safety data on cosmetic ingredients, enabling non-animal systemic toxicity assessments.

Materials and Methods

We used 93 SCCS scientific opinions (2009-2019) covering 88 cosmetic ingredients. Data was captured in Excel, converted to CSV, and transformed into RDF graphs (R2RML) for being machine-processable. The TOXIN KG focuses on organ-specific toxicological information, particularly the liver, through a specialized search tool. Additionally, it provides HESS *in silico* predictions from the OECD QSAR toolbox and Klimisch scores via the ToxRtool grading system. To ensure comprehensive representation, we enriched the ToXic Process Ontology (TXPO) with gene ontologies and biological pathways repositories, allowing semi-automatic inclusion of pre-2009 and post-2019 SCCS opinions via ontology-based annotation.

Results

Based on the search filters, 52 Annexed cosmetic ingredients showed at least one liver-related toxicity parameter. Among these, 16 compounds also bared structural alerts for hepatotoxicity, as the OECD QSAR toolbox predicted. The enriched TXPO integrated into TOXIN further enabled the identification of four

ingredients (Basic Red 51, Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165), Triclosan, and HC Yellow n° 13) that showed a link between observed liver effects in animals and specific toxicological pathways with associated genes. To gain more insight into their toxicity mechanism and eliminate potential species differences, further *in vitro* testing using human-relevant cell systems is necessary for these ingredients.

Conclusions

TOXIN KG provides several data types relevant for evaluating cosmetic ingredients' repeated dose (liver) toxicity. Furthermore, it serves as a valuable resource for guiding follow-up targeted *in vitro* studies to investigate potential species differences and generate new mechanistic data for developing IATA for liver toxicity. ■

14. Investigation of the role of adipocyte-derived exosomes in development of NASH using human stem cell-based *in vitro* models

P. Shaw, S. Sepehri, A. Heymans, T. Vanhaecke, R. M. Rodrigues

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of fatty liver disorders associated with metabolic determinants, ranging from hepatic steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma. The exact pathophysiology of NASH is not fully understood. Obesity-related adipose tissue (AT) dysfunction, inflammation, and adipocyte hypertrophy lead to excessive release of fatty acids and adipokines, which are taken up by the liver, exacerbating hepatic steatosis and inflammation. In this study, we aim to investigate whether adipocyte-derived exosomes contribute to the progression of NASH. This will be investigated *in vitro*, using stem cell-based models.

Methods

Human adipose-derived stromal cells (hATSc) and human skin-derived precursors (hSKP) were respectively differentiated towards adipocyte-like cells (hATSc-Adipo) and hepatocyte-like cells (hSKP-HPC). hATSc-Adipo were exposed for 24 hours to chemical cocktails mimicking the AT microenvironment in obese, hyperglycemic and hypertriglyceridemic individuals (palmitic acid, glucose, insulin) or in NASH patients (palmitic acid, insulin, glucose, IL1β, IL6, TGFβ, TNFα). Upon a recovery wash period of 24 hours, exosomes were isolated from the cell supernatants and added to hSKP-HPC.

Results

RT-qPCR analysis shows that the NASH cocktail upregulated leptin (LEP) and downregulated adiponectin (ADIPOQ), which are pro- and anti-inflammatory adipokines, respectively. An upregulation of multiple cytokines (IL1B, TNFA, IL6) and chemokines (CXCL5, CCL2) was also observed, suggesting that ATSc-Adipo exposed to the NASH cocktail responded in a similar way as inflamed AT. Exposure of hSKP-HPC to exosomes derived from these cells induced a moderate upregulation of de novo lipogenesis markers (SCD1, FASN) but a considerable increase in expression of pro-inflammatory cytokines (IL6, IL1A, CCL2, and CXCL5).

Conclusion

Our findings indicate that hATSC-Adipo can replicate distinct cellular characteristics of inflamed adipose tissue during obesity and NASH. We also found that exosomes secreted from those cells induce hepatic steatogenic and inflammatory responses, denoting a potential contribution to the progression of NASH. ■

15. Duodenal-derived organoids to investigate gut epithelium in nonalcoholic steatohepatitis (NASH) patients

A. Hadefi, M. Leprovots, G. Dinsart, M. Marefati, M. Vermeersch, D. Monteyne, D. Pérez-Morga, A. Lefort, F. Libert, L. Verset, C. Liefferinckx, C. Moreno, J. Devière, E. Trépo, M-I. Garcia

Nonalcoholic steatohepatitis (NASH) is a chronic and progressive disease that can evolve to advanced fibrosis, cirrhosis, liver failure or liver cancer and is associated to a higher risk of death related mainly to cardiovascular events. Although NASH constitutes the hepatic manifestation of the metabolic syndrome, the gut-liver axis appears to be intricately linked not only to the physiopathology but also to the development of this disease. In addition, recent evidence from mouse models suggest a disruption of gut epithelium, however, the literature is scarce in humans. Therefore, to better understand the status of NASH's duodenal epithelium, we generated Duodenal Epithelial Organoids from NASH and healthy patients in a pilot study. Organoids from healthy (CDEOs) and biopsy-proven NASH subjects (NDEOs) were obtained with similar efficiency: 71% (n=10/14) and 69% (=11/16), respectively. Despite both kinds of organoids showed comparable levels of stem cell markers (OLFM4, LGR5, AXIN2) and proliferation rates (KI67), they exhibited different morphology. Indeed, NDEOs more frequently maintained a cystic spheroid-like aspect as compared to CDEOs. To investigate CDEO and NDEO transcriptomes, bulk RNA sequencing was performed at passage 2. We identified 438 genes significantly modulated (225 upregulated, 212 downregulated) in NDEOs versus CDEOs (false discovery rate 0.05, log2-fold change 1). Investigation of associated biological processes revealed dysregulation of tissue homeostasis with downregulation of processes involved in lipid and carbohydrate metabolisms, as well as altered signal transduction. Moreover, components of the apical junctional complex and adherens junctions were also downregulated in NDEOs, suggesting altered intestinal barrier integrity in NASH organoids. These data were confirmed by in situ hybridization experiments and transmission and scanning electron microscopy. Altogether, this study reveals the advantage of organoid technology to investigate gut components and further explore potential new therapeutic strategies in the setting of NASH disease. ■

16* 3R-SMART: Information and training platform for methods to replace and supplement animal experiments

M. K. Valussi, C. Nordmann, F. Gumz, B. Hiebl, N. Linklater

The Directive 2010/63/EU firmly strengthens the adoption of the 3R principle (replacement – reduction – refinement) for the use of animals for scientific and educational purposes.

Against this background, the BMBF-funded project 3R-SMART (https://www.3r-smart.de) was designed as an information and training platform on alternative and supplementary methods to animal experiments.

In addition to showcasing specific examples of alternatives to animal testing, 3R-SMART also covers legal and ethical aspects of working with laboratory animals. Video or text-based content provides either a brief overview or more in-depth information. The information is tailored to different needs and is suitable for students, scientists and technical staff as well as for public institutions, companies and ethics committees.

Furthermore, 3R-SMART supports the 3R research activities of various stakeholders by enabling them to present their latest 3R findings on 3R-SMART in order to increase the reach of their research results.
In this way, 3R-SMART is constantly being expanded and developed.
A discussion forum has also been developed to exchange questions and information on the 3Rs and to network 3R scientists.
Interactive maps of the 3R Centres in Germany and Europe enable interested parties to obtain an overview of the 3R Centres and to find out more about the activities and focal points of the individual 3R Centres.

In order to disseminate and transfer knowledge about the 3Rs, 3R-SMART will make open educational resources (OER) available and also is planning to offer 3R seminars and other learning opportunities. In this context, work is being done in cooperation with LAS interactive (https://las-interactive.de) on a combined

continuing education portal (3R-Campus) on laboratory animal science and alternatives to animal experimentation (fee-based) for continuing professional development. ■

17. Innovative non-animal based testing platform to detect chemical-induced cardiotoxicity - A roadmap for regulatory uptake

S. Murugadoss, A. Schaffert, R. Gehring, T. Roos, N. Linzalone, G. Donzelli, M. Paparella and B. Mertens

Accounting for 32% of all global deaths in 2019, cardiovascular diseases (CVDs) are the leading cause of mortality worldwide1. However, the relationship between environmental chemical exposure and CVD development is poorly understood. Furthermore, current regulations do not address cardiotoxicity as a specific endpoint, resulting in ineffective prevention of CVD incidence and deaths caused by chemical exposures. With an aging population and chronic exposure to chemical mixtures, particularly in Europe, there is an urgent need to develop robust, non-animal based mechanistic strategies to assess the cardiotoxicity of chemicals.

In this context, the Horizon 2020 project ALTERNATIVE (grant no. 101037090) aims to develop a non-animal test platform to detect the cardiotoxicity of chemicals. The platform includes a 3D *in vitro* physiological system mimicking healthy and aged human cardiac tissue, as well as multi-omics analyses and an integrated Machine Learning (ML) risk assessment tool. One of the main challenges is facilitating the regulatory uptake of this innovative platform in order to provide a more robust basis for decision-making.

To facilitate regulatory uptake of the platform, we first reviewed existing regulations for chemicals, pesticides and biocides, and indicated how limitations in their coverage of cardiotoxicity as well as their coverage of the elderly population and chemical mixtures could be overcome using new approach methodologies (NAMs)3. The adverse outcome pathway (AOP) framework can provide mechanistic validation for NAMs by characterizing the mechanistic underpinnings of the toxicological responses observed in NAMs. Based on textbook knowledge, we have developed and introduced two putative AOPs leading to heart failure (AOP 479 and AOP 480) that are now included in the OECD workplan. Evidence from systematic reviews to support these AOPs is currently being assembled and assessed according to the OECD handbook.

Finally, an AOP-informed integrated approach to testing and assessment (IATA) will be drafted by integrating existing knowledge and other IATA components developed within this project, such as the 3D *in vitro* system, QSAR models, and PBK modeling. The outcome is expected to provide a basis for a practical regulatory approach to assess the potential of chemicals to induce cardiotoxicity. Subsequently, our IATA will be proposed for common elaboration within the ASPIS cluster, OECD working groups and regulatory stakeholders/policy makers, aiming to integrate our IATA into a larger framework for a NAM based assessment of systemic toxicity. A final guidance and strategy for validation will be drafted, which will be submitted to EURL ECVAM for review and to seek approval for further validation of this novel platform. Ultimately, we aim to improve the regulatory acceptance and uptake of NAMs for cardiotoxicity assessment, and contribute to the prevention of exposure to potential cardiotoxic chemicals. ■

18* Comparing enzymatic digestion for the highest recovery of neonatal porcine germ cells to generate testicular organoids

S. De Windt, D. Kourta and C. Wyns

Background

Immature human testicular tissue (ITT) for research is scarce, therefore animal models are still widely used. Testicular organoids (TOs) are promising to replace animal testing. TOs can also be considered for fertility restoration using pediatric

cancer cell-contaminated biopsies. However, TO generation requires prior efficient cell isolation. A good cell yield (numbers, viability and all cell types recovered) is indispensable, particularly for scarce germ cells. We hypothesized that the digestion protocol may influence the TO generation efficacy.

Objective

Comparing two enzymatic digestion protocols (P1 and P2) using two different ITT fragment sizes to determine if higher germ cell yields can be reached after differential plating.

Material and methods

Castration-derived neonatal porcine testes (n=3) were weighted and minced into 4 and 8 mm3 (representative volumes for cryobanked human ITT) fragments prior to enzymatic digestion. Cellular yield was assessed by trypan blue and Burker chamber before and after incubation overnight on normal plastic. Floating cells were then incubated four days on Poly-D-Lysine-coated plastic before assessing germ cells yield. Immunocytochemistry for SOX9, DDX4, ACTA2 and CYP19A1 was performed to identify cell types.

Results

Cellular viability was in all 4 tested conditions >92%. P1 revealed a trend of higher cell yield for 8 mm3 fragments, whereas P2 showed opposite results, although not statistically significantly different (p=0.75 and p=0.21, respectively). After Poly-D-Lysine incubation, the recovery of DDX4+ germ cells, using P1 was higher than P2 regardless of fragment volume. Poly-D-Lysine-coated plastic allowed an additional recovery (16.42% (P1) and 28.98% (P2)) of prior floating germ cells.

Discussion and conclusion

P1 allowed higher recovery rates of germ cells. Poly-D-Lysine differential plating enhanced recovery of germ cells which are critical for TO generation. Shorter incubation times and lower enzyme concentrations in P1 could reduce cell membrane damage and explain the differences in germ cell yields. ■

19* A Development of a robust high-throughput screenings assay for the evaluation of bacterial tyrosine ammonia lyases in the context of tyrosine-inherited meta bolic disorders

I. Nulmans, C. Laga, N. Salvi, L. Desmet, S. Lequeue, J. Neuckermans, J. De Kock

Background

Tyrosine-inherited metabolic disorders (TIMD) are caused by a deficient activity of one of the enzymes involved in the degradation of tyrosine (Tyr) and result in patient's inability to break down Tyr and the accumulation of toxic intermediates. TIMD are currently treated with dietary restrictions and/or nitisinone (NTBC). Although NTBC prevents the accumulation of toxic metabolites, it has several side effects including Tyr accumulation. Tyrosine ammonia lyase (TAL) enzymes could provide an alternative degradation pathway for these excessive Tyr levels. The aim of this study is thus to develop a robust high-throughput screenings (HTS) assay to evaluate the fitness of bacterial TALs in the context of TIMD therapy development.

Methods

The developed HTS assay is based on the spectrophotometric quantification of p-coumaric acid after conversion of Tyr into p-coumaric acid and ammonia by Flavobacterium johnsoniae TAL (FjTAL). Optimal growth conditions for high-level protein expression were determined by incubating transformed E. coli BL21 (DE3) cells at different temperatures during various incubation times. Afterwards, varied Tyr assay mix and bacterial lysate concentrations were evaluated at pH 9,2. Once optimal assay conditions were determined, the robustness was tested. To evaluate the functionality of the assay, the activity of two additional bacterial TAL enzymes, RsTAL and SeSAM8, were tested.

Results

Optimal FjTAL expression is obtained after incubation for 24h at 22°C. Ideal assay conditions consist of a 80/20 ratio of the 1 mM Tyr assay mix and bacterial

FjTAL lysate. During the robustness test, Z' values > 0,4 and signal window values > 2 were observed with only minor edge and drift effects under the optimized conditions. The HTS assay proved to be able to detect bacterial TAL activity as RsTAL (5,718.10-3 ± 0,21.10-3) and SeSAM8 (4,658.10-3 ± 0,37.10-3) activity was observed.

Conclusion

A robust, simple and cost-effective HTS assay was developed to evaluate the activity of bacterial TAL enzymes. ■

20* Human testicular organoids for high throughput applications: which is the ideal tissue source?

S. Silva, G. Richer, K. Papageorgiou, T. Vanhaecke, E. Goossens, Y. Baert

New approach methodologies should include human derived models for a safer and faster replacement of animals. In reprotoxicity and endocrine disruption, human testicular organoids can be an important tool[1]. In testicular organoid formation, prepubertal tissue is the most efficient[2-4]. However, human immature testicular tissue is scarce, making it irrelevant for high throughput applications. Conversely, transgender tissue is abundantly available, and our histological preliminary data point to exhibiting similar characteristics to immature tissue. To confirm further our preliminary data, we performed RNAseq of transgender (n=6), and cisgender prepubertal (n=3), pubertal (n=3) and adult (n=5) tissue. To evaluate whether transgender tissue allows organoid formation, we derived organoids from transgender donors (n=8), as well as cisgender prepubertal (n=3), pubertal (n=3) and adult (n=3) and compared their architecture and testosterone production.
Transgender tissue showed gene expression patterns fitting between immature and maturing testicular tissue. This finding confirms a regression in maturity from transgender tissue. Regarding transgender organoids cell content, protein markers confirmed the presence of the main specific testicular cells: spermatogonia, Sertoli, Leydig, and peritubular myoid cells. Although transgender organoids did not show a seminiferous tubule-like architecture, compartmentalisation was present: core composed of peritubular myoid cells and extracellular matrix proteins, and lining periphery of Sertoli, germ and Leydig cells, similarly to pubertal organoids, and in a smaller scale to prepubertal organoids. On the other hand, adult organoids showed no organisation and limited cell assembly. Regarding testosterone production, prepubertal cultures showed the highest levels of testosterone, while the adult cultures showed the lowest. Levels in transgender cultures were within prepubertal and pubertal levels.

Transgender testicular tissue shows signs of rejuvenation due to the hormonal treatment underwent by the donors and can form compartmentalised organoids. Although transgender testicular organoids did not have an ideal organisation, these can still be a valuable alternative to organoids derived from immature tissue in high throughput applications. Future research should investigate how to correct the organoid's architecture and promote germ cell differentiation. ■

21* A benchmark dose based strategy for evaluating the combined effects of genotoxicants

J. Sanders, R. Anthonissen, G. Johnson, T. Vanhaecke and B. Mertens

Until recently, chemical risk assessment was strongly focused on single compounds. However, humans are exposed to chemical mixtures instead of just a single compound. Consequently, there is a need to evaluate the combined effects of co-occurring chemicals on human health, including genotoxicity. Different types of combined effects have been described, whereby for non-genotoxic endpoints, the principle of additivity is assumed to generally apply.

This study aimed to investigate whether the principle of additivity is also justifiable for genotoxic mixtures. To this extent, two types of binary mixtures were evaluated for their potential to induce chromosome damage *in vitro*. The first consisted of two genotoxicants with a similar mode of action (MoA), i.e. ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS), two DNA-alkylating agents. The second mixture contained two genotoxicants with different MoAs, i.e. MMS and etoposide (ETP, a topoisomerase II inhibitor).

First, *in vitro* micronucleus data in TK6 cells for the individual compounds were collected in absence of S9 metabolic fraction. Next, benchmark concentrations of the two compounds inducing the same response level were calculated. Afterwards, the concentrations were used to select a range of binary mixtures expected to induce responses covering different parts of the concentration-response curve (low, moderate and high responses). The collected experimental data of the mixtures were compared to the responses predicted based on the data of the individual compounds using the PROAST dose-addition model in R.

The experimental results of the EMS-MMS mixtures were close to the fitted curve based on the data of the single compounds, indicating that the principle of additivity is applicable to mixtures of EMS and MMS. Analysis of the mixture results with MMS and ETP is currently being finalized. The outcome of this analysis will provide insights on whether or not additivity also applies to genotoxicants with different MoAs. ■

22* The role of intestinal epithelial cells in gut barrier dysfunction in Alcohol Use Disorder (AUD)

A. Toulehohoun

Introduction

More than 3 million deaths worldwide are linked to alcohol abuse. Alcohol abuse associated gut barrier dysfunction is thought to play an important role in the development of alcohol-associated liver disease. Although some aspects that contribute to this process have been elucidated, the role of intestinal epithelium, a major component of the gut barrier, and its alterations in gut barrier failure in Alcohol Use Disorder (AUD) remain poorly understood. Our preliminary data on duodenal epithelium in humans showed/indicate a disturbed proliferation- differentiation program in AUD patients. The present project aimed to understand the pathways underlying these alterations and design an Enteroid model of AUD in order to modulate these pathways.

Methods

Wnt/β-catenin and EGFR pathways involved in the proliferation and the Notch pathway involved in the differentiation in the crypt are investigated by Western Blot and qPCR. Enteroids were generated by isolating crypts from duodenal biopsies and cultured in 3D on a Matrigel (basement membrane) support for up to 14 days. They were then incubated with various concentrations of ethanol (40 mM, 70 mM, 100 mM). Cytotoxicity, viability and proliferation were assessed.

Results and discussion

Results showed an alteration of the Wnt pathway. On the other hand, only concentration 100 mM displayed a slight increase of proliferation on enteroids in ethanol presence without affecting cell viability. Further investigations will aim to reproduce the proliferation on AUD patients enteroids and modulate those pathways. ■

23* Unraveling tumour microenvironments: 3D models of liver cancer and metastasis

E. Knetemann, N. Eysackers, L. van Grunsven and I. Mannaerts

Introduction

Liver cancer is a leading cause of cancer-related deaths globally, with a significant portion of cancer metastases occurring in the liver. Unfortunately, the failure rate

of drugs in clinical trials currently stands at a staggering 90%. This is partly due to the use of preclinical *in vitro* or *in vivo* models which are not representative enough for the course of the disease. We aimed to develop a mouse-derived 3D scaffold-free *in vitro* models for liver cancer and metastases.

Methods

To construct our model, we isolated hepatic stellate cells (HSCs), Kupffer cells, liver sinusoidal endothelial cells, and hepatocytes from mouse livers using flow cytometry and density gradient techniques. These cells were then combined with either a mouse hepatoma (Hepa1-6) or a mouse colon adenocarcinoma (MC-38) cell line and seeded in 96-well cell-repellent plates. We performed cell viability assays and picrosirius stainings on day 2 or 7of culture.

Results

When the cancer cells are seeded together with the other liver cell types they survive and proliferate. However, when cultured without the presence of liver cells, both the Hepa1-6 and MC-38 cells experienced cell death. Moreover, we observed a tendency of the HSCs to activate towards a more cancer-associated fibroblast phenotype as evidenced by increased collagen deposition in the spheroids in the presence of the cancer cells.

Discussion

Using this model, we have shown that the liver microenvironment is essential for the support of liver cancer cell growth. Furthermore, we show that our model has the potential to mimic HSC to cancer associated fibroblast transdifferentiation. In the future, this model could be used as a screening tool for potential drug candidates before moving to *in vivo* models. Since we can generate 600 spheroids from one mouse liver for *in vitro* testing, we significantly reduce the reliance on animal testing. ■

24. Validation of an animal-free analytical method for the detection of bacterial endotoxins in human vaccines at Sciensano

F. Laurent

Endotoxins are lipopolysaccharides anchored at the outer membrane of gram-negative bacteria. These pyrogenic compounds can be introduced in pharmaceutical products during their manufacture and could induce severe physiological reactions in humans. This is why endotoxins are dosed, in order to assure quality and safety of products. Testing of endotoxins is well described in the Ph. Eur. and several detection methods exist, such as animal-derived Limulus amoebocyte lysate (LAL) assays, which are widely used. However, these have limitations such as the use of animals, a high lot to lot variability and interference of complex components such as beta-glucans. Since 2021, assay using recombinant factor C (rFC) is considered as alternative method in the Ph. Eur. This method inspired by the LAL assay has the advantage to be animal free and to avoid interference of beta-glucans. In case of a highly complex matrix, a ligand-based assay can help to obtain valid results by including a purification step before a “classical” rFC assay, thanks to the use of phage-derived receptor protein coated to the microplate’s wells.

Sciensano, as an Official Medicines Control Laboratory (OMCL) following OCABR guidelines, determines the concentration of endotoxin in the final lot of several vaccines for human use. During decades, LAL assay was used for this purpose. In 2023, the rFC assay has been validated for the endotoxin detection in the final lot of more than 10 different vaccines. During the validation, LAL, rFC and ligand-based assays have been compared. It has been shown that the most appropriate technique depends on the type of vaccine. However, the rFC assay is suitable for all vaccines tested at Sciensano. The sensitivity increases between the LAL and rFC technique except for one type. However, the expanded uncertainty of measurement remained the same between LAL and rFC (around 50%), which was not expected. ■

25. The right tool for the job: Why and how to adapt your science communication for in-person and virtual events

M. Piumatti, F. Busquet

Science communicators face increasingly daunting challenges as they strive to reach diverse audiences with different interests, simplify complex messages without sacrificing accuracy, and engage beyond their immediate community. Overcoming these hurdles requires communicators to employ a range of tools, as each is best suited to specific situations. While podcasts excel at describing the latest scientific discoveries, they lack direct audience interaction. In-person events, although more effective in some cases, introduce additional complexities. Unfortunately, there is no one-size-fits-all approach to mastering science communication. Trial and error is often necessary to determine the most effective methods for each situation and to find personal comfort. Engaging in discussions with fellow science communication practitioners and sharing experiences provides an excellent starting point for improvement.

In this proposed talk, I will delve into the various formats I have encountered and practiced throughout my science communication journey, highlighting my recent experiences with two innovative tools: the live-streaming show TOXstreams and the board game TATAbox, with a focus on the 3Rs. Drawing from my direct experiences, I will emphasize the differences between in-person and virtual events. For instance, I will discuss my involvement with the science outreach organization Pint of Science and my participation in the "I Love Science Festival" in Brussels. Additionally, I will share insights from my experimenting with podcasts and videos, specifically focusing on my live streaming show, TOXstreams, which combines elements from live talk shows, journalistic emissions, and stand-up comedy. As science communicators, it is crucial to recognize the uniqueness of each individual we engage with and to be proficient in using various tools to effectively reach our target audience. While perfection may not always be attainable, practicing and refining our skills will contribute to the growth of both ourselves and the science communication community as a whole. ■

26. Beyond chemicals: the adverse outcome pathway (AOP) framework embracing diverse stressors to bridge knowledge on mechanisms of adversity

L-A. Clerbaux, J. Filipovska, P. Nymark, V. Chauhan, K. Sewald, M. Sachana, A. Beronius, M-J. Amorim, C. Wittwehr, I. Leclercq

The Adverse Outcome Pathway (AOP) framework emerged to accelerate evidence-based chemical risk assessment by leveraging existing knowledge and data from new approach methodologies. Owing to its stressor-agnostic approach to modeling perturbations of biological pathways, the framework has gained popularity across various scientific fields. This has raised new questions that required revisiting some key AOP principles. Challenges regarding AOPs related to nanomaterials include non-specific molecular initiating events, limited understanding of nanomaterial biodistribution and needs for adaptations of the *in silico* modeling and testing systems. In terms of radiation, continued discussions on how best to incorporate dose, dose-rate, radiation-type and time effects into AOP and on how to account for ionizing events targeting multiple macromolecules. Recently, development of AOPs for COVID-19 required the inclusion of the SARS-CoV-2 replicative steps enabling to capture the essential events driving the disease while re-using knowledge of adversity triggered by chemicals. Developing immune-related AOPs to evaluate both the efficacy and toxicity of cell therapies necessitates addressing the cellular nature and therapeutic function of the stressor. Finally, COVID-19 AOPs can be inspirational for addressing toxicity and unique proprieties of emerging biological stressors like microbial pesticides.

Interestingly, the adaptations needed to expand the AOP applicability beyond chemicals revolve mainly around the initial interactions of the stressors with the biological systems. While downstream key events, such as inflammation, are shared by many AOPs initiated by various stressors. Hence, by embracing diverse stressors, the AOP framework might be seen as a powerful bridge between human toxicology, disease biology and ecotoxicology.

Here we aim to present the challenges and opportunities of the multidisciplinary AOP-aligned approach and of the adoption of the AOP framework to different types of stressors in order to support the increased uptake of AOP framework across scientific disciplines. ■

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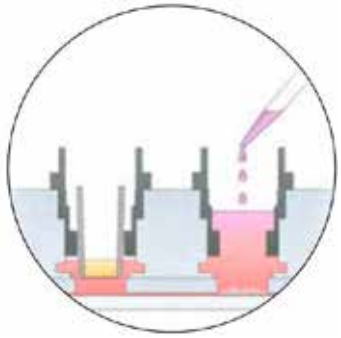

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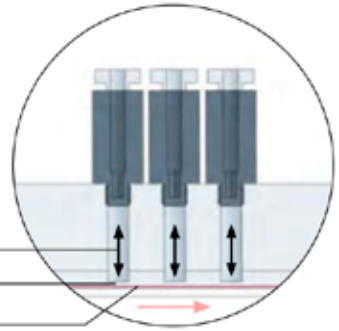

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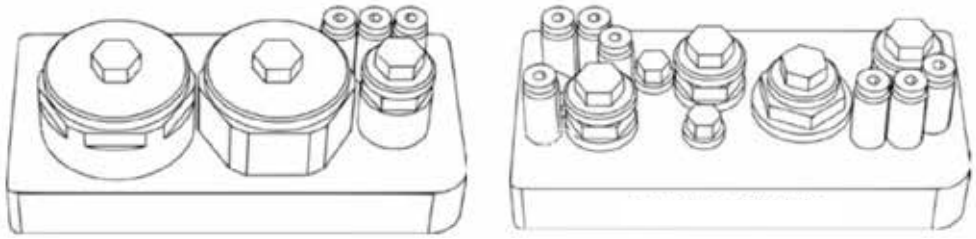

Villa Samson

Een beestig medicijn

In Villa Samson stimuleren we genezing door contact tussen mens en dier. In onze beestig leuke Villa kunnen gehospitaliseerde patiënten genieten van een moment met hun trouwe huisdier. Voor patiënten die zelf geen huisdier hebben, is het een plek waar genezing wordt gestimuleerd via therapie met dieren. De Villa is een warme, huiselijke plek net naast het UZ Brussel.

Door het contact tussen mens en dier mogelijk te maken, levert Villa Samson een positieve bijdrage aan het genezingsproces en het mentale en emotionele welzijn van patiënten.

Even weg uit de ziekenhuiskamer, een lekker kopje koffie en een knuffel van een trouwe viervoeter. Het doet wonderen voor de strijdvaardigheid en de mentale en fysieke weerbaarheid. Een bezoek aan Villa Samson is volledig gratis voor patiënten. En dat willen we zo houden. Jouw steun is essentieel voor het voortbestaan van onze unieke zorgmissie. Je zorgt er o.a. voor dat we de patiënten kunnen ontvangen, onze huiskatten kunnen verzorgen en de verwarming kunnen opzetten. Je bent een bron van hoop en troost voor velen!

Steun de unieke zorgmissie van Villa Samson met een online gift via www.villasamson.be of op BE75 3630 9458 5851 met melding 'Villa Samson'.

Wil je Villa Samson bezoeken? Of ons op een bijzondere manier steunen? Neem contact op met onze coördinator, Vicky De Baere.
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VILLA SAMSON

Thank you!

Organizing a 3-day symposium is undeniably a formidable undertaking. It involves a multitude of tasks, commencing with the creation of an engaging and interesting title. Furthermore, it necessitates the invitation of distinguished speakers renowned for their experience and expertise in the field. Equally important is the meticulous presentation of our young scientists, providing them with a platform to showcase their research findings so that the shift from *in vivo* to *in vitro* methods can be pursued in the near future in a dynamic way!

Our primary objective is to enhance human health, an aspiration intrinsically linked to the development and use of alternative research methods that ideally eliminate the use of experimental animals or at least reduce the numbers of animals involved and working in a humane way. Therefore, we are immensely grateful to all Colleagues and Friends who have made invaluable contributions to the planning and success of this Joint Symposium.

Our sincere thanks go of course also to all Speakers, Participants and Sponsors, as without them there would be no Symposium! Witnessing such a significant level of interest and engagement from our participants is truly heartwarming. Slowly but steadily, the pieces are falling into place, and we eagerly anticipate the remarkable work that lies ahead.

Wish you all the best and see you back at the next Symposium !

Prof. Tamara Vanhaecke
VUB, TWINALT, IC-3Rs

Dr. Birgit Mertens
Sciensano, RE-Place

Em. Prof. Vera Rogiers
VUB, TWINALT, RE-Place, IC-3Rs

Prof. Edyta Reszka
NIOM, TWINALT