Meeting Report

Non-Animal Models: Complexity for Interactions… Connecting Science

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The International *Fifth Virtual Summer School Lake Como School of Advanced Studies* "Non-Animal Models: Complexity for Interactions… Connecting Science" (https://namo.lakecomoschool.org/), held on May 15-16, 2024 and chaired by Francesca Caloni, Università degli Studi di Milano, Department of Environmental Science and Policy, was focused on innovative *in vitro* methodologies like 3D cultures, spheroids, organoids, and integrated microfluidic perfusion systems (MPS) and their interplay with other advanced technologies, also involving ethical considerations. The Summer School was attended by young scientists from all over the world with different scientific backgrounds.

Francesca Caloni gave a brief introduction. Non-animal methods have applications way beyond toxicology in multifaceted biomedical research; however, they need to overcome multiple barriers to gain acceptance and replace animal testing (Sewell et al., 2024).

Giulia Ranaldi, Food and Nutrition Research Centre, Council for Agricultural Research and Economics, CREA-AN, Rome held a presentation entitled "*In vitro* intestinal toxicity of micro- and nanoplastics". Fragmentation of plastics across their life cycle produces debris and particles of mixed morphology, chemical composition, and size, including in the micron and nanometer range, defined as micro-nanoplastics (MNPs). MNPs represent an emerging class of contaminants that have been detected in almost every biosphere environment. Despite MNPs constitute a potential public health concern, MNP risk assessment remains to be addressed since substantial data gaps regarding MNP classification, detection, exposure as well as toxicity have not been clearly determined. Human exposure has been demonstrated as plastic particles have been detected in human organs, blood, and in breast milk. Ingestion of MNPs from contaminated food and water represents the main way of human intake, thus understanding MNP exposure and the impact of MNPs on the gastrointestinal system is fundamental to evaluate possible harmful effects. *In vitro* intestinal systems represent a valuable approach to study intestinal toxicity and are also used for studying MNP intestinal impact. Intestinal absorption mechanisms, toxic effects, and intestinal barrier perturbation are under investigation using 2D intestinal differentiated *in vitro* cell lines, 3D intestinal organoids, as well as microfluidic systems. Although studies are still at a preliminary stage, results indicate the ability of MNPs to cross intestinal epithelia by endo- and transcytosis. Potential toxic mechanisms including oxidative stress, inflammatory challenge, and perturbation of barrier function have been suggested, and the role of the different cell types residing in the intestinal mucosa has been highlighted. However, experimental protocols are relatively heterogenous, and results are sometimes contradictory; therefore, further studies are necessary to better understand MNP effects on intestinal systems.

Helena Kandarova, CEM & FChFT Bratislava, held a lecture entitled "Challenges and complexities in medical device testing *in vitro*: from 2D to 3D models". The evaluation of biocompatibility is pivotal for ensuring the safety and efficacy of medical devices (Kandarova and Pôbiš, 2024). Traditional testing methods, which primarily utilize animal models and basic *in vitro* cytotoxicity tests as prescribed by ISO 10993, are foundational yet often inadequate in mimicking the complex biological interactions of human tissues. This shortfall becomes increasingly significant as medical devices grow more complex, integrating various materials intended for diverse application scenarios. Standard biocompatibility assessments such as cytotoxicity, irritation, and sensitization are mandated for most devices, but additional evaluations like genotoxicity, systemic toxicity, hemocompatibility, and implantation studies may be necessary based on the device's nature and intended use. Despite these requirements, the medical device industry continues to rely heavily on animal testing, with a slower adoption of alternative methods compared to other sectors. The field of tissue engineering has seen significant advancements leading to the development of three-dimensional human reconstructed tissue models, including spheroids, organoids, and planar systems. These innovative models have been incorporated into regulatory assessments for chemicals, cosmetics, and pesticides, and are increasingly recognized in the medical device sector. The recent integration of the 3D human epidermis model into ISO 10993-23, following successful validation trials, marks a pivotal development. The use of sophisticated *in vitro* models, enhanced by technologies such as microfluidics and 3D printing, suggests a potential shift in medical device safety assessments. These technologies enable the creation of physiologically relevant models that closely replicate human organ structures and dynamics, thus improving the accuracy of biocompatibility tests. The presentation discussed the challenges and complexities of medical device testing, but also the urgency for evolving regulatory frameworks to facilitate the adoption of these advanced methodologies, promoting more accurate, ethical, and scientifically rigorous biocompatibility testing.

Thomas Hartung, Center for Alternatives to Animal Testing (CAAT), Baltimore, focused his talk on "Alternatives to animal testing: The now, the new, and the next" The NOW: Despite growing discomfort and doubt in its scientific value, animal testing remains the dominant technology in toxicology, with some replacements by rather simple cell cultures. The NEW: The advent of stem cells and bioengineering has made human microphysiological systems (MPS) available, which replicate aspects

of organ architecture and function (Marx et al., 2020). They are ready for validation, but we have to adapt quality assurance and reporting as well as the validation process. The NEXT: The synergy of data generation and improvement of computers and algorithms has increased the power of artificial intelligence (AI) more than a billion-fold since the term was coined in 1956: Data in the world double every 18 months, i.e., 90% of all data were produced in the last three years; computers double in capacity every 24 months (Moore's law), and AI algorithms double in capacity every 3 months since 2010. For most human skill tests, AI performs better than 90% of us. For toxicology and safety pharmacology, AI promises support for data retrieval, evidence integration (systematic reviews, risk assessments), predictive toxicology of untested compounds, digital pathology, and support in reporting (Hartung, 2023a). The prospects of animal replacement with better accuracy in (human) prediction, ethical benefits, and cost-effectiveness are enormous (Hartung, 2023b). Beyond this, accelerated assessments with automated data analyses, real-time monitoring, and complex analyses come into reach with user-friendly prediction tools. These changes also promise to democratize knowledge, encourage open-access databases, algorithms, and publications. As a copilot for toxicology, it empowers researchers, regulators, consumers, and industry (Kleinstreuer and Hartung, 2024). Combining brain MPS with AI, the new field of organoid intelligence (OI) (Smirnova et al., 2023) was created, which seeks to establish learning and memory-in-a-dish, with opportunities to cover some of the most complex neurological hazards with functional endpoints.

The lecture presented by **Doris Wilflingseder**, Institute of Hygiene and Medical Microbiology, Medical University of Innsbruck and University of Veterinary Medicine, Department of Pathophysiology, Vienna, was titled "Infectious disease models 3.0". The growing spread of emerging infectious diseases, such as COVID-19 or resistant pathogens, indicates the need to speed up research on repurposing already approved drugs or testing novel innovative compounds. Since effective drugs or vaccines must induce both humoral and cellular responses against pathogenic challenges, novel alternative human approaches are needed including cellular and human immune components, and improved methods for delivery must be tested. Rapid developments in high-content screening as well as organotypic cultures provide groundbreaking new tools to study pathogen transfer at entry sites or to test novel treatment strategies. Therefore, we design optimized intelligent human barrier models combined with infection-relevant immune cells and humoral components to characterize and hinder overshooting host responses, pathogen entry, and initial transmission steps within a 3D system. These human systems offer improved power to test delivery methods, adjuvants, repurposing of drugs or novel vaccination approaches in high throughput. Using these models, we could demonstrate that: (i) C5aR inhibition of epithelial cells suppressed inflammation as assessed by local complement production, anaphylatoxin, and pro-inflammatory cytokine release, and maintained epithelial integrity in SARS-CoV-2-infected primary human airway epithelia (Posch et al., 2021), (ii) antiviral sprays effectively shielded airway epithelia from infection with different SARS-CoV-2 variants of concern (Zaderer et al., 2023), influenza A and B (Zaderer et al., 2024), and (iii) corticosteroids created a suppressive microenvironment and promoted fungal invasion in epithelial/immune respiratory models (Luvanda et al., 2021). These human systems offer the opportunity to evaluate novel therapeutic intervention strategies or test drugs or vaccination efficiency in a personalized manner.

Arno C. Gutleb, Environmental Sustainability Assessment and Circularity (SUSTAIN) Unit, Luxembourg Institute of Science and Technology, presented "Complex – more complex – most complex – but why?" In recent years *in vitro* systems have become increasingly complex in an attempt to better mimic potential interactions between different cell types in tissues. Such *in vitro* systems can consist of several cell types cultured in 3D orientation using inserts with porous membranes, membranes with a three-dimensional structure, 3D-scaffolds, and organ-on-a-chip format. Cell models can make use of cell lines, primary cells, and increasingly also induced pluripotent stem cells (iPSCs). Another level of complexity added to such models can come from the combination of *in vitro* models representing different organs or adding physical stress such as pressure, membrane tension, or change of oxygen levels. The final question, however, is what is the added value of such increasing complexity? The added value of combining different cells into a complex 3D *in vitro* model has been shown for a model representing the alveolar barrier including immune cells. Gene expression data showed an increasing correlation between transcriptional profiles observed in the alveolar barrier model when adding immune cells to the simple barrier model and normal human lung tissue (Marescotti et al., 2019).

Hassan Rashidi, NIHR Great Ormond Street Hospital Biomedical Research Centre, UCL Great Ormond Street Institute of Child Health, University College London, presented a lecture entitled "*In vitro* platforms to assess liver toxicity". Drug-induced liver injury (DILI) poses a significant challenge in drug development and patient safety assessment. Non-animal models offer promising alternatives for studying DILI mechanisms and drug toxicity in a controlled and ethically acceptable manner. However, the complexity of liver physiology and the intricate interactions between different hepatic cell types present challenges in recapitulating the dynamic microenvironment of the liver *in vitro*. The current landscape of non-animal models for studying DILI was discussed, including 2D and 3D cell culture systems, organ-on-a-chip platforms, and computational models. The advantages and limitations of these models in simulating hepatotoxicity, metabolism, and immune responses were explored, highlighting the need for multi-cellular and multi-organ interactions to accurately model DILI pathways. Additionally, recent advances in integrating liver models with other organ systems, such as the gut and immune system, to better mimic systemic drug effects and inter-organ crosstalk were discussed. Finally, future directions and challenges in refining non-animal models for DILI research were addressed, including standardization of protocols, incorporation of human-relevant endpoints, and validation against clinical data. Overall, non-animal models offer valuable tools for advanc-

ing our understanding of DILI mechanisms and improving drug safety assessment, but further efforts are needed to enhance their complexity and predictive capabilities.

During the Summer School, the participants were invited to address some questions related to the topics that were covered and to express their vision on adoption of NAMs in toxicology.

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