Meeting Report

Non Animal Methodologies (NAMs): Research, Testing, Assessment and Applications – *ecopa* Symposium 2019

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The *ecopa* Symposium 2019 was organized by the Italian Platform on Alternative Methods[1] (IPAM) at the Istituto Superiore di Sanità (Italian National Institute of Health, Rome, Italy) in November 2019 on behalf of the European Consensus-Platform for Alternatives[2] (*ecopa*). *ecopa* brings together European National Consensus Platforms that promote the 3R principle (Reduction, Refinement and Replacement of the use of animals in research) and strives for consensus among the four stakeholder groups: government and regulatory authorities, academia, industry, and animal protection and welfare organizations. Representatives of academia, institutional entities, non-governmental organizations, companies and research centers attended the event.

The opening session, chaired by **Philippe Hubert** and **Tuula Heinonen**, respectively President and Vice-President of *ecopa*, aimed to introduce the overall regulatory and scientific context of non-animal testing. **Gabriele Aquilina**, from the National Center of Chemicals, Cosmetics and Consumer Protection (CNSC) at ISS in Rome, introduced the OECD decision process that leads from an assay to a guideline. OECD Test Guidelines (TG) are fundamental references for scientists and regulators involved in risk assessment. The OECD Environment, Health and Safety (EHS) Programme is specifically dedicated to develop and co-ordinate activities on chemical safety and biosafety on an international level. In this framework, the Test Guideline Programme continuously revises the TGs in order to update existing, develop new and delete obsolete TGs. Proposals for the development of new or updated TGs can be submitted via a National Coordinator by member countries, scientists, industry or NGOs. The Working Group of National Coordinators (WNT) receives the submitted proposal that then is assessed by scientific experts of the member countries. Once approved by the WNT, the proposal is submitted to the EHS Joint Meeting for formal adoption.

Andrea Gissi, from the European Chemicals Agency (ECHA, Helsinki), gave an overview of the REACH Regulation (Registration, Evaluation, Authorization and restriction of Chemicals, CE n.1907/2006) and of the data requirements for the registration of a chemical. Under REACH, industry has to prepare registration dossiers containing, among others, hazard information on their substances. REACH requires the use of animal tests only as a last resort and offers the possibility for industry to instead fulfil the information requirements by using alternatives to standard testing, i.e., non-animal methods (NAMs). However, data from NAMs are accepted only when the reliability of their outcome is duly justified, ensuring the achievement of a high level of protection of human health and the environment. The level of acceptance of NAMs depends on the complexity of the property (endpoint) under analysis. For some "simpler" (from a toxicological point of view) endpoints, such as skin irritation, *in vitro* approaches are now the standard information requirements under REACH. For "middle level" endpoints, such as bioaccumulation, the use of non-animal methods is possible as adaptations, if properly justified. For the "most complex" endpoints, such as reproductive toxicity, NAMs should be used as supporting evidence. ECHA promotes the use of NAMs by supporting OECD TGs and REACH updates that follow the 3Rs principle, developing tools such as the Quantitative Structure Activity Relationship (QSAR) Toolbox[3], providing training, and participating in international projects that explore the use of non-animal methods in the regulatory context.

The lecture "3Rs principle overview and overall state of the art" was held by **Augusto Vitale** from the Center for Behavioral Sciences and Mental Health at ISS in Rome. He discussed the origin of the 3Rs principle (De Angelis et al., 2019), which was centered on the idea of merging the culture of laboratory techniques with the humane treatment of laboratory animals, i.e., promoting their positive mental states. He presented recent applications of the principle, which show that the concept is still relevant and modern today, such as the use of: i) very young zebrafish embryos (Replacement), ii) fluorescent techniques in parasitology that reduce the number of mice required for a study (Reduction), and iii) wireless systems that make restraint unnecessary in neurophysiology research using non-human primates (Refinement). The application of the 3Rs principle must be verified case-by-case, however it must be considered as a sort of *forma mentis* to approach animal laboratory research.

The second session, chaired by **Tuula Heinonen** and **Costanza Rovida**, introduced *in silico* and *in vitro* approaches. **Pietro Cozzini**, from the Molecular Modelling Laboratory of the Uni-

1 https://www.ipamitalia.org/

2 https://www.ecopa.eu/

3 https://echa.europa.eu/support/oecd-qsar-toolbox

versity of Parma, illustrated the opportunities offered by computational methods as alternatives to reduce animal tests, money invested, and time spent (Cavaliere et al., 2018). A huge number of compounds are present in our life and, in particular, in our food. Every year, 500 to 1,000 chemicals are discovered, and it is unrealistic to test all of them using *in vitro* and *in vivo* assays. To identify possible endocrine disrupting chemicals (EDCs), computational screening can extract the most probable interactors of nuclear receptors (NRs), which are known downstream EDC-mediators. These *in silico*-selected compounds should be prioritized for *in vitro* and *in vivo* tests. Some real case studies have illustrated the usefulness of this approach, such as on bisphenols (Cavaliere et al., 2020), pesticide residues in water, food contact materials, parabens and PCBs, food additives and drugs, in particular a drug for multiple sclerosis. In all cases, a mixed set of computational techniques was applied, including ligand-based screening, docking/scoring, consensus scoring and molecular dynamics, based on a reliable 3D database.

Federica Madia from the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) of the European Commission's Joint Research Centre introduced the JRC's non-animal approach to cancer. The rising incidence and prevalence of cancer identified by the WHO are of serious concern. Scientific advances of the past twenty years have helped to describe major properties of cancer, enabling more sophisticated therapies. It is also clear that the management of relevant risk factors, including chemical exposure, can significantly reduce cancer occurrence worldwide. Due to increasing global chemical production, including novel compounds, chemical exposure patterns are foreseen to change, posing high demands on chemical safety assessment, and creating potential protection gaps. Thus, the assessment of carcinogenicity potential needs to evolve to keep pace with changes in the chemical environment and cancer epidemiology. There is a need to address the most prevalent cancers, make better use of human data and clinical screening approaches, use biomarkers of exposure, and use human biomonitoring to prioritize chemicals. In addition, a novel methodology under investigation at JRC was presented that aims to extrapolate information from traditional data streams across toxicity endpoints together with data from new assessment methods as a more holistic human-relevant and 3Rs-impactful approach to carcinogenicity assessment (Madia et al., 2019).

The third session focused on the applications of the 3Rs principle was chaired by **Francesca Caloni** and **Francesco Nevelli**. **Stefano Lorenzetti** from ISS spoke on the toxicological application of the 3Rs concept in the EDC-related regulatory field. He presented a proof-of-principle approach devoted to screen EDCs based on their ability to modulate endocrine-dependent, cell-specific biomarkers (Lorenzetti et al., 2015). Most mechanistic-based assays for endocrine disruption rely either on binding to a NR or transcriptional modulation of a gene reporter via the NR promoter. This approach has two main limitations: i) ED, by definition a mode-of-action (MoA), is reduced to the identification of a mechanism; and ii) such a mechanism is decided *a priori*. These limitations can be overcome by the use of clinically relevant, functional biomarkers selected based on their cell specificity and endocrine dependency. In a reverse approach, i.e., from bedside to bench, hormone-dependent biomarkers are used as toxicological biomarkers to bridge non-animal to animal testing. Examples discussed included the androgen-dependent secretion of prostate-specific antigen (PSA) in human-derived prostate epithelial cell lines and estrogen-dependent secretion of βhCG in human-derived trophoblast-like cell lines.

Laura Goracci, from the H-EcoTox laboratory, University of Perugia, gave a talk entitled "3D-hepatolipidomics as a proof-of-principle for an *in vitro* ADMET tool in drug discovery". Two examples of the use of 3D-InSight™ human liver microtissues (MTs; InSphero, Swizerland) for untargeted hepatolipidomics were described. In the first, the effect of fusariotoxins (deoxynivalenol/DON, fumonisin B1/FB1 and T-2 toxin) on MTs was discussed, showing how the proposed method is sensitive to mycotoxin concentration and duration of treatment. In the second example, a cheminformatics approach to assess the risk of clinical hepatotoxicity based on a dataset of drugs belonging to five different chemical classes and with various drug-induced liver injury effects (Goracci et al., 2020) was discussed. In both cases, Lipostar software (Molecular Discovery, United Kingdom) was used to cover all the steps of the analysis (raw data file conversion, peak detection, statistical analysis, lipid identification, lipid pathways interpretation). The outstanding aspect of this study is the possibility to simultaneously monitor multiple interconnected variables (lipids). The workflow can be applied to other microtissues.

Alexandre Fouassier, from Ncardia BV (Leiden, The Netherlands), presented a lecture on the development of cellular models of neurons and cardiomyocytes, derived from pluripotent stem cells, to be used for the identification of new drugs and the study of their activity and pharmacological safety. Traditional methods to assess cardiotoxicity have relied on *in vitro* overexpressing human cell lines or *in vivo* animal models. These *in vitro* models often lack the complexity of human cardiomyocytes, whereas animal models may lack predictivity due to inherent species differences. Therefore, there is a need for more predictive and specific assays that allow for multiparametric assessment of potential cardiotoxic side effects of new drugs in humans. Human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are promising as an alternative model for cardiac safety assessment, and they have the potential to improve drug discovery efficiency (de Korte et al., 2020). A multiparametric assay to measure potential cardiotoxic drug effects *in vitro* was developed. Both acute and long-term effects of anticancer drugs on impedance, electrophysiology (microelectrode array), and cardiac troponin I (cTnI) release were determined simultaneously, generating mechanism-specific cardiotoxicity profiles that allow a better prediction of drug-induced cardiotoxicity.

Alberto Rainer, from the Campus Bio-Medico University of Rome, presented a lecture on "Bioprinting and biofabrication for the development of alternative models". He focused on computer-aided tissue engineering for the fabrication of skeletal muscle models (Costantini et al., 2017). With the advent of additive man-

ufacturing technologies, the fabrication of 3D biological constructs has been increasingly automated, allowing the generation of more rationally designed bio-fabricated products. This led to a new generation of 3D constructs that are finding increasing application as advanced *in vitro* models for drug development and toxicology. Among the available technologies, microfluidic-enhanced 3D printing (μ-3DP) exploits a microfluidic coaxial needle setup coupled to photocurable biopolymers for the high-resolution fabrication of cell-laden hydrogel constructs. Targeting skeletal muscle applications, the favorable combination of biochemical cues and geometrical confinement provided by μ3DP result in enhanced support of myoblast differentiation in terms of myotube number, rate of formation, and space distribution. Hence, μ-3DP represents a promising strategy for the scalable fabrication of 3D engineered skeletal muscle in the direction of the replacement of animal studies by *in vitro* models.

Eliana Marina Coccia, from the Immunology Unit at the Department of Infectious Diseases of ISS, presented a lecture on "Optimization and implementation of non-animal tests in the quality control of human vaccines: pyrogen testing by monocyte activation test". She focused on the application of the monocyte activation test (MAT; Ph. Eur. 2.6.30) to assess the presence of pyrogens in vaccines (Etna et al., submitted). The introduction and implementation of the MAT test in vaccine quality controls and batch release will contribute to a reduction of the use of rabbits, which are historically employed to detect pyrogens in injectable medicines and biologicals (rabbit pyrogen test). The MAT is a species-specific test for the detection of pyrogens in products for human use as it assesses the release of tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β) and IL-6 by human monocytes. A ready-to-use cell-based method was achieved thanks to the collaborative environment and close partnership established between public and private entities interested in developing non-animal methods covering key parameters for demonstrating vaccine batch consistency and, therefore, safety and efficacy.

The last session, chaired by **Isabella De Angelis** and **Philippe Hubert**, focused on the toxicology of mixtures. The lecture "New Approach Methodologies to face the opportunities and challenge posed by exposure from mixture of chemicals" was given by **Tuula Heinonen** (Finnish Centre for Alternative Methods, FICAM, Tampere). EU agencies have started to tackle the complexity of interactions among multiple exposures to chemicals and the multitude of combinations deriving from them. Sources of chemical mixtures include food, feed, consumer products, and environmental sources such as air, soil and water. Mixtures may be of various physical states such as liquid, gas and particles. In case of intentional mixtures, the safety assessment strategy follows that for single substances. However, when the quantitative and or qualitative composition of substances in mixtures varies, a safety assessment is practically impossible, especially with animal-based testing strategies. Another challenge comes from the combined toxic effect of substances in mixtures, which can be additive, synergistic or antagonistic. Thus, there is an urgent need for NAM-based phenotypic *in vitro* methods covering the critical adverse outcome pathways (AOPs) or MoAs and, if possible, qualified marker substances in mixtures.

The *ecopa* Symposium also included a poster session for young researchers. The *ecopa* Award for the best poster was presented to **Beatrice Battistini** as first author of the poster "Preliminary assessment of nanoparticles in tattoo ink. *In vitro* and *ex-vivo* approaches".

The symposium was closed by **Philippe Hubert** from the French National Institute for Industrial Environment and Risks (INERIS) with an outlook. He called for the pioneering work in the field of regulatory toxicology and ecotoxicology to move forward and address new challenges. One challenge is to foster the development of the 3Rs in upstream research activities, since some researchers feel the 3Rs, especially replacement, to be a threat for the maintenance of their activities. An open dialogue within *ecopa* is essential to address this challenge. Another challenge is to build and borrow NAMs from innovations in the life sciences (e.g., bioengineering) but also from other technological fields such as imaging, microfluidics, and machine learning. The symposium showed how important it is to open alternatives to other disciplines, for example, clinical data and human biomarkers, and to provide a forum where people with different training, disciplines and roles can meet. *ecopa* aims to be this forum.

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Acknowledegments

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Meeting Report

3R-related Research Funding: Insights from a Meeting Hosted by the German Centre for the Protection of Laboratory Animals (Bf3R)

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Introduction

In spite of extensive efforts towards implementation of the 3R principle, including the establishment of 3R centers and tightening of legislations on animal welfare, the overall number of animals used for scientific purposes remains high in Europe. One of the fundamental goals of 3R research foundations is to significantly reduce this number by promoting the further development and acceptance of alternative methods to animal experimentation. The different measures proposed to reach this objective must meet two main prerequisites: I) Transparent knowledge transfer between scientists and funders regarding funding opportunities and II) definition of criteria for assessing the success of funding.

With this in mind, the German Centre for the Protection of Laboratory Animals (Bf3R) at the German Federal Institute for Risk Assessment (BfR) held a symposium in Berlin on September 20, 2019, focusing on 3R-related research funding. The agenda included short lectures on nationwide funding programs, keynote lectures addressing particularly illustrative examples of third party-funded 3R research as well as current strategic challenges concerning open science, a world café, and a poster session. In addition to these events, which were open for all participants, the symposium also included a separate session for representatives of the funding institutions, academic and research institutes, and the Federal Ministry of Food and Agriculture. Here, topics such as measures for attracting promising project ideas, strategies for assessing the success and impact of funded projects, and incentives for the publication of negative results or replication studies were intensively discussed.

Information on 3R funding opportunities in Germany

The first thematic block included short presentations on current 3R-related research funding programs in Germany. The representatives portrayed the cornerstones of their funding programs, including information on the frequency of the calls for proposals, deadlines, structure and length of the application, and possible funding volume, as well as hints for a successful application. The participant institutions were the German Federal Ministry of Education and Research (BMBF), which was represented by the