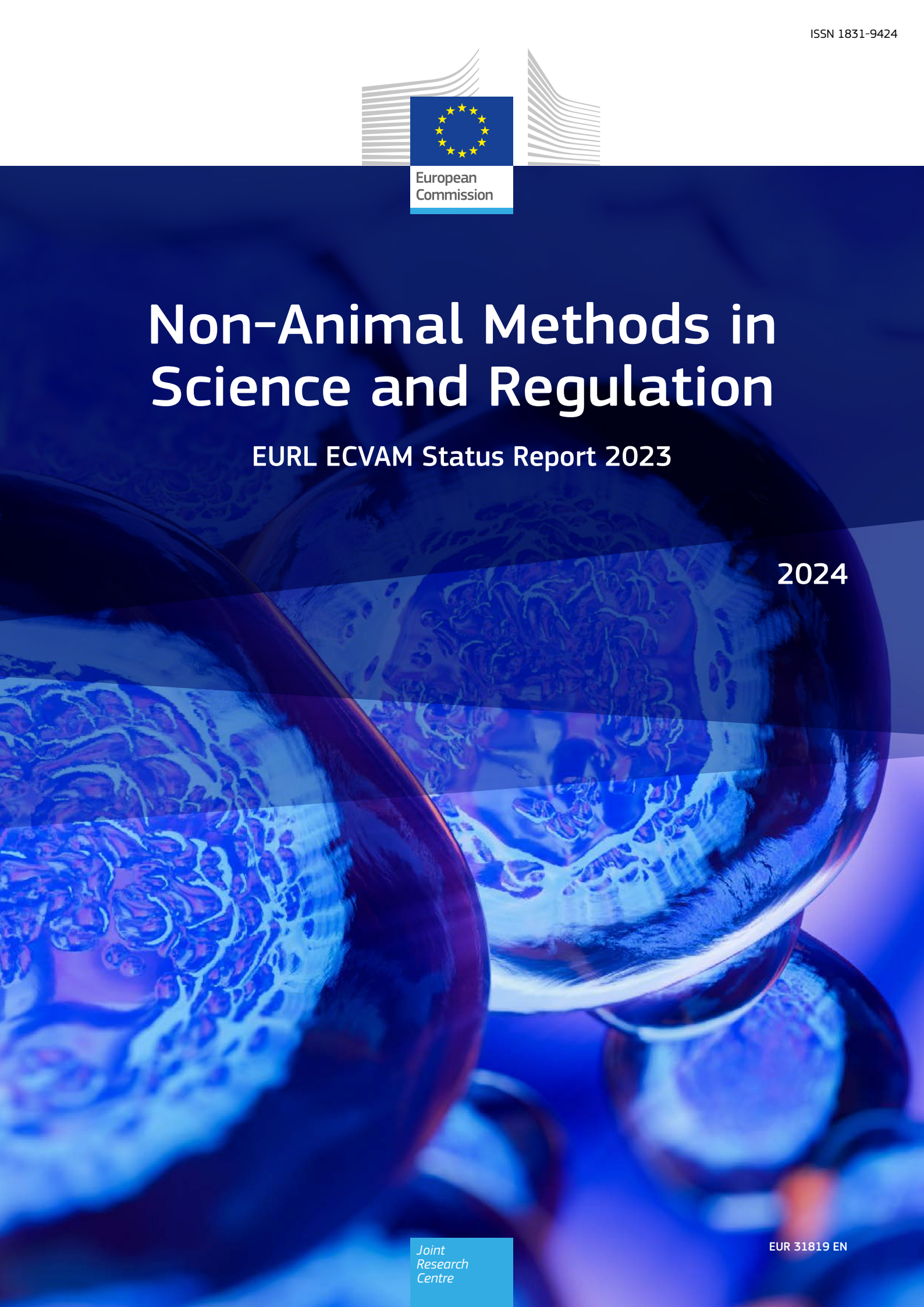
ISSN 1831-9424

European Commission

Non-Animal Methods in Science and Regulation

EURL ECVAM Status Report 2023

2024



Joint Research Centre

EUR 31819 EN

Manuscript completed in December 2023

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JRC136460

EUR 31819 EN

Print	ISBN 978-92-68-11756-9	ISSN 1018-5593	doi:10.2760/05390	KJ-NA-31-819-EN-C
PDF	ISBN 978-92-68-11755-2	ISSN 1831-9424	doi:10.2760/44273	KJ-NA-31-819-EN-N

Luxembourg: Publications Office of the European Union, 2024

CREDITS
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Chief editor: Zuang ,V.
Chapter editors: Worth, A., Corvi, R., Munn, S., Deceuninck, P.

How to cite this report: Zuang ,V. *et al.*, *Non-animal methods in Science and Regulation – EURL ECVAM Status Report 2023*, Publications Office of the European Union, Luxembourg, 2024, doi:10.2760/44273, JRC136460.

Non-Animal Methods in Science and Regulation

EURL ECVAM Status Report 2023

Contents

Abstract

The 2023 EURL ECVAM Status Report outlines research and development activities, along with initiatives that foster the implementation and utilisation of non-animal methods and approaches in scientific research and regulation. The Three Rs principle, which advocates for Replacement, Reduction, and Refinement of animal use in basic, applied, and translational research, as well as for regulatory purposes, is firmly established in EU legislation, with the ultimate goal of fully replacing animal testing.

New approach methodologies encompassing a range of innovative technologies, including *in vitro* methods employing 3D tissues and cells, organ-on-chip technologies, computational models (including machine learning and artificial intelligence), and 'omics (transcriptomics and metabolomics), are developed, evaluated, and integrated into assessment frameworks in order to enhance the efficiency and effectiveness of hazard and risk assessment of chemicals and products across various regulatory contexts. Furthermore, substantial efforts are directed at promoting the development and utilisation of non-animal approaches in fundamental and applied research, where the majority of animal testing occurs, as well as for educational purposes. The achievements and accomplishments documented in this report are the culmination of collaborative efforts with EURL ECVAM's dedicated partners and stakeholders.

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Executive summary

The 2023 EURL ECVAM Status Report provides an overview of the ongoing efforts to develop, validate, and apply non-animal methods in research and regulatory testing. It also highlights initiatives to promote the use of these methods in education and training programmes.

Development

The scientific community is making important strides in developing non-animal testing methods, driven by funding from the EU and collaborative research efforts. EURL ECVAM is at the forefront of these developments, providing valuable guidance, standardisation strategies, and support in promoting methods ready for regulatory acceptance. A few examples of such projects are provided hereafter.

The ASPIS cluster, powered by Horizon 2020 funding, includes three pivotal projects (PrecisionTox, ONTOX and RISK-HUNT3R) which aim to refine chemical safety assessment through New Approach Methodologies (NAMs) by leveraging genomics, *in vitro* and *in silico* techniques, and artificial intelligence (AI). More than 70 scientific organisations from 16 countries are involved in this initiative, which is designed to improve the accuracy, speed, and cost-effectiveness of chemical safety testing.

The ASPIS Regulatory Forum, facilitated by EURL ECVAM, aims to enhance dialogue between scientists and regulators to expedite the adoption of non-animal methodologies. Online sessions and workshops address various topics, such as the ASPIS Safety Profiling Algorithm (ASPA), to refine regulatory decision-making using NAMs.

EURION, consisting of eight Horizon 2020-funded projects, is entering its fifth year, transitioning from the discovery phase to selecting promising biomarkers and test methods for endocrine disruptor identification. This includes different projects focusing on thyroid hormone disruption, metabolic disruption, developmental neurotoxicity, and female reproductive toxicity. EURL ECVAM is actively supporting EURION through workshops and promoting method validation.

The PARC initiative, a seven-year Horizon Europe partnership with a 400 million Euro budget, involves nearly 200 institutions from 28 countries. It aims to develop and implement research to meet current and future chemical risk assessment needs. The JRC has established a formal collaboration with PARC to contribute to its governance and monitoring.

The VHP4Safety project, funded by the Dutch Research Council, seeks to improve the prediction of harmful effects of chemicals and pharmaceuticals, emphasising a holistic definition of human health. This project is developing a cloud platform and *in vitro* models for ADME processes, among other tools, to enhance safety assessment.

In Silico World (ISW), another H2020-funded project, focuses on overcoming barriers to the widespread adoption of In Silico Trials. The project has been developing solutions, providing validation data, and working with standard-setting bodies to establish new guidelines and promote the credibility of *in silico* methods.

Validation

Validation is a crucial component of the scientific process, especially in the context of 21st-century toxicology, which relies more and more on non-animal testing methods. By taking the lead, together with the United States and the Netherlands for updating OECD GD 34 on the validation and international acceptance of new or updated test methods for hazard assessment, EURL ECVAM is committed to modernising the validation process to ensure that new, more efficient non-animal methods are scientifically sound and suitable for regulatory use.

Regarding new test submissions, GENOMARK, uses a transcriptomics-based signature to distinguish between genotoxic and non-genotoxic chemicals in a hepatic cell line. Although promising, the method requires further development and reliability assessment before it can be adopted for regulatory purposes. The EURL ECVAM-coordinated validation study of *in vitro* methods for assessing thyroid hormone disruption arrived at its final stage. The EU-NETVAL network, consisting of 33 laboratories, supported the *in vitro* method validation process of 18 different methods. This study demonstrated the feasibility of assessing multiple methods simultaneously and emphasised the importance of method developer support, financial resources, and collaboration across EU-NETVAL laboratories and method developers. EURL ECVAM is also validating a high-throughput androgen receptor dimerization assay (AR2 assay) to identify androgen-disrupting chemicals. This assay uses a genetically engineered reporter cell line to measure luminescence as an indicator of androgenic effects.

The EURL ECVAM Scientific Advisory Committee (ESAC) provides independent scientific advice on the validity of non-animal methods. The ESAC oversees peer reviews

and has an open call for experts to join specialised subgroups. Two genotoxicity tests, the Reconstructed Skin Micronucleus (RSMN) and the Reconstructed Skin Comet (RS Comet) assays, have been validated by Cosmetics Europe and are undergoing an independent peer review by ESAC.

The EURL ECVAM Network for Preliminary Assessment of Regulatory Relevance (PARERE) facilitates updates on regulatory relevance of test methods and approaches and research in this field. EURL ECVAM is also involved in standardising complex test systems and technologies, such as Organ-on-Chip (OoC) devices. A Focus Group on OoC has been established to create a roadmap for standardisation, which includes best practices and regulatory considerations.

EURL ECVAM has contributed to OoC qualification efforts, including a workshop and participation in a ring trial for liver OoC devices. High-content imaging is another area where EURL ECVAM is working on standardisation to ensure data reliability and regulatory acceptance. Similarly, omics-based methods are being standardised, with EURL ECVAM taking part in initiatives like drafting the "Chemical Grouping – Application Reporting Module" and developing an omics data interpretation framework for regulatory application.

Overall, EURL ECVAM's efforts toward validating and standardising new non-animal testing methods are paving the way for their acceptance in regulatory toxicology, thereby reducing the reliance on animal testing.

Regulatory application

EURL ECVAM is actively involved in several EU and international regulatory activities to integrate non-animal science into chemical hazard and risk assessments. This involves collaboration with organisations like the OECD and UN GHS, as well as various EU regulatory agencies and stakeholders. EURL ECVAM is involved in advancing the application of non-animal testing methods in regulatory contexts, working on international guidelines, promoting new assessment frameworks, and addressing challenges in validation studies thereby contributing to the evolution of global chemical safety assessment practices.

In an attempt to increase the pace of chemical safety assessments and reduce the number of unassessed chemicals on the market, EURL ECVAM proposed "Chemicals 2.0," a new regulatory framework that would maintain current protection levels while simplifying the system, to be applied to all marketed chemicals, and supporting sustainability by design. Through a NAM Designathon challenge that is based on the vision of “Chemicals 2.0”, the European Partnership on Alternatives to Animal Testing (EPAA) invited the submission of NAM-based solutions to inform the development of such a future classification system for systemic toxicity to human health based on the activity and potential systemic availability of chemicals.

Alternatives in research and education

In 2020, the EU and Norway recorded over 5.5 million instances of animal use in biomedical research, a sector that accounts for 68% of all animal uses for scientific purposes. To address this, EU Directive 2010/63/EU requires that all scientific projects involving animals be evaluated with the Three Rs in mind.

EURL ECVAM launched various initiatives in 2023 to promote the use of non-animal models in biomedical research. These efforts included analysing the potential of advanced models to supplement ongoing animal-based projects and initiating a project to gather the most recent published models.

EURL ECVAM conducted a feasibility study by analysing non-technical summaries (NTS) of authorised projects on cardiovascular disease research. The data from these summaries allowed EURL ECVAM to pilot a thematic review aimed at enhancing the adoption of the Three Rs in this field.

A new project commenced to develop an automated database that collects and structures information on non-animal methods in biomedical research. This database is intended to become a comprehensive resource for various stakeholders, facilitating the transition towards human biology-based methodologies.

EURL ECVAM published an article and a technical report assessing the impact of EU-funded biomedical research in Alzheimer's disease, breast cancer, and prostate cancer using fourteen indicators. These publications aim to improve the translation of scientific innovation into societal impact.

EURL ECVAM released draft recommendations to enhance methodological clarity in life sciences publications. These recommendations target four stakeholder groups (researchers, research institutions and departments,

publishers and editors, and funders) and focus on capturing clear, accurate methodological details with reusable protocols.

The CIAO project successfully concluded, employing the Adverse Outcome Pathway (AOP) framework to model COVID-19 pathogenesis. This interdisciplinary and international collaboration resulted in disseminating COVID-AOPs, identifying knowledge gaps, and publishing several papers.

EURL ECVAM is reviewing innovations based on human data to understand ongoing European research projects and their challenges. The EC's EDITH[1] consortium is working towards developing a European 'Virtual Human Twin' (VHT), with EURL ECVAM examining how real-world data can be reused within the EU legal framework.

The JRC Summer School, titled "Non-animal Approaches in Science: Towards Sustainable Innovation", welcomed 120 international students. The program focused on non-animal methods and technologies, encouraging networking and active contributions to science without animal use.

EURL ECVAM developed a virtual reality (VR) application funded by the European Parliament to educate students about alternatives to animal testing. This application aims to provide an engaging learning experience for secondary school students, combining education on non-animal testing methods with STEM[2] concepts.

In summary, EURL ECVAM has made significant steps in promoting alternatives to animal testing in both research and education. By developing resources, conducting reviews, and engaging with scientific and educational communities, EURL ECVAM is contributing to a shift towards more humane and relevant scientific methodologies. These efforts are expected to enhance the impact of biomedical research and foster a greater understanding and acceptance of non-animal methods.

1 https://www.edith-csa.eu/

2 Science, technology, engineering, and mathematics

1. Introduction

The European Union (EU) is committed to minimizing animal testing through a framework of policies and legislation known as the Three Rs[3], replacement, reduction, and refinement. The ultimate goal is to replace animal testing with innovative, non-animal technologies wherever possible. However, in cases where animal testing remains necessary, it must adhere to the strict guidelines outlined in EU Directive 2010/63/EU on the protection of animals used for scientific purposes (EU, 2010).

Under this directive, animal testing can only be conducted if there are no viable alternatives and if the expected benefits outweigh the potential harm to the animals. Additionally, the use of animals must be ethically justified.

Replacing animal testing with advanced technologies is not only ethically sound but also aligns with the EU's commitment to scientific advancement and sustainable development with the aim of creating a safer world for both humans and the environment.

The development and utilisation of non-animal models and methods extend beyond regulatory testing, playing a crucial role in advancing basic, applied, and translational research. These innovative approaches provide scientists with more accurate and relevant tools to investigate complex biological processes, leading to breakthroughs in disease prevention and treatment.

The 2023 EURL ECVAM Status Report provides an overview of the ongoing efforts to develop, validate, and apply non-animal methods in research and regulatory testing. It also highlights initiatives to promote the use of these methods in education and training programmes.

EURL ECVAM, the European Union Reference Laboratory for Alternatives to Animal Testing, plays a crucial role in fostering the development of new approach technologies and advancing animal welfare. Its mandate, as defined in Directive 2010/63/EU, encompasses a wide range of responsibilities.

In the regulatory domain, EURL ECVAM supports a diverse range of EU policies covering a wide spectrum of chemicals and products, from industrial chemicals and plant protection products to biocidal products, medicinal products for human and veterinary use, toys, medical devices, and cosmetic products.

As an integral part of the European Commission's Joint Research Centre (JRC), EURL ECVAM is deeply committed to advancing animal welfare and promoting the development and use of non-animal alternatives in research and testing.

3 Three Rs and 3Rs are used interchangeably in this report


AI

2. Development

Scientists are making significant progress in developing non-animal testing methods, thanks to funding from the European Union and collaborations among researchers. EURL ECVAM is lending its expertise to these efforts in several ways, including providing guidance, sharing best practices for characterising and standardising methods, and assisting in the identification of promising methods that can be put into practice and accepted by regulatory bodies. The overall aim is to find and advance promising methods so that they can be used in real-world applications and gain regulatory approval.

2.1. Collaborative partnerships

2.1.1. ASPIS projects

With a view to developing NAMs for chemical safety assessment, three research projects funded under Horizon 2020 started their activities in 2021, led respectively by the University of Birmingham (PrecisionTox), the Vrije Universiteit Brussel (ONTOX) and Leiden University (RISK-HUNT3R). The three projects have joined forces in the so-called ASPIS cluster ("aspis" means "shield" in ancient Greek), which gathers more than 70 scientific organisations across 16 countries of the European Union, the United Kingdom and the United States. Its mission is to utilize all available knowledge across disciplines to improve the accuracy, speed, and affordability of chemical safety testing without the use of traditional laboratory animals. Building on advances in (i) comparative genomics, transcriptomics and metabolomics, (ii) robust *in vitro* and *in silico* methodologies and (iii) artificial intelligence (AI), ASPIS provides New Approach Methodologies (NAMs) to rapidly accelerate and improve chemical risk assessment in the EU and beyond. While the three 5-year projects are complementary to each other, they have common elements that form the basis of collaboration at cluster level. Recent highlights of the three projects are given in **Box 2.1, Box 2.2** and **Box 2.3**.

The JRC (through EURL ECVAM) has set up a formal collaboration with each of the three projects individually, and also contributes to activities at cluster level.

Box 2.1. PrecisionTox

The overarching goal of PrecisionTox is to propose a new assessment framework for health protection from exposure to chemicals based on observable mechanistic processes leading to toxicity. Emphasis is placed on uncovering the root causes of toxicity from the disruption of critical biological processes that are broadly shared among animals including humans by evolutionary descent, and by taking into consideration genetic variation in individual susceptibility. This is accomplished by making use of a suite of alternative biomedical model species that have already transformed our understanding of the human condition in relation to the root causes of disease. Moreover, PrecisionTox does not rely on the strength of science alone to engineer change, but also addresses the socio-technical and legal barriers to the uptake of new approach methodologies.

Recent highlights include:

- Implementation of improved automation solutions for higher-throughput and high-content imaging for phenotyping adverse effects.
- Cross-species comparison of dose-response and adverse phenotypes for nearly 100 chemicals that uncovers the phylogenetic signature of toxicity and the species conservation of adverse outcomes.
- Chemical grouping based on toxicological response profiles across the five model species and human cells according to chemical classes, modes of action and functional targets.
- Empirical evidence for the optimal dose and time of sampling for obtaining multi-omics signatures of the chemical modes of action across test systems.
- Extensive genetic variation and profound sexual dimorphism for genetic susceptibility to heavy metals (arsenic and cadmium chloride) and 4-methyl imidazole among a subset of 1038 wild-derived inbred and fully sequenced lines of the Drosophila Genetic Reference Panel.
- First report on the socio-technical barriers to the uptake of NAMs.
- Progress towards a proposed Group First, Regulate Better paradigm.

Website: https://precisiontox.org

Coordinator: John Colbourne, University of Birmingham, Centre for Environmental Research and Justice

Box 2.2. ONTOX

The vision of the highly interdisciplinary and intersectoral ONTOX consortium is to provide a viable and sustainable solution for advancing human risk assessment of chemicals without the use of animals in line with the principles of 21st century toxicity testing and next generation risk assessment.

Recent highlights from the Work Packages (WPs) include:

- WP1 advanced with physiological map creation; liver lipid metabolism and bile secretion maps are both standardised and annotated. The nephron map was standardised with annotation nearly complete. Progress on the neural tube closure map, with standardisation finished and annotation underway, while submap creation of the brain development is in progress. All maps are readily accessible via stable, permanent links on the MINERVA platform. Additionally, 40 foetal brain transcriptomes have undergone sequencing, mapping and quantification.
- WP2 developed a model to predict drug-induced cholestasis outcomes based on accessible clinical parameters and drug properties. It also developed quantitative systems models for common AOP KEs/KERs and drafted a comprehensive quantitative model of an entire AOP network, both capable of integrating with toxicokinetics.
- WP3 developed computational models to predict the capability of chemicals to interact with endogenous targets (enzymes, receptors) relevant for the MIEs of the project's AOPs. Models were developed with machine learning and multi-tasking methods and will ultimately be made available in the ONTOXHub.
- WP4 developed a High-throughput (HTP) Physiologically Based Kinetic (PBK) model to predict *in vivo*

simulations after oral uptake. In parallel, several *in vitro* distribution (mathematical) models to refine *in vitro* nominal to free concentration were also applied.

- WP5 revamped the concept of Probabilistic Risk Assessment with a White Paper discussing it in the context of the advent of AI-enabled methods; developed BioBricks, a one-line import command interface for all major public databases for chemical toxicity (also supported by US NIEHS and NSF); expanded the SysRev.com tool for automated systematic reviews to data extraction and automated feed into Physiological Maps and AOP Networks.
- WP6 developed probabilistic models for external exposure linked to kinetic modelling and developed a protocol for probabilistic risk assessment (integrating probabilistic exposure prediction, probabilistic hazard prediction and probabilistic risk assessment tools); established and maintained collaboration with a range of stakeholders to facilitate end-user acceptance by creating real live data and developing case studies to demonstrate the Probabilistic Risk Assessment approach.
- WP7 developed a machine-learning *in vitro* data-driven approach to identify a transcriptomic signature to predict chemical-induced cholestasis. Furthermore, the AOP networks on chemical-induced cholestasis and steatosis were updated using artificial intelligence-assisted data collection, subsequently accompanied by confidence level quantification.
- WP8 developed an AOP network for toxicant-induced kidney failure and developed a test battery of *in vitro* assays to monitor this.
- WP9 used human cell-based *in vitro* assays to study Key NeuroDevelopmental Processes (KNDP) crucial for brain development. With a focus on the adverse outcomes of 'Decreased cognitive function' and 'Impaired learning and memory', WP9 created physiological maps, an AOP network, and focused on characterising the *in vitro* assays for gaining confidence in the DNT *in vitro* battery (IVB). DNT assays were used to link KNDP to human neurodevelopmental disease. A computational model was developed to predict neural tube closure defects.
- WP10 steered the research and non-research aspects of the project through an established multi-level management structure, which resulted in a positive assessment of the 1st periodic report by the EC, strengthened the collaboration with the other two ASPIS consortia (PrecisionTox and RISK-HUNT3R) through continuous cluster co-coordination and established links with external projects (VHP4Safety, PARC).
- WP11 expanded the ONTOX data collection in BioStudies, which contains re-used as well as newly generated transcriptomics data from WP2, ToxTemp and DB-ALM-SOP entries from WP7-9, physiological maps from WP1 and an overview of important key events.
- WP12 supported the organisation of eight training sessions and three webinars to facilitate knowledge transfer within the consortium. Moreover, the ONTOX members were showcased at international conferences through short video interviews posted on the ONTOX LinkedIn page. Furthermore, a live-streaming event focusing on LGBTQIA+ community inclusion in science with various ONTOX partners took place and can be watched here (https://rb.gy/ytxps). In addition, short video productions, and three podcasts highlighting the ONTOX scientists and the EUROTOX activities were put online on the EUROTOX website (https://www.eurotox.com/podcast/). WP12 also contributed to establishing the ASPIS Academy, a network for young scientists within the ASPIS cluster (https://aspis-cluster.eu/aspis-academy/).
- WP13 established and released version 1 of the ONTOX Hub, the integration platform designed to provide access to registered NAMs developed within the ONTOX consortium, such as *in silico* tools, data, information, and *in vitro* testing, laboratory, and consulting services. The ONTOX Hub version 1 contains COSMOS Next Generation data (https://www.ng.cosmosdb.eu/), VEGA (WP3) *in silico* predictions, and the ONTOX-specific data from WP7, WP8, and WP9.
- WP14 played a pivotal role in disseminating the project's vision to advance human risk assessment of chemicals without animal testing. The WP managed the project website (https://ontox-project.eu/), engaged target audiences through various social media channels (Facebook https://www.facebook.com/ONTOX-EUProject/, LinkedIn https://www.linkedin.com/company/ontox/), posted four newsletters and contributed to the strategic planning and organisation of important project meetings, ensuring that the project goals and achievements reached a broad and informed audience.

Website: https://ontox-project.eu

Coordinator: Matthieu Vinken, Vrije Universiteit Brussel

Box 2.3. RISK-HUNT3R

RISK-HUNT3R aims to develop, validate and implement integrated approaches to lead the way toward next generation risk assessment (NGRA). The proposed approach is based on mechanism-based human-relevant *in vitro* and *in silico* systems (new approach methodologies). Through systematic and iterative evaluation of its NAM toolbox, the project will optimise a strategy to assess chemical exposure, toxicokinetics, and toxicodynamics.

Recent highlights include:

- assessing the impact of variability in *in vitro* clearance data on the output of PBK models and on the ability of *in vitro* systems to generate metabolites observed *in vivo*; combination of multiple QSAR models with kinetic data in the prediction of complex toxicity endpoints like liver cholestasis (Rodríguez-Belenguer *et al.*, 2023; Béquignon et *al.*, 2023)
- combination of *in silico* and *in vitro* NAMs to investigate the potentially toxic effects of neonicotinoids and nicotinoid metabolites on developing human neurons (Grillberger *et al.*, 2023); application of metabolomics and transcriptomics to elucidate the association of proteasome inhibition with Parkinsonian pathology (Suciu *et al.*, 2023); establishment of a transcriptomics-based method to improve early safety screening via weighted gene coexpression network analysis (WGCNA) to identify co-regulated gene clusters (Callegaro *et al.*, 2023); application of high-content imaging methods to investigate links between chemical modes of action and cell morphology (Cerisier *et al.*, 2023; Lejal *et al.*, 2023)
- use of network-based approaches was investigated, combining transcriptomics and proteomics networks in liver toxicology (Valls-Margarit *et al.*, 2023); extending the concept of a quantitative adverse outcome pathway (qAOP) for liver fibrosis to quantitative systems toxicology (QST); development of approaches to incorporate PBK and other kinetic models in qAOPs for liver toxicity and developmental neurotoxicity
- enabling high-quality descriptions of NAMs through the development of a web platform based on the ToxTemp template; development of FAIR principles for *in silico* models (Cronin *et al.*, 2023)
- inroads toward sustainable commercialisation, specifically on the SaferWorldbyDesign business and portal development
- scientific and training programs at prominent international conferences, such as the SOT 62nd Annual Meeting, EUROTOX 57th Congress, and the 12th World Congress on Alternatives and Animal Use in the Life Sciences.

Website: https://www.risk-hunt3r.eu

Coordinator: Bob van de Water, Leiden University

2.1.2. ASPIS Working Groups

ASPIS functions through eight Working Groups (WGs) composed of investigators from all three consortia (including early-stage researchers) who specialise in research domains that are relevant to the cluster's mission. Dr Jonathan Freedman from PrecisionTox serves as the ASPIS Working Group Coordinator to facilitate the development and reporting on ASPIS inter-working group projects and to link WG activities with the ASPIS leadership, various Scientific/Regulatory Advisory Boards and the Regulatory Forum.

Activities during the second and current years have focused the WGs on case studies (steatosis and conazoles) and on the continued development of chemical safety assessments based on knowledge of their modes of action. Recent highlights of the WGs are given in **Box 2.4**.

Box 2.4. Highlights from the ASPIS Working Groups

Chemical Selection

The goal of this WG is to coordinate chemical selection among the three projects. This WG is responsible for the development of ASPIS-wide case studies and assisting other WGs by providing information about test chemicals.

- Provision of an assembled chemicals list from the three consortia plus those of the JRC, US NTP and US EPA, thereby facilitating the work of EU-co-funded PARC projects.
- Provision of two cross-cutting case studies pursued by all WGs: (a) steatosis and (b) conazoles in collaboration with PARC.
- Development of a third case study on developmental neurotoxicants.

Communication and Dissemination

This WG harmonises dissemination activities and maximises the impact of ASPIS. Its objective is to effectively communicate scientific advances through a clear unified channel to regulatory stakeholders, policymakers, non-governmental organisations and the public.

- Provision of an internationally recognisable ASPIS identity (https://aspis-cluster.eu/).
- Provision of a stream of symposia at international meetings, webinars and news releases.
- Establishment of the ASPIS Academy (see below), following a survey completed by the ASPIS Early-Stage Researchers (ESRs).

ASPIS Academy

The ASPIS Academy is a network of ESRs focused on chemical hazard and risk assessment using NAMs. It promotes the careers of ESRs through specialised training, championing equal opportunity, and creating a platform devoted to the voices and aspirations of a new generation of regulatory scientists.

- Establishment of a cross-consortia ASPIS Academy Core leadership team.
- Organisation of training courses on science communication, artificial intelligence for reproducible research and grant writing.
- Organisation of poster sessions, networking opportunities and career development workshops at the annual ASPIS symposia.

Computational Approaches

This WG advances the development of *in silico* models to fill data gaps and to assess toxicity based on chemical properties and investigates *in silico* techniques that can be applied to ASPIS case studies and deployed from one project to another.

- Development of models of the toxicology of chemicals from the ASPIS case studies.
- Compilation and integration of relevant existing omics datasets from the three consortia.
- Development of a platform for consensus modelling, and pooling of datasets to broaden the chemical space and the interpretations of models.

Database

This WG is responsible for the F.A.I.R. management and sharing of project data. Its database initially contains physicochemical characteristics and toxicological information on chemicals used by ASPIS. It aims to create a central resource from the data generated by all three consortia such as:

- A beta-version release of the ASPIS database.
- Database resources including biology repositories, omics data and workflows, computational data, methodologies and Knowledge Graphs.
- Standardised processes for data acquisition including cutting-edge technologies using AI to mine the literature for toxicological and risk assessment information.

Kinetics and Exposure

This WG defines chemical exposure levels in the environment, human populations, target organs and *in vitro*. Its aim is to develop a tiered approach to exposure and kinetics assessment, integrated into a common, pragmatic guideline for risk assessors to use exposure, *in vitro* distribution kinetics, physiologically based kinetic and *in vitro-in vivo* extrapolation models.

- Comparative analysis of kinetic models and exposure approaches of each consortium and using the

ASPIS steatosis and conazoles case studies to further develop and improve kinetics assessment across the cluster.
- Development of *in vitro* biokinetic modelling approaches to calculate free and cell-associated chemical concentrations from nominal concentrations.
- Development of a tiered testing strategy beginning with *in silico* predictions, then progressing to experiments to address chemical absorption, metabolism and excretion – including ecotoxicity exposure and bioaccumulation testing strategies.

Omics

This WG promotes the transition of omics approaches into NGRA. For this, all data produced by ASPIS will be made compliant with the OECD Transcriptomics and Metabolomics Report Framework (TRF and MRF, respectively). These frameworks are designed to allow regulatory agencies to assess the quality of omics data used in hazard/risk assessment.
- OECD Reporting Framework in place for ASPIS.
- Research into the modes of action classification of substances based on gene expression associated with steatosis (case study).
- Progress at leveraging opportunities to harmonise omics datasets generated by the three consortia.

Quantitative AOP

This WG investigates models that quantify molecular initiating events (MIA) and/or key event relationships (KERs) within existing AOPs using public data and data produced by ASPIS while also establishing good practice. It aims to develop one or more common qAOPs, including those from linear and network AOPs.
- Development of a steatosis qAOP through data and model sharing, including modelling approaches to simulate steatotic Key Events.
- Development of a framework for validating qAOPs and providing qAOP input into regulatory decisions.
- Development of a prototype of a model to integrate steatosis qAOP and (toxico)kinetics.

Risk Assessment

This WG investigates the various uses of NAMs for chemical hazard and risk assessment. It coordinates joint activities and critically reviews ASPIS research in comparison to previous and other current EU-funded projects. It identifies gaps, limitations, and advantages of various NAMs, particularly with a view to identifying early targets for regulatory implementation.
- ASPIS case studies (i.e. steatosis and conazoles) are mapped to the objectives of the other WGs.
- Activities conducted by EU-ToxRisk and by the OECD are integrated with those of ASPIS.
- Maturing the ASPIS Safety Profiling Algorithm (ASPA) by incorporating elements from all three consortia into a NAMs chemical safety assessment workflow.

2.1.3. ASPIS Regulatory Forum

The ASPIS Regulatory Forum is a EURL ECVAM initiative to establish improved communication and debate between scientists and regulators with the purpose to facilitate regulatory uptake of non-animal methodologies and develop a common understanding of the problems in doing so. In 2021 and 2022, Forum meetings were organised as online sessions to facilitate wide participation and focus on a specific topic at each Forum meeting. In 2023, two alternative formats of the Forum took place: the "PARERE-ASPIS workshop on how to best achieve Regulatory Relevant Research", on 30 to 31 March at the JRC in Ispra (**Figure 2.1**), and a dedicated session on the role of standardisation supporting innovation in chemical safety assessment at the ASPIS Open Symposium, on 14 to 15 September in Ljubljana, Slovenia (**Figure 2.2**).

PARERE-ASPIS workshop on how to best achieve Regulatory Relevant Research

ASPIS presented the ASPIS Safety Profiling Algorithm (ASPA), a workflow and tool for regulatory decision-making using NAMs. The main outcomes of the discussion that followed together with PARERE were:
- CLP[4] classification must be included in the ASPA problem formulations, but the current criteria for systemic effects are not NAM-based. It was suggested to: (1) define criteria for hazards currently not covered by

4 Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures

animal studies, (2) introduce new hazard classes compatible with NAMs focussing on modes of action, and (3) start to experiment with a classification system based on key events without demonstrating adverse outcome.

- NAMs cannot provide the same type of information required in current safety assessments, but can for example provide bioactivity profiling of chemicals. Future work could explore how to use such information in safety assessment.
- ASPA is designed as a general safety assessment workflow with many regulatory applications. It could be a tool to support the strategy towards "one substance one assessment" (EC, 2020a).
- Detailed and clear guidance on how to apply ASPA will be crucial for successful implementation.

The main take-home message from the March workshop was that since ASPA should support regulatory decisions, we must embrace co-creation and let science and regulation develop to fit together. Therefore, chemical safety assessors (e.g. Member State authorities, agencies, industry) should be strongly involved in ASPA development to avoid scientific bias, especially regarding problem formulations that should be driven by regulation rather than scientific preferences.

To follow-up on the discussions at the PARERE-ASPIS workshop and allow time for PARERE members to consult on the development of ASPA with their Member State networks, an online meeting was organised on 6 June 2023.

While ASPA was initially developed by the scientific community, it was now considered timely to ask the regulatory community to contribute ideas on how to revise ASPA to make it suitable for making regulatory decisions using NAMs. Therefore, several actions were initiated to invite regulators to contribute directly to the further development of ASPA.

Figure 2.1. Participants of the PARERE-ASPIS workshop.

Regulatory Forum at the ASPIS Open Symposium on the role of standardisation supporting innovation in chemical safety assessment

The ASPIS Regulatory Forum session devoted to standardisation of NAMs was organised and chaired by EURL ECVAM at the ASPIS Open Symposium in September 2023 (Ljubljana, Slovenia). The session consisted of presentations, interactive questionnaires, and round-table discussions, focusing on the theme of standardisation supporting innovation in chemical safety assessment. Participants were asked to reflect on their current standardisation activities, both within and external to ASPIS projects, and identify areas of their work currently lacking standards which need further development. The session was highly interactive and provided an abundance of opportunities for participants to exchange knowledge on existing standards in their work, from exposure of test systems through to the reporting of NAM-based studies. Key areas that were highlighted as needing more standardisation included organ-on-chip, high-content imaging, omics, and artificial intelligence. An interactive questionnaire at the end of the session indicated that many participants were of the opinion that (i) standardisation is a vital component in accelerating the uptake of NAMs, and (ii) ASPIS is in a unique position to lead on standardisation efforts. This reinforces the importance of EURL ECVAM's role in advancing standardisation of NAMs across broad scientific communities.

Figure 2.2. Group discussions focused on the role of standardisation supporting innovation in chemical safety assessment at the ASPIS Open Symposium. *Photo courtesy of Agata Ormanin-Lewandowska, Project Manager of PrecisionTox.*

2.1.4. EURION

EURION, a cluster of eight European research projects funded by Horizon 2020, aiming to develop new test methods for the identification of endocrine disruptors is now into its fifth year of operation. Activities are switching from the discovery phase to the selection of most promising biomarkers/test systems and methods for use within testing strategies to identify endocrine disrupting chemicals. There are three projects focusing on thyroid hormone system disruption (ATHENA, SCREENED and ERGO), three on metabolic disruption (EDCMET, GOLIATH and OBERON) one on endocrine-mediated developmental neurotoxicity (ENDpoiNTS), and one on female reproductive toxicity, focusing primarily on the human ovary (FREIA).

Some projects have been active in connecting with regulators at national level, with the OECD Working Party of National Coordinators of the Test Guidelines Programme (WNT) and the OECD AOP community, and plan to bring forward project proposals on certain methods to the OECD. A few methods have been taken up by PEPPER[5] for validation: extension of H295R steroidogenesis assay (FREIA), *in vitro* assay for hepatic triglyceride accumulation (OBERON) and retinoic acid receptor (RAR)- and glucocorticoid receptor (GR)-dependent human neural progenitor cell proliferation arrest (ENDpoiNTs) (Grignard *et al.*, 2022).

EURL ECVAM has been active in supporting the cluster on many aspects, holding a number of workshops. These workshops introduced the cluster to the concepts of validation, including Test Readiness Criteria (adapted for endocrine relevant methods from (Bal-Price *et al.*, 2018)) to be used to self-assess the readiness of their methods to enter validation processes. The use of templates such as *ToxTemp* (Krebs *et al.*, 2019) was also encouraged, as a way of providing complete descriptions of their methods. In 2023, EURL ECVAM organised a workshop on Integrated Approaches to Testing and Assessment (IATA) to cover the principles of IATA, share progress and stimulate ideas and cross-cluster collaborations on IATA development.

Following budget neutral extensions of the projects applied due to COVID-19, the final meeting of the EURION cluster will take place in Brussels on 13 to 14 June 2024, where the project deliverables will be showcased to all stakeholders.

Website: https://eurion-cluster.eu/

2.1.5. PARC

The European Partnership for the Assessment of Risks from Chemicals (PARC) was established to support the development and implementation of a research and innovation programme to address current and future needs in relation to chemical risk assessment. PARC is a seven-year Horizon Europe public-public partnership, co-funded by the European Commission and the Member States with a budget of 400 million Euro. The partnership is coordinated by ANSES, the French Agency for Food Safety, Environmental Protection and Occupational Health.

As a multinational European project, PARC involves close to 200 institutions working in the areas of the environment or public health from 28 countries and three EU authorities, including the European Chemicals Agency (ECHA), the European Food Safety Authority (EFSA) and the European Environment Agency (EEA). Five Directorates-General of the European Commission (DG RTD, DG GROW, DG ENV,

5 Public-private platform for the validation of endocrine disruptors characterisation methods

DG SANTE and JRC) and the relevant ministries of the countries involved are contributing to the governance of PARC and monitoring its activities. In addition, the JRC has set up a formal collaboration agreement with PARC, so that JRC staff can be involved in various aspects of the work consistent with the JRC Work Programme.

The seven-year project formally started on 1 May 2022. Recent highlights are given in **Table 2.1**.

Table 2.1 Highlights from PARC activities considering NAMs.

Thematic Area	Highlights
Risk assessment	▸ During its first year of activity, PARC focused on selected priority outcomes related to immunotoxicology, non-genotoxic carcinogenesis, endocrine disruption, metabolic disruption and neurotoxicology. An inventory of Adverse Outcome Pathways has been established as well as a first inventory of NAMs for the chosen endpoints. ▸ Closing data gaps on mycotoxins and bisphenol A alternatives. ▸ Development of IATAs. ▸ A position paper on validation elaborated with JRC. ▸ Establishment of the PARC Roadmap towards implementation of NGRA as the default risk assessment approach across legislation. ▸ First steps in the development of a strategy and roadmap to assess aggregate exposure through different living environments, sources and routes. ▸ Strategy to perform mixture risk assessment based on human biomonitoring (HBM) data, knowledge developed for mixtures in regulatory risk assessment and in the field of toxicology, exposome and epidemiology. Its applicability was first tested on four prioritised chemical families (pesticides, metals, PFAS and mycotoxins) which are associated with specific effects of concern.
Tools and Resources	▸ Inventory of Physiologically based kinetic (PBK) models currently available for assessing internal exposure during the life course. ▸ Statistical analysis of HBM data (e.g. for mixture identification) has been implemented in the Monte Carlo Risk Assessment (MCRA) toolbox. ▸ Overview of i) sampling strategies, ii) sample preparation methods for bioassays and chemical analysis, iii) Effect Based Monitoring using bioassays, iv) chemical analytical methods including target, suspect and non-target screening, v) Effect Direct Analysis and iceberg modelling, and vi) future perspectives and needs for an Early Warning System.
Building capacities	▸ Contributed to the 1st edition of the Safe and Sustainable by Design (SSbD) Boot Camp organised on 25 to 27 October 2023 in collaboration with JRC. ▸ Participation in the EFSA-organised Knowledge Innovation Community meeting on NAMs (19 October 2023). ▸ Presentations to the OECD WPHA.
Science to Policy	Establishment of PARCopedia, PARC's online knowledge and community platform (www.parcopedia.eu).

Website: https://www.eu-parc.eu/

Coordinator: Pascal Sanders, ANSES.

2.1.6. Virtual Human Platform for safety assessment (VHP4Safety)

The Virtual Human Platform for Safety Assessment (VHP4Safety) is a five-year research project funded by the Dutch Research Council (NWO) programme 'Dutch Research Agenda: Research on Routes by Consortia (NWA-ORC)'. The VHP4Safety project started in June 2021, with the mission to improve the prediction of the potential harmful effects of chemicals and pharmaceuticals based on a holistic, interdisciplinary definition of human health, thereby accelerating the transition from animal-based testing to innovative safety assessment. VHP4Safety will integrate data on human physiology, chemical characteristics and perturbations of biological pathways, in order to incorporate: 1) human-relevant scenarios to discriminate vulnerable groups such as disease state, life course exposure, sex and age; 2) chemicals from different sectors: pharma, consumer products and chemical industry; and 3) different regulatory and stakeholder needs.

Recent highlights of the VHP4Safety project include:

- the development of the foundation of the Virtual Human Platform, the VHP cloud.
- the establishment of *in vitro* models that provide parameters for modelling of chemical absorption, distribution, metabolism and elimination (ADME) (i.e. intestinal, lung, skin, blood-brain barrier, kidney models).
- testing of the *in vitro* models with selected test chemicals has started.
- the development of AOPs for each of the three case studies (chronic kidney disease, neurodegenerative disease and life course exposure, and thyroid mediated developmental neurotoxicity).
- the design of a multi-stakeholder process, focusing on integration of stakeholders into work packages, communication and constructive technology assessment activities.

Coordinators: Anne Kienhuis, National Institute for Public Health and the Environment (RIVM); Cyrille Krul, HU University of Applied Sciences Utrecht; and Juliette Legler, Utrecht University

Project Manager: Esmeralda Krop, Institute for Risk Assessment Sciences, Utrecht University

Website: https://vhp4safety.nl/

2.1.7. In Silico World

In Silico World (ISW) is a four-year H2020-funded project which started in January 2021 with the mission to lower the barriers to a universal adoption of In Silico Trials. The term "In Silico Trials" refers to the use of computer modelling and simulation to evaluate the safety and efficacy of a medical product, whether it be a drug, a medical device, a diagnostic product or an advanced therapy medicinal product (Viceconti *et al.*, 2021). The ISW project aims to accelerate the uptake of *in silico* trials by lowering seven identified barriers: development, validation, accreditation, optimisation, exploitation, information, and training.

As recent highlights, ISW has:

- developed or further developed 11 solutions for *in silico* trials (five for the development of medical devices, four for medicinal products, one for vaccine development, and one for regenerative medicine products), contributing to the portfolio of available solutions. Two are already commercially available

for preclinical use through the InSilicoTrials[6] cloud-based platform, and a few more will be commercially available as products or services by other industrial partners, Materialise Motion[7] and Mimesis[8], before the end of the project.

- made available three validation data collections (TBValid[9], StentValid[10], and HFValid[11]) in open access; four more will be published on Zenodo before the end of the project.
- worked closely with ASME and ISO/IEC to support the creation of a new ISO/IEC workgroup and to ensure that the risk-based approach to model credibility assessment applied to medical devices, first proposed in the ASME VV-40:2018, is recognised also in future EU-harmonised standards. Key references include Musuamba *et al.*, 2021; Curreli *et al.*, 2023; Pappalardo *et al.*, 2022. ISW also submitted two requests for qualification advice for two In Silico Trials to EMA, and will produce in Q1/2024 a full report of this experience, including the sharing in Open Access of all the documentation. In addition, the ISW Community of Practice was essential in the development of the Toward Good Simulation Practice[12] open access book published by Nature-Springer.
- conducted substantial work on Responsible Research & Innovation (RRI). On the communication side, there have been more than 180 communication activities including papers, conferences, scientific events, workshops, and press releases. For example, in April 2023 a RRI Workshop[13] was organised in Bologna. Tailored content was produced for social media, where over 700 communication items were published.
- carried out an in-depth analysis of the ethical and legal aspects of *in silico* trials. This led to a second report (Biasin, 2023) summarising the main legal challenges surrounding *in silico* EU trials.

Coordinator: Marco Viceconti, University of Bologna

Website: https://insilico.world/

ISW Community of Practice: https://insilico.world/community/join-the-community-of-practice-channels/

2.1.8. Mechanistic analysis of repeated dose toxicity studies

In a two-year EURL ECVAM-funded study (2020-2022), the Free University of Amsterdam (VU Amsterdam), together with Edelweiss Connect GmbH, collected and analysed toxicological information provided by repeated dose systemic studies. The aim of the study was to "reverse engineer" repeated dose toxicity studies by describing how they capture the key characteristics (KCs) of repeated dose systemic toxicants. The concept of KC was interpreted within the context of this study as a property of a particular chemical that is likely to be implicated in the initiation and/or development of organ toxicity in repeat dosing scenarios. A key feature of the project was to refine and organise the mechanistic evidence in terms of KCs, and to create logical and plausible associations with specific organ toxicity.

An extended list of chemical KCs causing liver, lung, cardiovascular and kidney toxicities in repeated dose studies were drawn up using a combined literature and data driven approach. Case study chemicals, known to cause chronic toxicity, helped to generate a shortened list of KCs per organ, giving a rationalisation of the target organ specificity of these chemicals (**Figure 2.3**).

The approach and the data elaborated should prove helpful in improving mechanistic understanding of toxicological observations and for designing/optimising new experimental methods.

The results of the project are available through a JRC technical report (Jennings *et al.*, 2023) and the collection of *in vivo* data have been included in the JRC Data Catalogue[14].

6 https://insilicotrials.com/
7 https://www.materialise.com/en/healthcare/hcps/pressure-measurements-orthotics
8 https://www.mimesis.srl/
9 https://zenodo.org/record/8307759
10 https://zenodo.org/record/7752991
11 https://zenodo.org/record/7555270
12 https://link.springer.com/book/9783031482830
13 https://insilico.world/press-release/a-workshop-on-responsible-research-innovation/
14 https://data.jrc.ec.europa.eu/dataset/4824023e-ad0f-4126-a6a4-f0e7b0bc330a

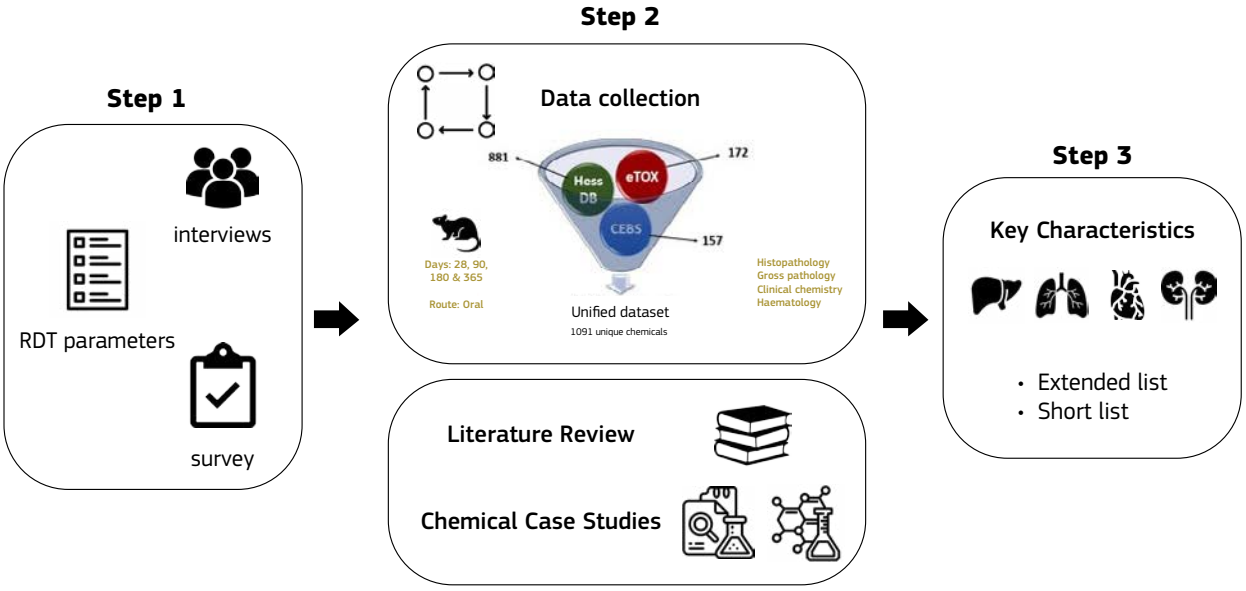

Figure 2.3. Stepwise approach followed to make use of large *in vivo* RDT datasets: Step 1. Selection of the most relevant RDT parameters to identify relevant mechanistic information contributing to target organ toxicities repeated dose studies. Step 2. Identification of organ-specific KCs derived from existing RDT data and their linkage to time of exposure. Step 3. Profiling selected chemicals in relation to the identified KCs.

2.2. Applications of NAMs and modelling

2.2.1. Physiologically based kinetic models

Physiologically based kinetic (PBK) models are playing an increasingly important role in *in vitro* to *in vivo* extrapolation and chemical risk assessment (Chang *et al.*, 2022). These models simulate what the body does to the chemical, in terms of how the chemical is absorbed, distributed, metabolised and eliminated, so called ADME processes. The human health risks of a chemical are directly linked to its levels in body tissues, which vary over time as the chemical is absorbed and removed.

To accelerate the acceptance and use of PBK models, the JRC and the US Environmental Protection Agency (EPA) led an international working group at the Organisation for Economic Cooperation and Development (OECD) to draft a guidance document on the characterisation, validation and reporting of these models (OECD, 2021). To further promote the guidance document, dedicated training courses were held at the 2023 Society of Toxicology (SOT 2023[15]) meeting in Nashville, USA, and at the 57th Congress of the European Toxicologists and European Societies of Toxicology (EUROTOX 2023[16]), in Ljubljana, Slovenia.

Going beyond traditional applications in drug development and chemical risk assessment, one area of application where PBK models have been rarely used is forensic science. To explore opportunities in this area, Fairman *et al.*, 2023 reviewed PBK models developed for illicit drugs and environmental chemicals that could be applied for forensic interpretation, highlighting the gaps, uncertainties, and limitations.

2.2.2. Introducing time variables in toxicology

EURL ECVAM participated in an ECETOC workshop titled "Chronos and Kairos – Understanding time in biology: Time4NGRA[17]". The workshop explored the need for approaches to study the influence of time and level of biological organisation in next generation risk assessment (NGRA) based on NAMs. More specifically, there were discussions on how to integrate the influence of exposure time window, duration, frequency and damage accrual rate in developing and interpreting *in vitro* models, quantitative adverse outcome pathways (qAOPs) and quantitative *in vitro* to *in vivo* extrapolation (QIVIVE).

15 https://www.toxicology.org/events/am/AM2023/continuing-education.asp
16 https://www.eurotox2023.com/programme/
17 https://www.ecetoc.org/event/chronos-and-kairos-understanding-time-in-biology-time4ngra/

3. Validation

Validation is an intrinsic part of the scientific process aiming at building confidence in new methods. While established validation principles remain relevant, the evolving landscape of 21st century toxicology demands a more streamlined and efficient validation approach to accommodate the wide array of non-animal testing methods. This chapter delves into EURL ECVAM's efforts towards modernising and enhancing the validation process. These include rigorous scientific evaluation through EURL ECVAM's validation process, support from EURL ECVAM networks, and initiatives dedicated to standardising complex test systems and technologies like Organ-on-Chip platforms. The chapter further explores facets of scientific validation and standardisation across various projects, ranging from those spearheading the development and optimisation of novel testing methodologies (see **Section 2**) to those contributing to the development of international standards (see **Section 4**).

3.1. Test method submissions

3.1.1. GENOMARK

GENOMARK is a transcriptomics-based signature aimed at providing insights into the molecular events underlying the genotoxic mode of action of different types of test chemicals. The test method is based on the measurement of expression of 84 genes to differentiate between genotoxic and non-genotoxic chemicals in the metabolically competent hepatic cell line HepaRG™ and a prediction model using a supervised machine learning algorithm is applied to classify the test chemicals as genotoxic or non-genotoxic (Thienpont *et al.*, 2023). EURL ECVAM considers the submitted method a valuable tool to potentially de-risk chemicals misleadingly identified as positive within the standard *in vitro* genotoxicity battery. Such an approach may therefore reduce unnecessary animal testing that, otherwise, would be conducted to follow up on positive *in vitro* results. However, EURL ECVAM recognises that additional work is ongoing since the submitters are conducting further method development and optimisation (e.g., by increasing the throughput of the method, developing new prediction models and developing a quantitative approach). Therefore, a final version of the method's Standard Operating Procedures (SOPs) is not yet available and some additional aspects (e.g. assessment of reliability) still need attention to establish the scientific validity of the method with a view to its use in a regulatory context.

3.2. Validation studies

3.2.1. Thyroid methods validation study

The multi-laboratory study, aiming at validating 18 *in vitro* methods for different modes of action relevant for the thyroid hormone signalling pathway, has been finalised and a validation report has been published.

The validation report provides details on the sites where chemicals can modulate thyroid hormone signalling (**Figure 3.1**), the study organisation, the mechanistic methods, the experimental design, the partners involved, the chemicals selected, and the overall outcome of the validation study (Bernasconi *et al.*, 2023). For two methods investigating sodium iodide symporter activity and monocarboxylate transporter 8 activity, work to generate the data and study reports is still ongoing by the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL).

For several methods, the SOPs and study reports were made publicly available in the online tracking system for alternative methods towards regulatory acceptance (TSAR[18]), and will gradually be added for all methods. Study data and reports have been assessed for most of the methods by the OECD thyroid disrupting methods expert group (TDM-EG), deciding if the method is promising for inclusion in the OECD test guidelines programme, and if and how further validation activities should continue (see **Section 4.1.4**).

The validation study for thyroid methods, has been quite a challenge for the 15 EU-NETVAL laboratories, the 14 method developers and the validation coordination team. The following are some lessons learnt from this validation study: it is possible to assess many methods simultaneously, support from the method developer for trouble shooting and training during implementation of the method is essential and available financial support for ingredients purchase and hire of personnel greatly helps to speed up the validation process.

In summary, for 11 out of 18 methods a data set was produced that is valuable to judge on the methods' validity and data for two additional methods will be upcoming. Further assessment and validation was stopped for five methods because of lack of resources (four methods) or poor method performance (one method). Also, a mechanism is in place through the OECD TDM-EG (see **Section 4.1.4**) and PARC (see **Section 2.1.5**) to use the results from this validation study for the development of testing strategies to address thyroid hormone system disruption.

Block#	Description
1	Central regulation (HPT axis).
2	Thyroid Hormone (TH) syntheses.
3	Binding and transport in serum.
4	Metabolism and excretion (by hepatic deiodnases, glucuronidation and sulfation).
5	Local cellular concentrations (TH selective membrane tranposters e.g. monocarboxylate transporter 8 (MCT8)).
6	Cellular responses (activation of specific nuclear receptors TRα and TRβ).
7	Relevant short term assays integrating multiple MoAs.
8	Integrative cellular assays

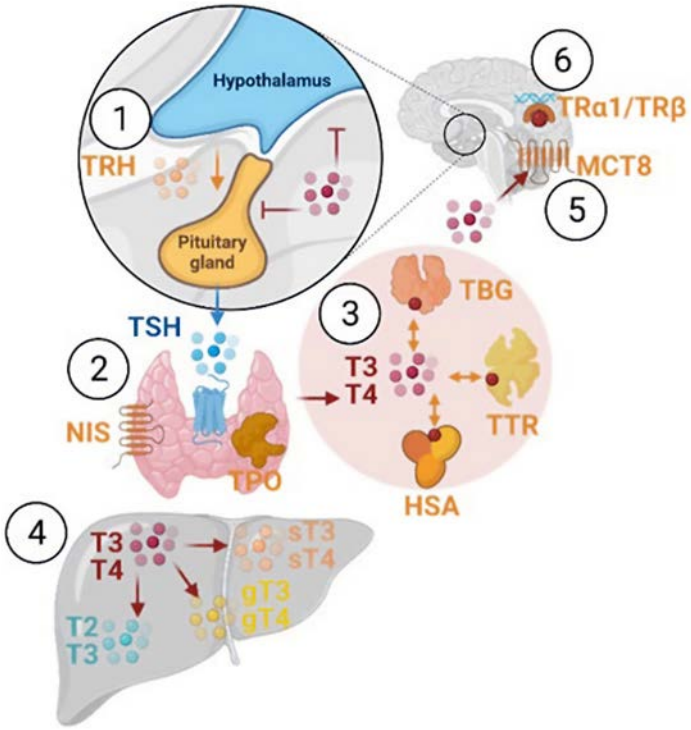

Figure 3.1. Figure from the validation study report showing the sites where the thyroid hormone system can be disrupted (Bernasconi *et al.*, 2023).

18 https://tsar.jrc.ec.europa.eu/

3.2.2. Validation of a high-throughput in vitro assay to identify androgen-disrupting chemicals

To support the identification of endocrine disrupting chemicals, EURL ECVAM is commencing a study aiming to validate a high-throughput androgen receptor dimerization assay (known as the AR2 assay) developed by the US Environmental Protection Agency (Brown *et al.*, 2023). The AR2 assay, which screens for androgenic effects of chemicals, is intended to feature in a test battery to facilitate the assessment of pesticides in the US. The AR2 assay is available in both agonist and antagonist modes and employs a genetically engineered reporter cell line (HepG2-AR2). The cell line expresses the androgen receptor fused to either of two subunits of NanoLuc® luciferase. In the presence of an agonist, the androgen receptor undergoes homodimerization, which brings the subunits together reconstituting the NanoLuc® luciferase for subsequent measurement of luminescence in the presence of a substrate. The study is envisioned to complement several international efforts focused on using non-animal data to identify endocrine disrupting chemicals.

3.3. EURL ECVAM Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL)

The European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL), coordinated by EURL ECVAM, includes 33 highly qualified laboratories across Europe, to support the *in vitro* method validation process. Members in this network have been appointed by the Member States in response to Directive 2010/63/EU (Article 47).

The network has been active in the past few years with the EURL ECVAM coordinated validation study of thyroid hormone disruption assays (see **Section 3.2.1**). About 50% of EU-NETVAL's capacity (15 EU-NETVAL facilities) was involved in this study, validating 18 *in vitro* methods simultaneously.

In September 2023, the 6th EU-NETVAL meeting was organised at the JRC (**Figure 3.2**). Speakers from EFSA, DG RTD, PEPPER and OECD were also invited. The first day of the meeting focused on method validation topics, including the need for validated methods, organisational and funding challenges in validation studies, the transferability of methods including methods with complex test systems and endpoints. A highlight of the meeting was a panel interview and discussion on perspectives from Contract Research Organisations (CROs) and industrial end-users. The second day concentrated on the recently completed validation study of Thyroid Hormone System Disruption assays. Eleven facilities that participated in this study presented their results, challenges and lessons learned. Moreover, current activities related to these *in vitro* methods at OECD and within the PARC project were discussed.

Figure 3.2. Participants of the 6th EU-NETVAL meeting at the JRC.

3.4. EURL ECVAM Scientific Advisory Committee (ESAC) peer reviews

The EURL ECVAM Scientific Advisory Committee (ESAC) is a formal expert group of the European Commission that is charged with providing EURL ECVAM with independent scientific advice. In particular, the ESAC acts as a scientific peer-review body by providing EURL ECVAM with its opinion on the adequacy and outcome of formal validation studies carried out to assess the reliability and relevance of non-animal methods/approaches, typically in the context of regulatory safety assessment. The ESAC may also provide scientific advice on other scientific issues of relevance to the work and mission of EURL ECVAM. The ESAC's tasks are:

a) to assess the scientific validity of non-animal methods/approaches intended for a given purpose;
b) to advise the JRC on other scientific matters related to the work of EURL ECVAM and the protection of animals used for scientific purposes;
c) to share its knowledge and experience on non-animal methods/approaches used in science.

3.4.1. Continuously open call for applications for the selection of members of ESAC sub-groups

Peer-reviews and other work of the ESAC are normally facilitated by specialised ESAC sub-groups set up by the JRC. On 25 October 2022, the JRC launched a continuously open call for applications for the selection of members of the sub-groups operating under the ESAC, which is available at: https://ec.europa.eu/transparency/expert-groups-register/core/api/front/calls-application/77960/download.

Experts who are interested in participating in ESAC peer reviews are invited to apply to this continuously open call. In order to ensure the smooth functioning of ESAC sub-groups, the JRC has established a list of suitable experts (expert pool) from applicants complying with the eligibility criteria referred to in this call. The members of a specific sub-group that are not members of the ESAC shall be selected by the JRC from the expert pool on the basis of the selection criteria referred to in this call and of their qualifications/expertise related to the specific question(s) under review.

Each ESAC sub-group must comprise at least one ESAC member who shall chair it but may be composed of any combination of ESAC members and sub-group members that are not members of the ESAC. ESAC sub-groups typically consist of up to 10 members in total. Members of ESAC sub-groups shall be individuals appointed in a personal capacity who shall act independently and in the public interest (Type A members).

3.4.2. Genotoxicity in reconstructed skin models

The Reconstructed Skin Micronucleus (RSMN) and the Reconstructed Skin Comet (RS Comet) assays are two genotoxicity tests for topically exposed substances. Both tests have been fully validated by Cosmetics Europe.

The RSMN is an adaptation of the well-established *in vivo* and *in vitro* micronucleus tests (OECD TG 474 (OECD, 2016a) and OECD TG 487 (OECD, 2023e)). The RSMN was designed in the EpiDerm™, a three-dimensional reconstructed human epidermis model that resembles the structure, morphology, and xenobiotic metabolism of the human epidermis. This model is obtained from normal human epidermal keratinocytes derived from neo-natal foreskin tissue on specially prepared tissue culture inserts.

The RS Comet is an adaptation of the alkaline comet assay, which measures DNA strand breaks in eukaryotic cells and for which an OECD *in vivo* mammalian test guideline is already available (OECD TG 489, (OECD, 2016b)). The RS Comet was designed in a three-dimensional reconstructed human skin model: the Phenion® full-thickness skin model. This model resembles the fully differentiated epidermis and the underlying dermis. The Phenion® full-thickness skin model is composed of human primary keratinocytes and fibroblasts cultured under air-liquid-interface conditions on a collagen scaffold.

RSMN and RS Comet are recommended to be used within regulatory genotoxicity hazard identification testing strategies to follow up positive results from the classical *in vitro* test battery. Moreover, the test submitter proposes to use the two tests together to cover all the genotoxicity endpoints that need to be addressed for regulatory purposes (gene mutation, clastogenicity, and aneugenicity).

EURL ECVAM has assessed the completeness and quality of the information provided for the two tests (available

from TSAR) and the EURL ECVAM Scientific Advisory Committee (ESAC) is currently conducting an independent peer review to assess the scientific validity of both assays.

3.5. EURL ECVAM Network for Preliminary Assessment of Regulatory Relevance (PARERE)

The 12th meeting of the Preliminary Assessment of Regulatory Relevance network (PARERE, see **Box 3.1**) was held on 30 March 2023 at the European Commission's Joint Research Centre, Ispra, Italy. Activity updates were provided by EU Member States, the European Commission's Scientific Committee on Health, Environmental and Emerging Risks (SCHEER), EU agencies and Commission services. Updates from EURL ECVAM included new test (pre)submissions (see **Section 3.1**), the revision of the chapter on skin sensitisation of the Globally Harmonized System of Classification and Labelling of Chemicals (GHS; see **Section 4.4.3**); follow-up activities to the OECD project in the area of developmental neurotoxicity (DNT, see **Section 4.1.1**), innovating chemical safety assessment ("Chemicals 2.0") and the related EPAA project (see **Section 4.2** and **Section 4.3.1**); follow-up to the EURL ECVAM thyroid hormone validation study and activities at the OECD thyroid disrupting methods expert group (see **Sections 3.2.1**, **3.3** and **Section 4.1.4**); test readiness criteria applied within the EU Horizon 2020 project EURION (see **Section 2.1.4**); updates on the ECVAM Scientific Advisory Committee (ESAC, see **Section 3.4**) and updates on the revision of OECD GD 34 on the validation and international acceptance of new or updated test methods for hazard assessment (see **Section 3.6**).

The PARERE meeting was followed up by a PARERE-ASPIS workshop on how best to achieve regulatory relevant research on 30 March (afternoon) and 31 March. Part 1 of the workshop presented and discussed regulatory relevant research undertaken within the ASPIS cluster (EU H2020 funded project; see **Section 2.1.1**), whereas Part 2 was dedicated to the JRC recommendations for more regulatory relevant research.

Box 3.1. Preliminary Assessment of Regulatory Relevance (PARERE) network

The Preliminary Assessment of Regulatory Relevance (PARERE) network is a trans-sectoral network of regulators from EU Member States, representatives from EU agencies, and relevant policy services of the EC. It was established by EURL ECVAM through Directive 2010/63/EU to provide advice on the regulatory relevance and suitability of alternative approaches proposed for validation.

PARERE members are consulted on several occasions over the year, either on the regulatory relevance of individual methods or approaches that are submitted to EURL ECVAM for validation, peer review, or evaluation, or on other topics such as EURL ECVAM Recommendations, and methods and approaches developed within research projects funded by the EU Framework Programme for Research and Innovation.

The PARERE network helps to ensure that alternative approaches to animal testing are relevant to the needs of regulators and that they will be accepted for use in regulatory testing.

More information on the PARERE network can be found here: https://joint-research-centre.ec.europa.eu/eu-reference-laboratory-alternatives-animal-testing-eurl-ecvam/alternative-methods-toxicity-testing/advisory-and-consultation-bodies/parere-eurl-ecvam-network-preliminary-assessment-regulatory-relevance_en

3.6. Revision of OECD Guidance Document 34 on the validation and international acceptance of new or updated test methods for hazard assessment

Guidance Document (GD) 34 (OECD, 2005) has been an important reference to support the validation and international acceptance of test methods for hazard and risk assessment since its publication in 2005 (based on principles agreed in an OECD workshop held in 1996). Since then, toxicological science has evolved tremendously and so has the number and type of methodologies available to assess the hazardous properties of chemicals. While the fundamental principles of validation enshrined in GD 34 still hold true today, several aspects related to the validation process are not reflecting the current state of the art. In fact, there is widespread recognition that the processes used in the past decades for validation and international acceptance need to be updated to encourage timely uptake of fit for purpose and biologically relevant NAMs, while ensuring that GD 34 provides practical guidance for validation of all types of methods. Therefore, the JRC submitted a new project proposal (SPSF) to the OECD WNT to update GD 34 and guarantee that it will continue to be a valuable asset in the years to come. The project was approved by the WNT during its annual meeting in April 2023 and will be co-led by the EU (JRC), the United States and the Netherlands.

The project will address key considerations that have been identified by the WNT in a workshop on emerging technologies held in December 2022 and any follow-up discussions/workshops within the WNT, as well as by various recent publications addressing the need to revisit the processes used to establish scientific confidence in NAMs and achieve regulatory acceptance (Bal-Price *et al.*, 2018; Browne *et al.*, 2019; Burgdorf *et al.*, 2019; Casati *et al.*, 2018; EFSA, 2022; ICCVAM, 2018; ICCVAM, 2023; Parish *et al.*, 2020; Patterson *et al.*, 2017; Patterson *et al.*, 2021; Petersen *et al.*, 2023; Piersma *et al.*, 2018a; Piersma *et al.*, 2018b; Sewell *et al.*, 2017; US CPSC, 2022; US EPA, 2018; US FDA, 2017; US FDA, 2021; van der Zalm *et al.*, 2022). These key considerations will be further discussed within a new project group that was established by the OECD for this project. The proposed revision will also include a simplification and general revisions of the document, where possible and appropriate.

The project group had its first meeting (virtually) in December 2023 and will meet face-to-face in Paris in April 2024.

3.7. ICATM satellite meeting on validation and establishment of confidence in new approach methodologies

The International Cooperation on Alternative Test Methods (ICATM) held a satellite meeting during the 12th World Congress on Alternatives and Animal Use in the Life Sciences (see **Section 4.6**) to discuss the validation and establishment of confidence in new approach methodologies. The meeting aimed to develop a collective ICATM position on the key elements necessary for an updated OECD GD 34, which focuses on validation standards for regulatory application (see **Section 3.6**). During the meeting, the partners discussed various aspects of validation, including quality systems for data integrity, the importance of accurate method descriptions, the need for ring trials, the concept of applicability domain, the potential for a repository of technically validated methods, and the possibility of a stand-alone guidance document on technical validation. The partners agreed that technical validation should focus on the scientific and biological relevance of non-stand-alone mechanistic methods, which are combined in defined approaches (DAs) or integrated approaches to testing and assessment (IATAs) for regulatory application. They emphasised the importance of method readiness, reproducibility, and well-designed transferability studies. The partners also discussed the validation of DAs, highlighting the differences between DAs and IATAs. They agreed that DAs can be validated and included in OECD test guidelines, while IATAs require a more flexible approach due to the use of different information sources and expert judgment. Other topics of discussion included the need for clear criteria for selecting reference chemicals, the importance of demonstrating reproducibility and proficiency in testing, and the need for a quality system or standard for non-GLP labs. The partners suggested to focus on validation only and remove international acceptance from the guidance document since this part repeats many concepts already described in the validation part. Overall, the partners recognised the need to update OECD GD 34 to reflect current scientific progress, including the emergence of defined approaches, computational models, and new technologies. They agreed that the updated guidance should be high-level, simple, and easily implemented, and that validation should be viewed as a flexible and modular approach adaptable to different needs and contexts. The discussions at the ICATM satellite meeting provided valuable insights into the key elements necessary for the validation and establishment of confidence in new approach methodologies. These insights will inform the development of an updated OECD GD 34 and contribute to the advancement of alternative test methods in regulatory applications (see also **Section 3.6**).

3.8. Standardisation of complex test systems and technologies

Biotechnology is advancing at an unprecedented pace, making complex systems and technologies available for use in industry and regulatory domains, as well as for biomedical research. Standardisation and harmonisation can help to define common terminology, protocols, criteria for design and characterisation, but also analysis and data reporting. By enabling the development of reference materials and reference chemical lists, quality control procedures, best practices and standards promote active use of the information generated with these technologies by companies and regulatory bodies.

3.8.1. Organ-on-chip

To address the growing need for standards in the field of OoC, in March 2022, the European Committee for Standardization (CEN) and the European Committee for Electrotechnical Standardization (CENELEC) decided to establish a Focus Group on OoC (FGOoC). This group is analysing the landscape of standards for OoC and defining a roadmap on OoC standardisation for the coming years. This roadmap will list the main relevant terms and their definitions to establish a common language that can be used by the OoC community. Best practices and standardisation needs will be described for biosciences, including cell model selection, culture, use of extracellular matrix and culture media, and engineering, discussing the design and fabrication of microfluidics, laboratory equipment, and microelectronics. The FGOoC is also discussing and describing best practices for experimental design and data management. The roadmap will also include regulatory, legal, and ethical considerations of OoC development and use.

Qualification of organ-on-chip for use in the pharmaceutical sector

The pharmaceutical sector stands out as the primary domain where OoC technology is currently being implemented, especially for internal decision making during the drug development process.

EURL ECVAM is involved in different activities around OoC qualification. EURL ECVAM acts as observer member of the EMA 3Rs working party[19] that has the role to advise EMA committees on all matters concerning the use of animals in the regulatory testing of medicines. An initial activity prioritised by the 3Rs working party refers to the identification of qualification criteria to establish the scientific credibility of OoC. To support this endeavour, EURL ECVAM conducted a mapping exercise of current relevant documents, including guidance documents/guidelines from regulatory agencies (EMA, 2017a; EMA, 2016; EMA, 2017b; ICH, 2020; ICH, 2005; ICH, 2013), and scientific peer-review papers (Hartung *et al.*, 2004; Parish *et al.*, 2020; Patterson *et al.*, 2021; van der Zalm *et al.*, 2022) on the topic of qualification and validation. This has allowed to identify the main, high-level features that were commonly described in those documents.

To promote the use of OoC devices for regulatory applications, EURL ECVAM created, in collaboration with the European Society of OoC Regulatory Advisory Board, a repository of resources for developers and end-users to support validation and qualification of these new technologies (Batista Leite *et al.*, 2021)

In addition, on 26 June 2023, EURL ECVAM, organised a workshop as a pre-conference event of the Microphysiological Systems (MPS) World Summit 2023. The "Heads on! Designing a qualification framework for Organ-on-Chip" workshop had the aim of collecting the developers and end-user perspectives on what features a qualification framework for OoC should consider. Practical solutions and the stakeholders who should be involved were also discussed. During the workshop, the developed proposals were pitched to a panel composed of three experts from the pharmaceutical industry, regulatory authority and academia (Piergiovanni *et al.*, 2024).

Liver organ-on-chip ring trial

The Human Liver Ring Trial (HLRT) is an industry-led experimental study to evaluate the reproducibility and robustness of an OoC device for use in the pharmaceutical sector. The HLRT study will build confidence on the TissUse liver OoC, for the early identification of Drug-Induced Liver Injury (DILI), metabolism and intrinsic clearance of selected reference drugs (including low-clearance compounds).

Primary hepatocytes will be treated for 14 days with five reference compounds to test DILI. In addition, three reference compounds will be tested for drug metabolism. The HLRT will measure biomarkers (albumin, urea), viability (ATP, LDH) and parent drug concentrations (by LC-MS/MS). A digital-twin model of the biological liver-chip will be developed to simulate hepatic metabolism, compound

19 https://www.ema.europa.eu/en/committees/working-parties-other-groups/chmp/3rs-working-party

binding to plastic, compound metabolism and kinetics. The developed software platform will comprise an advanced mathematical description of the underlying biological processes in liver-on-chips. This combined experimental and computational strategy will enable more human-relevant estimations of both *in vitro* clearance and EC50 values for pharmacokinetics and pharmacodynamics studies, respectively.

The following companies opted to become member of the HLRT: UCB, Bayer, Orion Pharma, Servier, AstraZeneca, Sanofi, ESQLabs and TissUse. EURL ECVAM will contribute to the study by providing expertise, knowledge, and insights.

3.8.2. High-content imaging

Imaging technologies are playing an increasing role in biomedical sciences, as one of the main tools to investigate a variety of endpoints in cell-based assays. Recently, the use of imaging has started to gain a prominent role also in regulatory toxicology, with the example of the developmental neurotoxicity *in vitro* battery (OECD, 2023a). Most of the cell-based assays in the neurotoxicity battery are using high-content imaging to capture relevant morphological characteristics and measure dimension and migration of cells in time. It is therefore crucial to start discussing challenges in regulatory acceptance, to ensure practical implementation and reliability of high-content imaging and facilitate transferability and validation. The development and use of standards can support all key steps of imaging (image acquisition, data analysis and interpretation) to ensure that the data produced with this technology would be fit for use in a regulatory context. With the view of bridging between developers, end-users, and regulators, EURL ECVAM is currently performing a gap analysis on standardisation. Particular attention is posed on GLP implementation, proficiency testing and reference materials, instrument calibration and maintenance, and existing best practices in reporting and metadata.

3.8.3. Omics-based methods

Despite growing interest in using omics-based approaches for chemical safety assessment, their uptake and routine use in this area is scarce. One significant barrier is the lack of standardisation of omics-based methods. For this reason, EURL ECVAM is currently working towards standardisation of omics-based methods (such as transcriptomics and metabolomics) through both in-house activities and collaborations with other ongoing initiatives. At OECD level, EURL ECVAM is actively participating in drafting the "Chemical Grouping – Application Reporting Module", which aims to describe how omics data should be reported for the purpose of chemical grouping. This activity therefore complements the recently completed OECD Omics Reporting Framework (Harrill *et al.*, 2021). EURL ECVAM is also involved in a new task force formed by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), which focuses on the development of an omics data interpretation framework for regulatory application. This represents one of the greatest challenges posed when working with omics data. Internally, EURL ECVAM is also addressing standardisation needs at key stages, from development through transferability and optimisation, to application of omics-based *in vitro* methods. This work envisions development of a comprehensive support package to bring together knowledge resources, tools, and guidance to aid the implementation of standardisation in a systematic, efficient, and collaborative manner. The primary goal of this package is to assist stakeholders undertaking method development using omics technologies and subsequently support application of these methods in a regulatory context, but the package may also have wider application across the omics field to support general development in this space.

4. Regulatory application

EURL ECVAM contributes to many EU and international activities, working with the OECD, UN GHS and EU regulatory agencies, and collaborating with a range of stakeholders with different interests, in order to bring forward emerging non-animal science and technologies into practical application within the context of regulatory hazard and risk assessment of chemicals. Here is a flavour of the ongoing work in these many different fora over 2023.

4.1. Test methods and integrated approaches to testing and assessment

4.1.1. Developmental neurotoxicity - in vitro battery

At present there is no *a priori* requirement for pesticides, biocides or other chemicals, to be tested for neurotoxicity (NT) and developmental neurotoxicity (DNT) prior to registration but testing can be triggered based on observed neurotoxic effects in repeat-dose testing, a known neurotoxic mode of action, or a structure-activity alert (OECD, 2007; OECD, 2018).

In the last years, EFSA has engaged with the scientific community including EURL ECVAM for the development of NAMs that could contribute to the NT and DNT risk assessment in a cost and time effective manner. EFSA now aims to expand the use of NAMs in these areas to be applied in the weight of evidence assessment of pesticides.

To this end, in 2023 a call for tender was launched by EFSA with the overarching goal of advancing the understanding of how chemical exposures impact on brain development in order to implement this knowledge for the protection of human health. The overall objective of the call is to identify test systems to be used in a DNT screening programme, prioritise chemicals to be tested, develop high throughput methods using molecular or cellular approaches and to produce proof-of-concept case studies to conclude on risk assessment. The call is also aiming at progressing the regulatory uptake of DNT NAMs through an initial evaluation of their laboratory transferability and reproducibility[20].

4.1.2. Towards achieving a modernised science-based approach for carcinogenicity testing

Decades of research have described the limitations of the rodent cancer bioassay leading to international initiatives to seek alternatives and establish approaches that predict carcinogenic potential without the rodent cancer bioassay. Among these initiatives, EURL ECVAM contributed to a paper (Hilton *et al.*, 2023) outlining challenges and opportunities that authorities should consider when building a roadmap that leads to global acceptance and incorporation of fit-for-purpose, scientifically defensible new approaches for human-relevant carcinogenicity assessment of agrochemicals.

Following a pilot case study on carcinogenicity of agrochemicals, the OECD Working Party on Hazard Assessment (see **Box 4.1**) has called for more integrated approaches to testing and assessment (IATA) through

20 https://etendering.ted.europa.eu/cft/cft-display.html?cftId=13967

case studies evaluating human carcinogenicity using a variety of approaches across different chemical sectors.

In particular, the assessment of non-genotoxic carcinogens is considered a regulatory gap, prompting the OECD to set up an expert group to develop an IATA for non-genotoxic carcinogens (Jacobs *et al.*, 2016). The expert group agreed on a general AOP-like structured IATA, including assay blocks, based on hallmarks of cancer (Jacobs *et al.*, 2020) and conducted critical reviews of assays in the various blocks identified. This led to several papers published in a special issue of the International Journal of Molecular Sciences on advances in mechanism-based toxicity and hazard assessment of non-genotoxic carcinogenic chemicals (Sovadinová *et al.*, 2021; Desaulniers *et al.*, 2021; Ohmori *et al.*, 2022; Pillo *et al.*, 2022; Oku *et al.*, 2022; Colacci *et al.*, 2023). Currently, the work is progressing, including the preparation of a non-genotoxic carcinogenicity IATA draft regulatory framework and further publications to support the future IATA. Cooperation between this expert group and the OECD Case study project mentioned above is important and offers the opportunity for mutual feedback.

4.1.3. Relative metal/metalloid release using a simple simulated gastric fluid

The EC (through EURL ECVAM) is leading a project for the development of an OECD test guideline that describes how to conduct a test using a simple simulated gastric fluid (0.032M HCl) to generate relative metal/metalloid release data for metals and metalloids in massive and powder forms. An OECD expert group composed of regulators, industry representatives and academics is supporting the project.

The last WNT commenting round on the draft TG was launched in December 2021 and comments were received beginning of February 2022 together with a testing proposal from the Netherlands, aiming at clarifying a possible role of the particle size on the metal/metalloid release. Since then, EURL ECVAM has been trying to address these comments on particle size, as well as issues raised by Canada in June/July 2022 on (i) the need for the TG vis a vis other existing standard methods (i.e., ASTM D5517 and US EPA 1340), (ii) the pH and fluid composition, (iii) the biological relevance, (iv) the loadings used and possible saturation, (v) the modifications made to the protocol after the ring trial, (vi) the reproducibility, and (vii) the validation status of the method.

New data on particle size were presented and discussed with the Netherlands. Given the positive feedback received by the Netherlands, it was decided to present those data to the wider expert group.

Throughout 2022 and 2023, the EC engaged in multiple bilateral meetings with Canada to address their questions and concerns regarding the draft TG. While these discussions were not entirely successful, the EC remained open to considering Canada's feedback and proposed revisions to the TG in an attempt to reach a consensus. However, Canada deemed these revisions insufficient and put forward more extensive changes that the EC found unacceptable. These proposed revisions, if implemented, would significantly diminish the value of the 0.032 M HCl method, raising concerns about its scientific validity and justification from the EC's perspective.

As a result, the EC decided that it would be preferable not to establish a TG that explicitly and unjustifiably limits its own applicability. Given the prolonged deadlock and the absence of a clear resolution, the OECD secretariat organised an expert group meeting in October 2023 to address Canada's concerns and determine the project's future. During this meeting, the data on the impact of particle size on metal release was presented to the expert group and received positive feedback. Canada reiterated its comments, and the EC provided an update on the ongoing discussions. While several experts expressed their views on these issues, a solution remained elusive.

In an effort to find a solution, the UK proposed bilateral discussions with Canada. However, if a resolution cannot be reached, the project will be discontinued.

Regulatory and policy issues related to the use of the metal release data are discussed in parallel within a Competent Authorities for REACH and CLP (CARACAL) bioelution subgroup. No meeting of the subgroup took place in 2023.

Box 4.1. Highlights from the OECD Working Party on Hazard Assessment

The 7th Meeting of the OECD Working Party on Hazard Assessment (WPHA) was held as a hybrid meeting on 27 to 28 June 2023. Topics included country updates, progress on OECD-related IT Tools (IUCLID, eChemPortal, QSAR Toolbox, OHTs), an update on ECHA's Robust Study Summaries project, the IATA Case Studies Project, work on the use of validated effect biomarkers to address human and environmental exposures to mixtures, progress on the Omics Application Reporting Module (ARM) for Chemical Grouping, and an update on OECD Guidance Document 194 on Grouping.

The WPHA had previously endorsed two IATA case studies from the 8th (2022) review cycle, which were declassified in March 2023. At the 7th meeting, the WPHA approved the IATA Considerations document (OECD, 2023c). More information can be found here: https://www.oecd.org/chemicalsafety/risk-assessment/iata/

An important outcome was the endorsement of the QSAR Assessment Framework (QAF) (OECD, 2023b), the development of which was led by the EU (ECHA) and Italy (ISS). The QAF is a systematic and harmonised framework for evaluating Quantitative Structure Activity Relationship (QSAR) models and their predictions, including QSAR "results" based on the predictions of multiple QSAR models. Further information can be found here: https://echa.europa.eu/-/new-qsar-assessment-framework-supports-alternatives-to-animal-testing

The JRC (EURL ECVAM) provided an update on the development of a Guidance Document to Improve the Use of Academic Data in Regulatory Assessments (see also **Section 4.5.3**).

Three new proposals were approved for: a) developing guidance on best practices and standardisation of omics samples; b) developing proteomics modules for the OECD Omics Reporting Framework (OORF); and c) completion of the Enrichment Analysis Reporting Module of the OECD Omics Reporting Framework (EARM).

Andrea Gissi (ECHA)

"The OECD (Q)SAR assessment framework (QAF) further improves the regulatory assessment of (Q)SAR results. This project was an important step of ECHA efforts to increase the confidence on computational modules and reduce reliance on animal testing as quickly as possible. With clear criteria and instructions how to apply them, registrants will be able to assess whether a given property can be reliably predicted, and regulators will gain confidence and transparency in their evaluations. Furthermore, (Q)SAR developers and users will be able to produce models and results that are more fit for the intended regulatory application. ECHA believes that the OECD QAF will significantly increase the use of the QSAR predictions for simpler endpoints. The framework is in line with ECHA's current evaluation practices and rules for (Q)SAR results, and REACH registrants can expect references to the QAF in future assessments of QSARs by ECHA."

4.1.4. OECD thyroid disruption methods expert group

On behalf of the OECD WNT, the Thyroid Disruption Methods Expert Group (TDM-EG) continued its assessment of data generated by EU-NETVAL laboratories with methods that are relevant for thyroid hormone system disruption. The assessments were completed for 10 methods investigating deiodinase-1 activity, thyroperoxidase activity, tyrosine iodination, thyroid hormone transport in serum, thyroid hormone glucuronidation, thyroid hormone receptor (in)activation and a method incorporating several modes of action using zebrafish eleutheroembryos.

During meetings in May and October 2023, the TDM-EG recommended further validation efforts for several methods to confirm with a limited amount of chemicals the methods' transferability to other laboratories and between laboratory reproducibility. Methods relevant for deiodinase-1 activity and thyroid hormone transport in serum will be further validated by PEPPER. For the validation of the other methods, the OECD is making efforts to find interested OECD member countries.

The plan is to hold the next meeting of the TDM-EG in May 2024.

4.1.5. OECD stakeholder workshop on organisational and financial aspects of validation

At the 35th meeting of the Working Party of National Coordinators of the OECD Test Guidelines Programme (WNT, see **Box 4.2**), it was agreed to hold an OECD Stakeholders' workshop on operational and financial aspects of validation. The workshop took place on 14 to 15 December 2023 and followed up on the 2022 WNT workshop to prepare the test guidelines programme for emerging technologies (OECD, 2023d).

The objectives of the workshop were to:

- Develop a good understanding of the global validation stakeholder community, including differences across regions.
- Understand the needs, drivers, and difficulties of validation for regulatory purposes, and how the stakeholder community is responding.
- Discuss and determine acceptable and good operational practices for validation moving forward with the uptake of emerging science and techniques.

A stakeholder survey on practical, organisational, and financial aspects of validation took place from July to September 2023 to collect different perspectives on issues that were subsequently discussed at the workshop. The workshop clarified the landscape of stakeholders involved in validation activities, including method developers, partner laboratories, organisations that provide financial support to validation activities, validation bodies, regulatory agencies, and standard-setting organisations. The workshop also provided case examples of what works well and does not work in validation, clarified practical aspects of validation that may need to be reflected in GD 34 revisions or elsewhere, and discussed how to increase trust in validation processes, practices, and resultant methods. A workshop report will be published in 2024.

Box 4.2. Highlights of the 35th meeting of the WNT

The OECD test guidelines (TG) for chemicals are internationally agreed-upon testing methods used by governments, industry, research labs, and academia to assess the safety of chemicals and products. They are used in regulatory safety testing and chemical notification and registration. The OECD regularly updates the TGs to reflect scientific developments and member countries' regulatory needs. Scientists from government, academia, and industry provide input through OECD-wide networks of National Coordinators.

The OECD test guidelines programme (TGP) and Mutual Acceptance of Data (MAD) agreement are the main tools for ensuring globally harmonised regulatory safety testing of chemicals. This supports an open global market, avoids non-tariff barriers to trade for the chemicals industry, and protects workers, consumers, and the environment. OECD is committed to animal welfare and implements the 3Rs principles in developing TGs. The MAD system saves governments and industry around 309 million euros each year (OECD, 2019) and thousands of animals by avoiding duplicate testing. The Working Party of National Coordinators of the Test Guidelines Programme (WNT) oversees the programme. The Joint Research Centre (JRC) acts as a National Coordinator for the OECD TGP, representing the European Commission and EU, and is a member of the WNT.

The 35th meeting of the WNT was held on April 25 to 28, 2023. The following new and updated Test Guidelines (TG), as well as other types of documents, were approved:

- New TG hydrophobicity index of manufactured nanomaterials
- Revised TG 240 Medaka Extended One-Generation Reproduction Test
- Revised TG 218-TG 219: sediment-water Chironomus test
- GD on Juvenile Medaka anti-androgen screening assay
- DRP on inclusion of thyroid-related endpoints in fish TGs
- Revised TG 442E skin sensitisation on IL-8 Luc assay (Annex 3)
- Initial recommendations on evaluation of data from DNT *In Vitro* Battery
- Study report on applicability of TG 442D for nanomaterials
- Updated Performance Standards 216 for TG 492B RhCE methods for eye hazard potential
- Validation and Peer-review reports for EpiSensA
- Corrected TGs 125, 316, 442C, 456, 458, 487, 497, 437, 438, 460, 491, 492, 494, 496, 498.

In addition, thirteen new project proposals were included into the TGP's workplan.

More information can be found on the OECD website of the TGP: www.oecd.org/chemicalsafety/testing/oecd-guidelines-testing-chemicals-related-documents.htm

4.2. Chemicals 2.0 - a vision for chemical safety in the EU

While contributing to the work of the REACH revision in the context of the Chemicals Strategy for Sustainability (EC, 2020b), it became clear that non-animal approaches do not generally fit for safety assessment as set up under the current legislative framework. In addition, it is too time-consuming to finalise the assessments of individual chemicals (EEB, 2022), while the majority of the chemicals on the market are not subject to any safety assessment at all (EEA, 2019).

Therefore, EURL ECVAM suggested a new future regulatory framework for chemicals – Chemicals 2.0 (Berggren *et al.*, 2023b), where the idea is to keep the levels of protection as today by calibrating the new system based on harmonised classified chemicals under the CLP Regulation (EC, 2008), so that the chemicals already classified as hazardous under the current system would be also caught by the new system (**Figure 4.1**). The new classification system should not consider observable adverse effects but cover essential toxicodynamic and toxicokinetic mechanisms leading to such effects without making predictions. The idea is that this simplified system would be possible to apply to all chemicals on the market and thereby improve the protection of human health and the environment, while identifying chemicals to be used for replacement of hazardous chemicals, supporting sustainability by design.

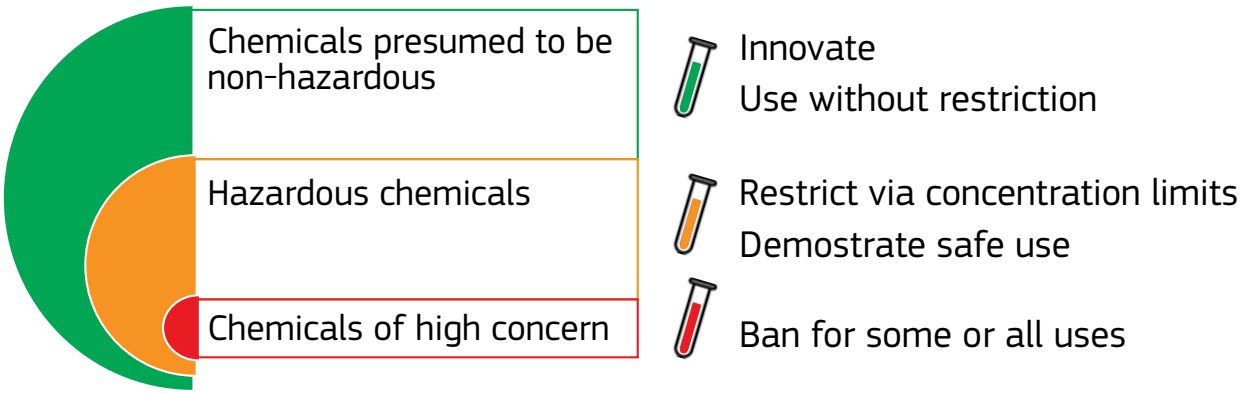

Figure 4.1. The principle of equivalent protection underpins the proposed transition to Chemicals 2.0. In this approach, NAMs are judged by their utility to reach the same decisions, rather than predict the same adverse outcomes.

4.3. EPAA promotion of the regulatory acceptance of alternatives to animal testing

The European Partnership for Alternative Approaches to Animal Testing (EPAA), a partnership between seven industry sectors, different Commission services and EU agencies, is continuing its activities which aim to replace animal testing by innovative, non-animal testing methods, to reduce the number of animals used and to refine procedures where no alternatives exist or are not sufficient to ensure the safety of substances (the '3R principle').

At the core of the EPAA work are the scientific projects, which synergistically combine the expertise available across industry sectors, academia, NGOs and regulatory agencies. EURL ECVAM co-chairs the project platform, and a number of individual projects. The status of these projects is summarised in **Box 4.3** showing that several projects have been successfully completed in 2023.

In addition, the EPAA seeks to promote its activities through international collaboration, knowledge sharing, its annual conference and stakeholder dialogue, including the continued and valuable input received from the mirror group. The aim of the annual conference was to give participants insight on the EPAA achievements in 2023, announce the EPAA Refinement Prize winner, and to discuss challenges and opportunities for protection of people and the environment through NAMs.

Read more at EPAA Annual report:
https://single-market-economy.ec.europa.eu/document/download/2612b269-bff4-4060-b19b-d93d2f25f7d2_en?filename=Annual%20report-2023-WEB.pdf

EPAA annual conference:
https://single-market-economy.ec.europa.eu/events/epaa-annual-conference-2023-protection-people-and-our-environment-through-nams-2023-11-15_en

4.3.1. EPAA project on non-animal science in regulatory decisions for chemical safety in the EU

The EPAA project on opportunities to use non-animal science in regulatory decisions for chemical safety in the EU builds on the actual experience of EPAA partners in the use of NAMs for decision-making and on the exchange of this experience between the industry sectors and Commission partners.

As follow up of the 'deep-dive' workshop on NAMs in November 2021 that kicked-off the discussions (Westmoreland *et al.*, 2022), two initial working groups were established to address the challenges identified around regulatory frameworks and knowledge sharing.

Working Group 1 focuses on addressing the gap between scientific research and regulatory use through reflecting on frameworks for the use of NAMs for regulatory decisions. It has explored the feasibility of two published conceptual frameworks: the ECETOC framework for chemical safety assessment incorporating NAMs within REACH (based on Ball *et al.*, 2022) and the "Chemical 2.0" framework, a cross-sector approach to safety assessment and risk management (see **Section 4.2**).

From the discussions, the group identified three main topics for follow up:

- to examine exposure-based approaches in the context of REACH, building on the concept of "classification of exposures";
- to survey existing weight of evidence approaches and evaluate their potential use to characterise chemical hazards, and
- to investigate a tiered approach as an alternative classification system for risk management/classification and labelling without using animal data.

Given the current priorities and expertise within the group, it was agreed to explore a new concept of future use of NAMs for hazard classification through a NAM Designathon challenge that is based on the vision of "Chemicals 2.0" (see **Section 4.2**). During the autumn 2023, EPAA invited the submission of NAM-based solutions to inform the development of a future classification system for systemic toxicity to human health based on the activity and potential systemic availability of chemicals[21]. The future system will divide chemicals into three classes of high, medium or low concern. The deadline for proposals was the end of 2023. In March 2024, all contributors will meet to compare and contrast the different solutions, and to plan a follow-up phase to further develop and test the new classification system.

Working Group 2 is progressing the implementation of a NAMs User Forum with the aim of addressing the lack of cross-sector, scientific consensus on NAMs' use for chemical regulatory testing. The first User Forum workshop was held on 7 to 8 December 2023 (Helsinki) hosted by ECHA and included discussions on different toxicological endpoints and NAMs through selected case studies.

Box 4.3. Status of EPAA projects

Clostridial vaccines – completed
Aim: replacement of alternatives to *in vivo* tests required for in-process testing of Clostridium septicum vaccines (Behr-Gross *et al.*, 2021).

Monoclonal antibodies – completed
Aim: to reduce the use of animal studies in the non-clinical safety studies during the drug development programmes of monoclonal antibodies for humans (Chien *et al.*, 2023).

Acute toxicity – completed, but dissemination is still ongoing
Aim: Evaluate if clinical signs (evident toxicity) are predictive of mortality at higher dose levels in acute oral toxicity studies and are an appropriate alternative to death as an endpoint (Sewell *et al.*, 2023).

Prediction of Carcinogenic Potential of Agrochemicals – final manuscript under preparation
Aim: enhance the prediction of carcinogenic potential of agrochemicals in humans using mechanistic information together with 90-days repeated dose toxicity data to reduce or replace the need for the 2-year carcinogenicity studies.

Rabies vaccines – start of phase 3
Aim: replace the current *in vivo* potency test for the release of human rabies vaccines (US National Institute of Health mice intracranial challenge test) with an *in vitro* antigen (G glycoprotein) quantification assay using an ELISA technology.

Skin sensitisation User Forum - ongoing
Aim: build confidence in the use of NAMs for skin sensitisation in decision-making through knowledge exchange.

Harmonisation of 3Rs in Biologicals - ongoing
Aim: facilitate harmonisation and international convergence of 3Rs in regulatory testing requirements for biological products (Cirefice *et al.*, 2023)

Non-animal science in regulatory decisions for chemical safety – ongoing (See **Section 4.3.1**)

Read more: https://single-market-economy.ec.europa.eu/system/files/2023-11/Annual%20report-2023-WEB.pdf

21 https://single-market-economy.ec.europa.eu/calls-expression-interest/epaa-launches-designathon-human-systemic-toxicity_en

4.4. Classification and Labelling

4.4.1. Inclusion of new hazard classes in the CLP Regulation

The Classification, Labelling and Packaging (CLP) Regulation (EC, 2008) requires that companies communicate the hazards presented by chemicals they place on the market. In this way, the EU aims to protect workers, consumers and the environment from the hazards posed by these substances as well as ensuring the free movement of substances, mixtures and articles.

New hazard classes for classifying, labelling and packaging (CLP) substances and mixtures were introduced via a revision of the CLP regulation in December 2022, and entered into force on 20 April 2023[22]. The new hazard classes are:

- Endocrine disruption for human health - ED HH in Category 1 (known or presumed ED) and Category 2 (suspected ED)
- Endocrine disruption for the environment - ED ENV in Category 1 (known or presumed ED) and Category 2 (suspected ED)
- PBT (persistent, bioaccumulative, toxic), vPvB (very persistent, very bioaccumulative)
- PMT (persistent, mobile, toxic), vPvM (very persistent, very mobile)

ECHA is now in the process of updating the CLP guidance to include guidance on application of the new CLP criteria for the ED hazard classes. A stakeholder consultation on the draft guidance is underway through the so-called 'Partner Expert Group'. This will be followed up with a consultation of ECHA's Committees and will be concluded via consultation of the European Commission and the Member State Competent Authorities. The aim is to finalise the guidance by mid-2024. A similar process is ongoing for an update of the guidance to include the application of the new criteria for the PBT/vPvB and PMT/vPvM hazard classes[23].

4.4.2. Inclusion of new hazard classes in UN GHS

Since the CLP Regulation is based on the UN's Globally Harmonised System of Classification and Labelling of Chemicals (GHS) a proposal to introduce the new hazard classes into the UN GHS was made by the EU delegation to the UN GHS Sub-Committee in December 2022. The proposal was accepted and the EU is now leading a new UN informal working group on 'potential hazard issues' (PHI-IWG) to consider the need to develop global criteria for a number of different hazard classes[24].

The PHI-IWG has developed a work plan for the 2023-24 biennium starting with those hazard classes introduced into the CLP regulation via the recent update, (endocrine disruptors for human health and the environment, PBT/vPvB, and PMT/vPvM) (see **Section 4.4.1**), but also considering other critical hazards such as neurotoxicity, immunotoxicity, and hazardous to the terrestrial environment which was approved at the 44th session of the UN GHS sub-committee of experts in July 2023[25].

As a first step a mandate to the OECD was drawn up to request a review of the science needed for classification and labelling of substances and mixtures that have endocrine disrupting properties, using the current IPCS/WHO definition of endocrine disruptors as a basis and considering the extent to which current GHS hazard classes may already address endocrine disruptors. OECD accepted the mandate and has set up an ad hoc expert group, which started its work in September 2023, aiming to provide a response to the UN GHS's request by the end of 2024. JRC is co-chairing the ad hoc expert group.

The PHI-IWG also agreed to develop a mandate for the OECD in relation to 'persistence and mobility' within the new PMT/vPvM criteria, and start the work on criteria for PBTs/vPvBs and hazardous to the terrestrial environment in different work streams with different co-leads. Delegates that have volunteered so far to co-lead on the different hazard endpoints are as follows:

- DE-EU co-leading on persistence and mobility
- US-ICCA co-leading on immunotoxicity.

The work related to neurotoxicity and immunotoxicity will be initiated later, but still within the current biennium if resources allow.

22 https://environment.ec.europa.eu/publications/clp-delegated-act_en
23 https://echa.europa.eu/support/guidance/consultation-procedure/ongoing-clp
24 https://unece.org/info/events/event/368936
25 https://unece.org/info/events/event/373701

4.4.3. Revision of the UN GHS chapter on skin sensitization

In the biennium 2021-2022 EURL ECVAM contributed to the work of the informal working group on the use of non-animal test methods (NATM) for classification of health hazards by revising Chapter 3.4 to include criteria based on non-animal data (from *in chemico*, *in vitro* methods and non-standard methods[26]) for classification of skin sensitising substances. The revised chapter was published in July 2023 in the tenth revision of the GHS[27].

The informal working group - NATM has been working since then on amending Chapter 3.4 to include non-animal methods for the classification of mixtures and to develop a guidance section specific to mixtures providing a detailed overview of factors to be considered in the classification of mixtures. The IWG-NATM is aiming to get consensus from the group on the revised text by March 2024 with a view to submitting a formal proposal for adoption at the UN GHS sub-committee meeting in July 2024.

4.4.4. Revision of the UN GHS classification criteria for germ cell mutagenicity

EURL ECVAM is leading the informal working group on germ cell mutagenicity on behalf of the European Union since 2021. The objective of the working group is to review the classification criteria, but also update the Chapter 3.5 on germ cell mutagenicity in general and according to the current state of science, including newly available test guidelines, as well as add guidance.

The discussion on merging category 1A and 1B and defining common criteria for category 1 stalled during 2023 after initially gaining considerable support in the working group, thus no consensus was reached to present such a proposal to the GHS sub-committee. The issue is to be revisited at a later stage. The discussion on the criteria of category 2 will now be started.

A new section including text on the use of non-testing methods[28] for classification was imported into Chapter 3.5 from the recently revised Chapters 3.2, 3.3, 3.4 on topical toxicity. The introduction of this text triggered considerable discussion within the working group, primarily because germ cell mutagenicity is considered as a more critical endpoint than local effects to skin and eye and skin sensitisation, and there is thus the perception that this could lead to less protection. The working group will also consider how and if to introduce classification criteria based solely on *in vitro* methods.

Read more: Report of the Sub-Committee of Experts on the Globally Harmonized System of Classification and Labelling of Chemicals on its forty-fifth session: https://unece.org/sites/default/files/2023-12/ST-SG-AC10-C4-90e.pdf

26 Non-standard methods, according to GHS Chapter 3.4.5.3.6, are validated but not yet adopted in chemico/*in vitro* methods as well as *in vivo* test methods which do not comply with internationally agreed guidelines

27 https://unece.org/transport/documents/2023/07/standards/ghs-rev10

28 Non-testing methods include computer models predicting qualitative structure activity relationships (structural alerts, SAR); quantitative structure-activity relationships (QSARs); computer expert systems and read-across using analogue and category approaches.

4.5. Data and knowledge management

4.5.1. AOP Knowledge Base

The AOP-Wiki is the central repository for Adverse Outcome Pathway (AOP) related knowledge. AOPs are a framework for organising scientific knowledge about how chemicals, other stressors, and biological processes can lead to adverse effects in humans and other organisms. The AOP-Wiki currently holds more than 400 AOPs and is used by researchers, regulators, and other stakeholders around the world.

A group of experts, led by EURL ECVAM, is working to develop a new version of the AOP-Wiki, known as Wiki 3.0. This new version will be designed to meet the needs of a growing user base and to support the latest advances in AOP science.

Wiki 3.0 will introduce a range of new features, including:

- A more intuitive user interface that is accessible to users with a wide range of technical expertise.
- A clearer and more pronounced way of managing and displaying information about test methods used to assemble AOPs.
- Improved FAIRness (Findability, Accessibility, Interoperability, and Reusability) of the Wiki.
- Improved ways to trace who contributed which knowledge and what their rationale was.
- Tagging of certain free text elements (stressors, references, and others) to increase searchability.

This added and improved functionality is expected to increase trust in the Wiki content, so that regulators will feel more comfortable applying AOP knowledge.

Actual development work on Wiki 3.0 is expected to start in late 2024, and it is a significant undertaking. However, it has the potential to improve the way that AOPs are developed, applied, and communicated. By making AOP knowledge more accessible, reliable, and FAIR, Wiki 3.0 will help to accelerate the development of new and safer chemicals and products.

4.5.2. Use of chemical monitoring data from IPCHEM to build indicators

IPCHEM, the Information Platform for Chemical Monitoring, is a single access point where EU authorities, national and regional authorities, and researchers can find and share information about where chemicals are found in the environment and at which concentrations. Users can discover information on chemical concentrations in air, water and soil, food and animal feed and in humans (**Figure 4.2**). IPCHEM will be integrated in the future common open data platform on chemicals (CDPC), which will facilitate the sharing, access and re-use of different types of information on chemicals coming from different sources.

On top of continuously integrating new data and updating already included datasets, the focus of the JRC's IPCHEM team is to use data to address policy questions. Lately, the team explored the use of EU-scale monitoring datasets to develop indicators to track progress in reducing exposure to chemicals and related risks. Such indicators are used under the various strategies such as the Chemicals Strategy for Sustainability to evaluate the progress towards policy targets. Examples include the use of pesticide residue data in soil in the context of evaluating risk to soil organisms (Vieira *et al.*, 2023). Other examples under development are exploring the use of human biomonitoring data to look at the trends of risks from mixtures of chemicals found in humans, and the use of pesticide residues in food to look at trends in dietary exposure of humans.

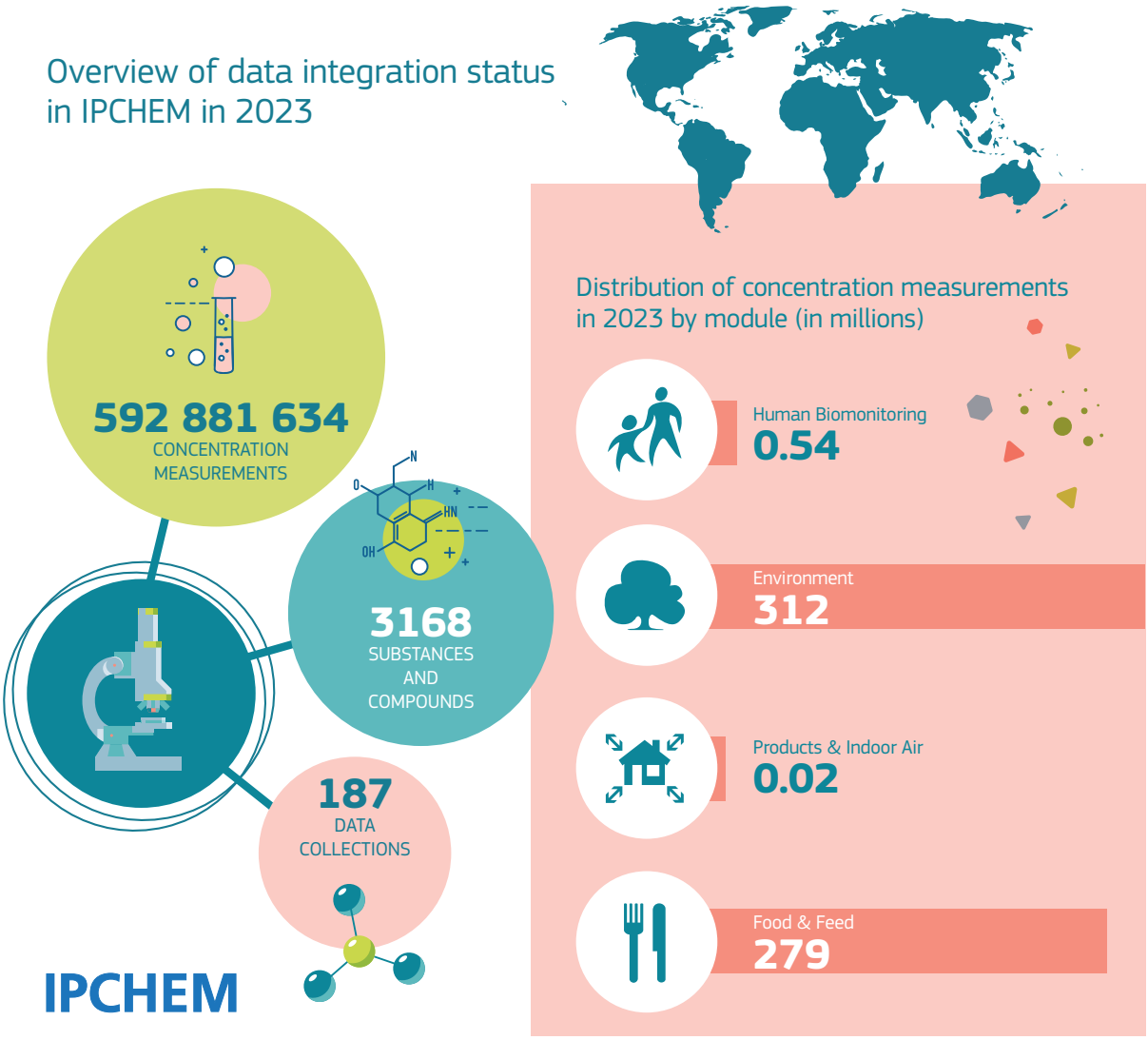

Figure 4.2. Overview of data integration status in IPCHEM in 2023.

4.5.3. Improving the use of academic data in risk assessment

Chemical legislation across countries and policy areas requires assessors to consider all relevant and reliable scientific data in performing safety assessments. Much of these data, published worldwide, are generated using non-standard methods that include an increasing amount of data obtained using non-animal approaches. Such data is highly heterogeneous in their methodological quality and reporting standards. Consequently, assessors struggle to implement transparent, rigorous and efficient workflows to identify and evaluate this deluge of data.

The JRC is leading an expert group in the OECD Working Party on Hazard Assessment to develop and implement a Guidance Document to improve the use of academic data in regulatory assessments. The work is ongoing, and the outcome is expected in 2024. In 2023, the expert group carried out four case studies and two surveys. The case studies explored current workflows implemented by assessors in four representative regulatory scenarios. Through the survey, the expert group collected evidence on the state of play and recommendations from assessors, publishers, editors and data repository managers (https://ec.europa.eu/eusurvey/publication/OECD-WPHA_Survey_Publication_Academic_Data_Regulatory_Use). The information collected is currently informing the development of the guidance document. The aims of this guidance are to define good practice for researchers to maximise the regulatory utility of published scientific data. It also aims to identify opportunities to harmonise and reuse elements of assessment workflows. This includes tools for the evaluation of non-standard data and templates to facilitate the reuse of results from screening and evaluation workflows.

4.6. World Congress on Alternatives and Animal Use in the Life Sciences (WC12)

EURL ECVAM participated in the 12th World Congress on Alternatives and Animal Use in the Life Sciences (WC12) in Niagara Falls, Canada, on 27 to 31 August. Its contributions were key to several of the main themes of WC12. Besides the plenary sessions, there were six parallel sessions mirroring the main themes and many activities and opportunities to interact with other participants. The sessions represented forefront research on alternatives to animal testing and implementation of such methodologies in legislation, education and in different industrial sectors, based on ethical reasons as well as supporting scientific progress towards a sustainable future.

A key note speaker from EURL ECVAM gave a talk entitled, "Regulatory acceptance, unpacked and unplugged", explaining the challenges and opportunities to translate non-animal approaches into chemical safety assessment and referring to several unit activities.

Another EURL ECVAM representative contributed with two oral presentations under the theme "Regulatory acceptance and global harmonisation", one looking at the introduction of NAMs in the UN Globally Harmonised System for Classification and Labelling of Chemicals (see **Section 4.4**); and the other on a future vision of method validation underlining that it's not all about ring trials (see **Section 3.6**). A further presentation was given under the same theme on how to evolve the regulatory framework based on innovative and scientifically valid methods. EURL ECVAM also presented and participated in two panel discussions: "Putting All the Chips on the Table! Doubling Down on Regulatory Acceptance of MPS" and "The 3Cs: Curating & Cultivating Change for Faster Integration of NAMs in Animal-Free Policy Making".

Within the theme "Ethics, welfare, policies and regulations", an oral presentation was made on how to develop indicators to monitor progress on the implementation of the Three Rs (see **Section 5.1.3**). A future vision of a more efficient chemical safety assessment applying animal-free approaches was laid out, based on a recent paper on Chemicals 2.0 (see **Section 4.2**), which is also the basis for the EPAA Designathon (see **Section 4.3**).

In a session on "Next-gen education", the EURL ECVAM initiative on how to introduce the Three Rs in school and high school education was described, including how EURL ECVAM carried out training online for teachers together with the European Schoolnet including learning scenarios (see **Section 5.2**).

Other topics explored by EURL ECVAM speakers included the credibility of computational models in biology and toxicology under the theme of "Human centred biomedical research", as well as oral presentations on how to accelerate transition to animal-free biomedical research, and non-animal methods in neuroscience. Finally, a session on animal-free reagents, methods and standards was chaired by EURL ECVAM.

5. Alternatives in research and education

In 2020, the EU and Norway witnessed over five and a half million uses of animals for biomedical research related to human purposes (excluding ethology/animal behaviour/animal biology under basic research and animal welfare, and animal diseases and disorders under translational and applied research; **Figure 5.1**). This makes biomedical research the predominant sector utilising animals for scientific purposes, accounting for around 68% of all animal uses in research and testing. Therefore, incorporating the principles of the Three Rs into research practices represents a formidable challenge that necessitates specific approaches.

As per Directive 2010/63/EU, all scientific projects must undergo evaluation, taking into consideration the 3Rs, before receiving approval. Replacement proves to be a particularly arduous task due to the inherent nature of scientific experimentation itself, which involves developing new methods, testing hypotheses, and exploring novel ideas. To make progress in this direction, it is crucial to shift mindsets and increasingly incorporate non-animal approaches in basic, applied, and translational research.

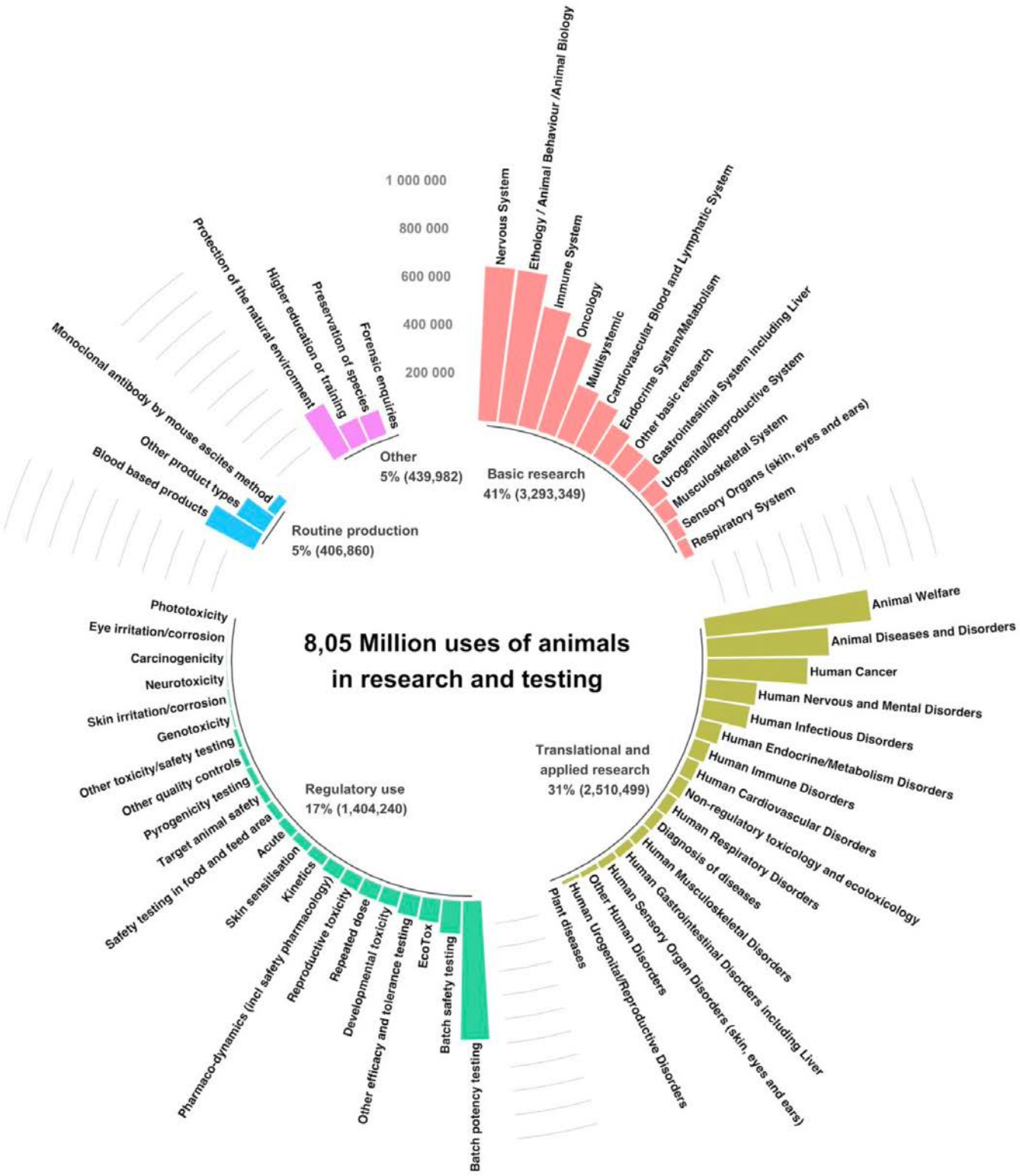

Figure 5.1. Statistics on the use of animals for scientific purposes for EU and Norway, including re-uses, in 2020.

In 2023, EURL ECVAM, built upon the seven reviews of advanced non-animal models in biomedical research[29] (see **Box 5.1**) to identify how to foster the adoption of non-animal methods in biomedical research. It also continued the knowledge sharing of alternative approaches to animal testing by promoting its educational resources and organising its fourth JRC Summer School.

29 https://joint-research-centre.ec.europa.eu/eu-reference-laboratory-alternatives-animal-testing-eurl-ecvam/biomedical-research_en

5.1. Biomedical research

In 2023, EURL ECVAM launched new initiatives aimed at enhancing the recognition and utilisation of advanced non-animal models in biomedical research. These initiatives involved analysing the potential of these models to provide supplementary information to approved research projects that currently use animals and starting a project to collect most recent published models.

5.1.1. Thematic review on the state of 3Rs implementation in cardiovascular diseases, a feasibility study

Directive 2010/63/EU on the protection of animals used for scientific purposes mandates that Member States (MS) publish non-technical summaries (NTS) of authorised projects involving animals within six months from their approval. The information to be provided includes the projects' objectives, the number and type of animals to be used, the severity of the harm inflicted, and the demonstration of compliance with the 3Rs. The language used should be understandable by the public to increase transparency on animal-based research. Since January 2021, NTS are submitted to a central database. Additionally, the directive stipulates that the European Commission should conduct periodic thematic reviews on available non-animal approaches with the potential to replace or reduce the numbers of animals used for scientific purposes (article 58).

EURL ECVAM conducted a feasibility study through an analysis of the information found in the NTS approved for cardiovascular disease research. The projects were classified according to the subject matter investigated, using as basis the categories found in the dataset coming from its report (Celi *et al.*, 2022), and were deemed to be of sufficient quality to carry out a pilot thematic review related to improving the adoption of the Three Rs in cardiovascular disease biomedical research.

After presenting its conclusions to the 25th National Contact Point meeting on 10 to 11 October 2023, EURL ECVAM decided to launch together with DG ENV a pilot thematic review on the state of 3Rs implementation in cardiovascular diseases.

5.1.2. European Parliament pilot project on the development and use of artificial intelligence/ machine learning approaches for biomedical models review updates

In November 2023, EURL ECVAM kicked-off a new project to develop an automated database that collects and structures information on non-animal models in use for biomedical research, using automated approaches to mine the vast body of published literature. This will enable the creation of an up-to-date, state of the art knowledge source collating non-animal models applied to biomedical research, allowing the extension of already collected models both in time (from 2019 up to now) and in scope with the addition of new diseases categories (including infectious diseases, metabolic diseases and gastrointestinal disorders). Moreover, it will allow interested parties (e.g., scientists working in biomedical research, Member States evaluation committees, educational institutions, etc.) to search easily for information on available non-animal models in specific categories.

By understanding and sharing information on successful non-animal models in biomedical sciences, it is expected that the transition of the scientific community towards human biology-based methodologies will be encouraged, facilitated and potentially accelerated. In fact, the use of human biology-based models and methods is vital to improve the relevance of biomedical research, to enhance the likelihood that results will translate to patients and to accelerate the transfer of research results into clinical and public health practices. It is also expected that such databases represent a valuable and sustainable resource for stakeholders such as Member State Competent Authorities responsible for project evaluation or research project funding organisations.

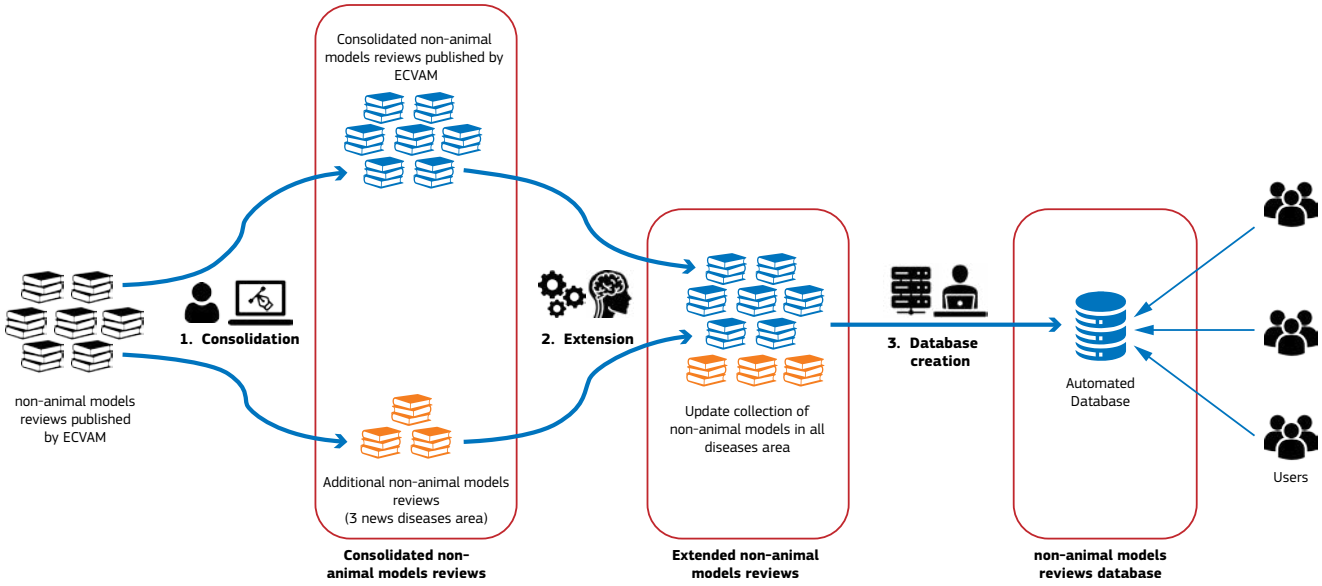

Figure 5.2. Implementation objectives of the artificial intelligence driven database of non-animal models in biomedical research.

Though the automation of the selection process and the organisation of identified non-animal models, extension in time and scope should continue beyond the project and continuously deliver new models (**Figure 5.2**).

5.1.3. EURL ECVAM indicators to assess the impact of EU-funded research into Alzheimer's disease, breast cancer and prostate cancer

In 2023, EURL ECVAM published a scientific article on "Gauging innovation and health impact from biomedical research: survey results and interviews with recipients of EU-funding in the fields of Alzheimer's disease, breast cancer and prostate cancer" (Pistollato *et al.*, 2023) and a technical report on the assessment of the impact of EU-funded biomedical research in the areas of Alzheimer's disease, breast cancer and prostate cancer through the use of fourteen indicators (Gastaldello *et al.*, 2023a).

The article discusses the main findings coming from a survey carried out in 2020 and addressed to former and current participants of EU-funded research projects in the fields of Alzheimer's disease, breast cancer and prostate cancer, complemented by the in-depth interviews of thirty participants and followed by a round table discussion with DG JRC, RTD and SANTE organised at the end of 2021. It proposes a set of priority actions that could be considered to help improving the translation of scientific innovation of biomedical research into societal impact.

The report describes the development and refinement of these indicators and presents a case study of their use applied to EU-funded biomedical research. The publication summarises and further develops work initiated in 2020 about the retrospective monitoring of outputs and societal impact of biomedical research in the three above-mentioned areas funded by the EU framework programmes FP5, FP6, FP7 and H2020 in the previous 20 years. The report is accompanied by a data table accessible from the JRC data catalogue, which can be exploited by interested stakeholders to delve deeper into the findings and/or carry out tailored-made analyses (Gastaldello *et al.*, 2023b).

The report classified outputs according to funding programme and disease area of research, but did not take the type of methods and approaches employed into consideration. To further the analysis in this direction, in 2023, EURL ECVAM in collaboration with the Humane Society International, started work on using some of the indicators to assess the impact of methods on the outputs of EU-funded research. Projects were therefore categorised based on whether they used methods employing animals, methods not employing animals, or a combination of both. This work will inform on the relationship between methods and outputs/impact, and possibly shape future funding strategies to improve their returns in terms of societal impact.

5.1.4. Promoting Reusable and Open Methods and Protocols (PRO-MaP): draft recommendations to improve methodological clarity in life sciences publications

Detailed, accessible methods are essential for reproducibility, trust in science and scientific advancement, yet many studies suggest that the reporting of methodological details in life sciences research publications is often incomplete. This may be due to a lack of incentives or reporting standards, or other cultural or educational factors.

In 2023, EURL ECVAM published a pre-print (Leite *et al.*, 2023) together with the participants of the "Scientific methods and protocols: roadmap to increase clarity in life sciences peer reviewed publications" workshop that took place in June 2022. It contains recommendations for Promoting Reusable and Open Methods and Protocols (PRO-MaP) to increase and improve the reporting of detailed, reusable and open methods and reusable step-by-step protocols in the life sciences. These draft recommendations outline actions that four stakeholder groups such as researchers, research institutions and departments, publishers and editors, and funders, can take to achieve these goals. While some recommendations address study design and reporting guidelines, the primary focus is on capturing clear, accurate, methodological detail, e.g. with re-usable step-by-step protocols.

During summer 2023, Charité Berlin led a set of consultations amongst the four stakeholder groups, including experts in improving methodological reporting, to finalise the recommendations. Feedback received during consultation sessions will be incorporated to the pre-print and a publication prepared to encourage organisations and individuals from each stakeholder group in collaboratively working to improve the reporting of detailed methods and reusable step-by-step protocols in the life sciences.

5.1.5. CIAO – applying the AOPs in the biomedical research to model the COVID-19 pathogenesis

The CIAO project (Modelling the pathogenesis of COVID-19 using the Adverse Outcome Pathway Framework) was successfully concluded in 2023. Initiated by EURL ECVAM in the first weeks of the pandemic, the project quickly developed into a large scale interdisciplinary and international collaboration of 70+ scientists from 50+ renowned research, academic and government organisations world-wide. Amidst the deluge of COVID-related information, the CIAO project aimed to distill and organise this vast knowledge into manageable streams of evidence. By doing so, the project sought to identify critical gaps in our understanding, pinpoint relevant biomarkers, and elucidate the mechanisms by which diverse factors, such as age, impact clinical outcomes. Adverse Outcome Pathways (AOPs) are an OECD-led knowledge management framework well acknowledged in toxicology, and the CIAO project succeeded in extending this proven conceptual approach for structuring knowledge to a viral disease. The COVID-AOPs were disseminated via the publicly accessible AOP-Wiki website and led to the publication of numerous papers (Carusi *et al.*, 2023; Hogberg *et al.*, 2022; Shahbaz *et al.*, 2022).

Developing evidence-based pathways starting from binding of the virus to its receptor and leading to the diverse disease outcomes (e.g. respiratory distress, anosmia) via a series of key events at the different levels of biological organisation (molecular, cellular, organ, tissue, individual) required input from several scientific disciplines. Hence, within CIAO, AOPs were effective in supporting a true interdisciplinary collaboration. The CIAO way of collaboration, i.e. to assemble a community across various scientific domains, deploy a conceptual framework as the basis for a shared language, collect and disseminate knowledge at a central location, make the exploration of synergies between disciplines the default mind-set, has raised significant interest, both at EURL ECVAM and outside. Applying the lessons learned in CIAO, both in terms of results and process, to other science and research areas will be the legacy of the project.

5.1.6. Re-use of human health data in biomedical research

Mathematical models offer the opportunity of reducing the use of animals in biomedical research, while providing innovative tools which can be used as clinical decision support systems, for personal health forecasting or as methodologies for the development of personalised medical products. Computational models can be defined either as knowledge-driven, when they are based on prior mechanistic knowledge on cause-and-effect relationships of the phenomenon of interest or as data-driven, when they rely directly on data without making any causal assumption, such as artificial intelligence methods. Both approaches are effective depending on the question of interest and many models are built as a combination of those approaches. Independently of the modelling approach, such models require human health data such as medical images, electrophysiological signals or genomic data, for their development and validation.

EURL ECVAM is working on reviewing the current innovations in biomedical research based on human data, to provide a clear picture of the ongoing research projects at the European level and the current challenges in the field. Among the research projects, the EC launched the EDITH[30] consortium with the mission of defining a roadmap to build a European 'Virtual Human Twin' (VHT) by identifying the main barriers towards its implementation. The VHT aims to be an integrated multiscale and multi-discipline representation of quantitative human physiology and pathology designed to accelerate the development of patient-specific predictive computer models. Besides the lack of high-quality data available in open access, major barriers in the re-use of human health data for the development and validation of VHT are the lack of guidance of data anonymisation and data sharing, the lack of harmonisation and standardisation among different collectors and the lack of clear and consolidated regulatory pathways. As those challenges are linked to specific regulations, EURL ECVAM will also contribute to assess how real-world data can be re-used in line with the EU legal framework (Data governance act (EU, 2022), Artificial Intelligence act[31]) and initiatives (European Health Data Space[32]).

Box 5.1. EURL ECVAM reviews of advanced non-animal models in biomedical research

EURL ECVAM has released a series of studies aimed at assessing the current and emerging non-animal models used in biomedical research across seven disease domains, including respiratory tract diseases, breast cancer, neurodegenerative disorders, immuno-oncology, immunogenicity testing for advanced medicinal therapy products, cardiovascular diseases, and autoimmune diseases.

30 https://www.edith-csa.eu

31 https://www.europarl.europa.eu/news/en/headlines/society/20230601ST093804/eu-ai-act-first-regulation-on-artificial-intelligence

32 https://health.ec.europa.eu/ehealth-digital-health-and-care/european-health-data-space_en

The objective of these studies is to identify and elucidate specific research scenarios where traditional animal models have been replaced by innovative non-animal techniques. These novel approaches encompass *in vitro* methodologies employing human cells and engineered tissues, as well as *in silico* methods utilising computer modelling and simulation.

The expectation is that by understanding and sharing information on successful use-cases of alternative models in biomedical research, the transition towards non-animal approaches can be better facilitated and potentially accelerated.

Encouraging the uptake of alternative methods is important to tackle such considerable reliance on animal experiments for carrying out research. Furthermore, non-animal methodologies offer the promise of more accurately replicating human physiology compared to many traditional animal models, ultimately enhancing our comprehension of human-specific biology and disease.

Each disease domain is covered by a technical report describing the specificities of the models identified in this area, an executive summary providing an overview of the non-animal methods development as well as a list of models containing references to peer reviewed articles. Reports can be downloaded from the JRC science hub and individual datasets from the JRC data catalogue, EURL ECVAM collection[33].

5.2. Education and training

5.2.1. JRC Summer School 2023: towards sustainable innovation

The aim of the JRC Summer School is to share knowledge and experience on the latest non-animal approaches in science and to promote their use in biomedical research, regulatory applications and drug development. It is specifically tailored for post-graduate students and early-career scientists working in the biosciences field and focused on non-animal methods and technologies. Hundred-twenty students from all over the world participated at the fourth edition of the JRC Summer School entitled "Non-animal Approaches in Science: Towards Sustainable Innovation" at the JRC in Ispra, Italy, 23 to 26 May 2023 (**Figure 5.3**; (Berggren *et al.*, 2023a)).

The summer school is organised every second year, and this was the fourth edition with a highly engaging and interactive programme combined with lectures by both invited and in-house experts across several scientific fields, with plenary sessions followed up with round table discussions. The students themselves took the lead in debate sessions, explored how to introduce the Three Rs in university education and prepared poster presentations. The JRC Summer School is not only a Three Rs training, it is about creating networks across sectors, across the globe, and even across generations. We observed how students from the earlier editions are now sending their own PhD students to attend, or are themselves invited as speakers. The emerging science without the use of animals is a game changer, because it forces us to understand more of the underpinning biology, and the summer school students themselves are actively contributing to this progress when interacting and challenging both speakers and other students with multi-disciplinary background in discussions and debates.

Figure 5.3. Participants of the JRC Summer School 2023.

33 https://data.jrc.ec.europa.eu/collection/id-0088

5.2.2. EURL ECVAM laboratory virtual reality

The EURL ECVAM laboratory virtual reality (VR) application is a project funded by the European Parliament. The aim of the project is to develop a digital learning application for students between 14 and 18 years old, which provides awareness for alternatives to animal testing. In 2023, EURL ECVAM completed the storytelling and launched, with the support of the publication office, an implementation contract with Markenfilm Space that will design it with young people media habits in mind (**Figure 5.4**).

Students will explore the laboratory setting through a playful experience. They will work with a professor and learn about alternatives to animal testing. Specifically, they will complete a series of experiments and tasks, which will enable them to evaluate a novel non-animal method for testing chemicals. The VR experience should take 20 minutes to complete and is expected to make a significant contribution to science education by providing students with a fun and engaging way to learn about science, technology, engineering and mathematics in general and alternatives to animal testing in particular. The application will have educational purposes and it will be used in the context of school lessons.

After the VR prototype is developed, selected users will evaluate its functionality and users' understanding of the concepts. The findings of the user testing will enable to improve the application before it is released to the public. Oculus Quest 2 as well as a web application will be used to run the VR that will be also accessible to visually and hearing-impaired users.

This virtual reality application will be added to the resources on the 3Rs already developed by EURL ECVAM for primary and secondary pupils (see **Box 5.2**).

Figure 5.4. A view of the virtual EURL ECVAM laboratory.

Box 5.2. EURL ECVAM educational resources for primary and secondary students

In 2023, EURL ECVAM finalised the Three Rs project implemented by EU Schoolnet focusing on introducing the principles of the Three Rs in primary and secondary education. The goal was to inspire students to think critically about science, become aware of the scientific progress made for a future science without animal use, and build the skills to debate nuanced and complex topics such as animal testing.

With the learning activities of the project, students will develop science literacy skills by exploring topics such as ethics in science, how the European Union is protecting the welfare of laboratory animals, and what high-tech non-animal tools are available as alternatives.

All resources are available on the Scientix website[34] and can be downloaded freely. Teachers will find engaging teaching materials to include in their school lessons: career sheets, podcasts and interviews with experts working in the field as well as interesting learning scenarios.

Ten learning scenarios are available for primary school and seven for secondary school pupils. These scenarios are completed by additional resources linked to existing pedagogical trends and the 21st century skills to help teachers to integrate the Three Rs in their curriculum and prepare students for future careers in the field.

The materials can be used as part of one lesson, or throughout several lessons. Learning scenarios can be also implemented as project-based interdisciplinary activities that could be implemented in collaboration with several teachers. While generally intended for STEM classes, materials can be implemented in non-STEM classes with small adaptations.

Last, teachers will also find information from their peers presenting their own experience of implementing some of the learning scenarios in their classroom.

34 https://www.scientix.eu/projects/steam-partnerships/3rs

6. Conclusions

The 2023 EURL ECVAM Status Report showcases a strong commitment to advancing the development, validation, and regulatory application of non-animal methods in scientific research and testing. Through collaborative projects, EURL ECVAM is leading the charge in promoting NAMs that offer more ethical, cost-effective, and potentially more accurate alternatives to traditional animal-based models. EURL ECVAM's commitment to update OECD GD 34 and to the validation and standardisation of innovative and complex methods and technologies demonstrates EURL ECVAM's pivotal role in ensuring that non-animal methods meet the rigorous standards necessary for regulatory acceptance. EURL ECVAM's initiatives are also aligned with the objectives of the European Citizens Initiatives "Stop Vivisection[35]" and "Save cruelty free cosmetics – commit to a Europe without animal testing[36]", as well as the European Parliament's resolution[37], which call for a shift away from animal testing towards more humane and scientifically advanced alternatives. The report's focus on regulatory applications of non-animal methods reflects a proactive response to these initiatives, highlighting efforts to integrate NAMs into the regulatory frameworks that govern chemical hazard and risk assessments. Furthermore, EURL ECVAM has taken significant steps to integrate non-animal approaches into education and training programmes, which is crucial for nurturing a culture of innovation and ethical scientific practices among the next generation of researchers. Projects like the JRC Summer School and the development of a virtual reality application for students underscore the importance of incorporating alternatives into the educational mainstream. The 2023 EURL ECVAM Status Report, therefore, not only reflects the progress made in the field of alternatives to animal testing but also emphasises the ongoing need for research, validation efforts, collaboration, and education to fully realise a future where non-animal methods are the gold standard in scientific testing and research. With continued support and commitment from the scientific community, regulatory bodies, and the public, the vision of a Europe that champions cruelty-free and better science and respects animal welfare can become a reality.

35 http://www.stopvivisection.eu/

36 https://europa.eu/citizens-initiative/initiatives/details/2021/000006_en

37 https://oeil.secure.europarl.europa.eu/oeil/popups/ficheprocedure.do?reference=2021/2784(RSP)&l=en

References

Bal-Price, A., Hogberg, H. T., Crofton, K. M., Daneshian, M., FitzGerald, R. E., Fritsche, E., Heinonen, T., Bennekou, S. H., Klima, S., Piersma, A. H., Sachana, M., Shafer, T. J., Terron, A., Monnet-Tschudi, F., Viviani, B., Waldmann, T., Westerink, R. H. S., Wilks, M. F., Witters, H., Zurich, M. G., and Leist, M. (2018). Recommendation on Test Readiness Criteria for New Approach Methods in Toxicology: Exemplified for Developmental Neurotoxicity. *Altex-Alternatives to Animal Experimentation*, 35, 306-352. doi:10.14573/altex.1712081

Ball, N., Bars, R., Botham, P. A., Cuciureanu, A., Cronin, M. T. D., Doe, J. E., Dudzina, T., Gant, T. W., Leist, M., and van Ravenzwaay, B. (2022). A framework for chemical safety assessment incorporating new approach methodologies within REACH. *Archives of Toxicology*, 96, 743-766. doi:10.1007/s00204-021-03215-9

Batista Leite, S., Beken, S., Brendler-Schwaab, S., Corvi, R., Daskalopoulos, E. P., Delrue, N., Fitzpatrick, S., Piergiovanni, M., Tarazona, J., van Engelen, J., and Whelan, M. (2021). Resources for Organ-on-Chip validation and qualification - EUROoCS RAB. European Commission, Joint Research Centre (JRC) [Dataset]. Retrieved from https://data.jrc.ec.europa.eu/dataset/7bcb1db5-5c7e-460b-b79e-ca5f642514a4

Behr-Gross, M. E., Siklodi, B., Le Tallec, D., Halder, M., Manou, I., Sinitskaya, N., Bruckner, L., Dalmadi, B., Kiss, L., and Redhead, K. (2021). Collaborative study for the validation of cell line assays for in-process toxicity and antigenicity testing of Clostridium septicum vaccine antigens - Part 2: Optimisation of cell line assays. *Pharmeur Bio Sci Notes*, 2021, 101-156. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/34078535

Béquignon, O. J. M., Gómez-Tamayo, J. C., Lenselink, E. B., Wink, S., Hiemstra, S., Lam, C. C., Gadaleta, D., Roncaglioni, A., Norinder, U., van de Water, B., Pastor, M., and van Westen, G. J. P. (2023). Collaborative SAR Modeling and Prospective In Vitro Validation of Oxidative Stress Activation in Human HepG2 Cells. *Journal of Chemical Information and Modeling*, 63, 5433-5445. doi:10.1021/acs.jcim.3c00220

Berggren, E., Ahs Lopez, E., Barroso, J., Carpi, D., Chinchio, E., Cusinato, A., Deceuninck, P., Franco, A., Gastaldello, A., Gribaldo, L., Langezaal, I., Malinowska, J., Mennecozzi, M., Milcamps, A., Munn, S., Palosaari, T., Piergiovanni, M., Sampani, S., Selfa Aspiroz, L., Valsesia, D., and Whelan, M. (2023a). *Report on the outcome of the JRC Summer School 2023 on Non-Animal Approaches in Science*, Publications Office of the European Union, Luxembourg, doi:10.2760/891000, JRC135377

Berggren, E., and Worth, A. P. (2023b). Towards a future regulatory framework for chemicals in the European Union-Chemicals 2.0. *Regulatory Toxicology and Pharmacology*, 142. doi:10.1016/j.yrtph.2023.105431

Bernasconi, C., Langezaal, I., Bartnicka, J., Asturiol, D., Bowe, G., Coecke, S., Kienzler, A., Liska, R., Milcamps, A., Munoz-Pineiro, M. A., Pistollato, F., and Whelan, M. (2023). *Validation of a battery of mechanistic methods relevant for the detection of chemicals that can disrupt the thyroid hormone system*, EUR 31456 EN, Publications Office of the European Union, Luxembourg, 978-92-68-01257-4, doi:10.2760/862948, JRC132532

Biasin, E. (2023). *In Silico World D9.2 In-depth analysis of legal and ethical requirements*, doi:10.5281/zenodo.7991232

Brown, E. C., Hallinger, D. R., and Simmons, S. O. (2023). High-throughput AR dimerization assay identifies androgen disrupting chemicals and metabolites. *Frontiers in Toxicology*, 5. doi:10.3389/ftox.2023.1134783

Browne, P., Delrue, N., and Gourmelon, A. (2019). Regulatory use and acceptance of alternative methods for chemical hazard identification. *Current Opinion in Toxicology*, 15, 18-25. doi:10.1016/j.cotox.2019.02.003

Burgdorf, T., Piersma, A. H., Landsiedel, R., Clewell, R., Kleinstreuer, N., Oelgeschlager, M., Desprez, B., Kienhuis, A., Bos, P., de Vries, R., de Wit, L., Seidle, T., Scheel, J., Schonfelder, G., van Benthem, J., Vinggaard, A. M., Eskes, C., and Ezendam, J. (2019). Workshop on the validation and regulatory acceptance of innovative 3R approaches in regulatory toxicology - Evolution versus revolution. *Toxicology in Vitro*, 59, 1-11. doi:10.1016/j.tiv.2019.03.039

Callegaro, G., Schimming, J. P., Pinero Gonzalez, J., Kunnen, S. J., Wijaya, L., Trairatphisan, P., van den Berk, L., Beetsma, K., Furlong, L. I., Sutherland, J. J., Mollon, J., Stevens, J. L., and van de Water, B. (2023). Identifying multiscale translational safety biomarkers using a network-based systems approach. *iScience*, 26, 106094. doi:10.1016/j.isci.2023.106094

Carusi, A., Clerbaux, L. A., and Wittwehr, C. (2023). How to Catalyze a Collaboration. *Issues in Science and Technology*, 39, 46–50. doi:10.58875/WXQO5887

Casati, S., Aschberger, K., Barroso, J., Casey, W., Delgado, I., Kim, T. S., Kleinstreuer, N., Kojima, H., Lee, J. K., Lowit, A., Park, H. K., Regimbald-Krnel, M. J., Strickland, J., Whelan, M., Yang, Y., and Zuang, V. (2018). Standardisation of defined approaches for skin sensitisation testing to support regulatory use and international adoption: position of the International Cooperation on Alternative Test Methods. *Archives of Toxicology*, 92, 611-617. doi:10.1007/s00204-017-2097-4

Celi, S., Cioffi, M., Capellini, K., Fanni, B. M., Gasparotti, E., Vignali, E., Positano, V., Haxhiademi, D., Costa, E., Landini, L., Daskalopoulos, E., Piergiovanni, M., Dura, A., Gribaldo, L., and Whelan, M. (2022). *Advanced non-animal models in biomedical research – Cardiovascular diseases*, EUR 30334/5 EN, Publications Office of the European Union, Luxembourg, 978-92-76-56985-5, doi:10.2760/94608, JRC130702

Cerisier, N., Dafniet, B., Badel, A., and Taboureau, O. (2023). Linking chemicals, genes and morphological perturbations to diseases. *Toxicology and Applied Pharmacology*, 461, 15:461:116407. doi:10.1016/j.taap.2023.116407

Chang, X. Q., Tan, Y. M., Allen, D. G., Bell, S., Brown, P. C., Browning, L., Ceger, P., Gearhart, J., Hakkinen, P. J., Kabadi, S. V., Kleinstreuer, N. C., Lumen, A., Matheson, J., Paini, A., Pangburn, H. A., Petersen, E. J., Reinke, E. N., Ribeiro, A. J. S., Sipes, N., Sweeney, L. M., Wambaugh, J. F., Wange, R., Wetmore, B. A., and Mumtaz, M. (2022). IVIVE: Facilitating the Use of Toxicity Data in Risk Assessment and Decision Making. *Toxics*, 10. doi:10.3390/toxics10050232

Chien, H. T., Prior, H., Andrews, L., van Aerts, L., Cauvin, A., Clarke, D. O., Datta, K., Dempster, M., Dybdal, N., Freebern, W., de Haan, L., Herzyk, D., Hey, A., Kissner, T., Kronenberg, S., Leach, M. W., Lee, D., Schutte, K., Sewell, F., Trouba, K., Ulrich, P., Weir, L., and van Meer, P. (2023). Re-evaluating the need for chronic toxicity studies with therapeutic monoclonal antibodies, using a weight of evidence approach. *Regulatory Toxicology and Pharmacology*, 138. doi:10.1016/j.yrtph.2022.105329

Cirefice, G., Schütte, K., Spreitzer, I., Charton, E., Shaid, S., Viviani, L., Rubbrecht, M., and Manou, I. (2023). The future of pyrogenicity testing: Phasing out the rabbit pyrogen test. A meeting report. *Biologicals*, 84. doi:10.1016/j.biologicals.2023.101702

Colacci, A., Corvi, R., Ohmori, K., Paparella, M., Serra, S., Carrico, I. D., Vasseur, P., and Jacobs, M. N. (2023). The Cell Transformation Assay: A Historical Assessment of Current Knowledge of Applications in an Integrated Approach to Testing and Assessment for Non-Genotoxic Carcinogens. *International Journal of Molecular Sciences*, 24. doi:10.3390/ijms24065659

Cronin, M. T. D., Belfield, S. J., Briggs, K. A., Enoch, S. J., Firman, J. W., Frericks, M., Garrard, C., Maccallum, P. H., Madden, J. C., Pastor, M., Sanz, F., Soininen, I., and Sousoni, D. (2023). Making in silico predictive models for toxicology FAIR. *Regulatory Toxicology and Pharmacology*, 140. doi:10.1016/j.yrtph.2023.105385

Curreli, C., Di Salvatore, V., Russo, G., Pappalardo, F., and Viceconti, M. (2023). A Credibility Assessment Plan for an In Silico Model that Predicts the Dose–Response Relationship of New Tuberculosis Treatments. *Annals of Biomedical Engineering*, 51, 200-210. doi:10.1007/s10439-022-03078-w

Desaulniers, D., Vasseur, P., Jacobs, A., Aguila, M. C., Ertych, N., and Jacobs, M. N. (2021). Integration of Epigenetic Mechanisms into Non-Genotoxic Carcinogenicity Hazard Assessment: Focus on DNA Methylation and Histone Modifications. *International Journal of Molecular Sciences*, 22. doi:10.3390/ijms222010969

EC (2008), Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. Retrieved from https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32008R1272

EC (2020a), Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions - Chemicals Strategy for Sustainability Towards a Toxic-Free Environment. Retrieved from https://eur-lex.europa.eu/resource.html?uri=cellar:f815479a-0f01-11eb-bc07-01aa75ed71a1.0003.02/DOC_1&format=PDF

EC (2020b), Report from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions: Chemicals Strategy for Sustainability Towards a Toxic-Free Environment, COM(2020) 667 final. Retrieved from https://eur-lex.europa.eu/resource.html?uri=cellar:f815479a-0f01-11eb-bc07-01aa75ed71a1.0003.02/DOC_1&format=PDF

EEA (2019), The European environment - state and outlook 2020: Knowledge for transition to a sustainable Europe. Retrieved from https://www.eea.europa.eu/soer/publications/soer-2020

EEB (2022), The Need for Speed: why it takes the EU a decade to control harmful chemicals and how to secure more rapid protections. Retrieved from https://eeb.org/wp-content/uploads/2022/07/Need-for-speed_Report_Final-1.pdf

EFSA (2022). Development of a Roadmap for Action on New Approach Methodologies in Risk Assessment. *EFSA Support Publ*. doi:10.2903/sp.efsa.2022.EN-7341

EMA (2016), Guideline on the principles of regulatory acceptance of 3Rs (replacement, reduction, refinement) testing approaches, EMA/CHMP/CVMP/JEG-3Rs/450091/2012. Retrieved from https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-principles-regulatory-acceptance-3rs-replacement-reduction-refinement-testing-approaches_en.pdf

EMA (2017a), Essential considerations for successful qualification of novel methodologies, EMA/750178/2017. Retrieved from https://www.ema.europa.eu/en/documents/other/essential-considerations-successful-qualification-novel-methodologies_en.pdf

EMA (2017b), Guidance for individual laboratories for transfer of quality control methods validated in collaborative trials with a view to implementing 3Rs, EMA/CHMP/CVMP/3Rs/94436/2014. Retrieved from https://www.ema.europa.eu/en/documents/scientific-guideline/guidance-individual-laboratories-transfer-quality-control-methods-validated-collaborative-trials-view-implementing-3rs_en.pdf

EU (2010), Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, 2008/0211 (COD). Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L0063

EU (2022), Regulation (EU) 2022/868 of the European Parliament and of the Council of 30 May 2022 on European Data Governance and amending Regulation (EU) 2018/1724 (Data Governance Act). Retrieved from https://eur-lex.europa.eu/eli/reg/2022/868/oj

Fairman, K., Choi, M. K., Gonnabathula, P., Lumen, A., Worth, A., Paini, A., and Li, M. (2023). An Overview of Physiologically-Based Pharmacokinetic Models for Forensic Science. *Toxics*, 11. doi:10.3390/toxics11020126

Gastaldello, A., Bernasconi, C., Campia, I., Daskalopoulos, E., Deceuninck, P., Pistollato, F., and Whelan, M. (2023a). *Building indicators to assess the impact of EU-funded research into Alzheimer`s disease, breast cancer and prostate cancer*, EUR 31435 EN, Publications Office of the European Union, Luxembourg, 978-92-68-00311-4, doi:10.2760/63620, JRC132705

Gastaldello, A., and Deceuninck, P. (2023b). EURL ECVAM indicators to assess the impact of EU-funded research into Alzheimer's Disease, Breast Cancer and Prostate Cancer. European Commission, Joint Research Centre (JRC) [Dataset]. Retrieved from https://data.jrc.ec.europa.eu/dataset/a96ad0dd-f23a-4ee4-a839-04928c1c5cf3

Grignard, E., de Jesus, K., and Hubert, P. (2022). Regulatory Testing for Endocrine Disruptors; Need for Validated Methods and Integrated Approaches. *Frontiers in Toxicology*, 3, 19;13:821736. doi:10.3389/ftox.2021.821736

Grillberger, K., Cöellen, E., Trivisani, C. I., Blum, J., Leist, M., and Ecker, G. F. (2023). Structural Insights into Neonicotinoids and N-Unsubstituted Metabolites on Human nAChRs by Molecular Docking, Dynamics Simulations, and Calcium Imaging. *International Journal of Molecular Sciences*, 24, 13170. doi:10.3390/ijms241713170

Harrill, J. A., Viant, M. R., Yauk, C. L., Sachana, M., Gant, T. W., Auerbach, S. S., Beger, R. D., Bouhifd, M., O'Brien, J., Burgoon, L., Caiment, F., Carpi, D., Chen, T., Chorley, B. N., Colbourne, J., Corvi, R., Debrauwer, L., O'Donovan, C., Ebbels, T. M. D., Ekman, D. R., Faulhammer, F., Gribaldo, L., Hilton, G. M., Jones, S. P., Kende, A., Lawson, T. N., Leite, S. B., Leonards, P. E. G., Luijten, M., Martin, A., Moussa, L., Rudaz, S., Schmitz, O., Sobanski, T., Strauss, V., Vaccari, M., Vijay, V., Weber, R. J. M., Williams, A. J., Williams, A., Thomas, R. S., and Whelan, M. (2021). Progress towards an OECD reporting framework for transcriptomics and metabolomics in regulatory toxicology. *Regulatory Toxicology and Pharmacology*, 125, 105020. doi:10.1016/j.yrtph.2021.105020

Hartung, T., Bremer, S., Casati, S., Coecke, S., Corvi, R., Fortaner, S., Gribaldo, L., Halder, M., Hoffmann, S., Roi, A. J., Prieto, P., Sabbioni, E., Scott, L., Worth, A., and Zuang, V. (2004). A modular approach to the ECVAM principles on test validity. *ATLA Alternatives to Laboratory Animals*, 32, 467-472. doi:10.1177/026119290403200503

Hilton, G. M., Corvi, R., Luijten, M., Mehta, J., and C. Wolf, D. (2023). Towards achieving a modern science-based paradigm for agrochemical carcinogenicity assessment. *Regulatory Toxicology and Pharmacology*, 137. doi:10.1016/j.yrtph.2022.105301

Hogberg, H. T., Lam, A., Ohayon, E., Shahbaz, M. A., Clerbaux, L. A., Bal-Price, A., Coecke, S., Concha, R., De Bernardi, F., Edrosa, E., Hargreaves, A. J., Kanninen, K. M., Munoz, A., Pistollato, F., Saravanan, S., Garcia-Reyero, N., Wittwehr, C., and Sachana, M. (2022). The Adverse Outcome Pathway Framework Applied to Neurological Symptoms of COVID-19. *Cells*, 11. doi:10.3390/cells11213411

ICCVAM (2018). A Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States. doi:10.22427/NTP-ICCVAM-ROADMAP2018

ICCVAM (2023). Validation, Qualification, and Regulatory Acceptance of New Approach Methodologies. A Report of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Validation Workgroup. Retrieved from https://ntp.niehs.nih.gov/sites/default/files/2023-08/VWG%20Report%20Draft_for%20public%20comment_08Aug2023.pdf

ICH (2005), The nonclinical Evaluation of the Potential for delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals - S7B. Retrieved from https://database.ich.org/sites/default/files/S7B_Guideline.pdf

ICH (2013), Photosafety evaluation of pharmaceuticals - S10. Retrieved from https://database.ich.org/sites/default/files/S10_Guideline.pdf

ICH (2020), Guideline on detection of reproductive and developmental toxicity for human pharmaceuticals - S5(R3), EMA/CHMP/ICH/544278/1998. Retrieved from https://www.ema.europa.eu/en/documents/scientific-guideline/ich-s5-r3-guideline-detection-reproductive-and-developmental-toxicity-human-pharmaceuticals-step-5-revision-4_en.pdf

Jacobs, M. N., Colacci, A., Corvi, R., Vaccari, M., Aguila, M. C., Corvaro, M., Delrue, N., Desaulniers, D., Ertych, N., Jacobs, A., Luijten, M., Madia, F., Nishikawa, A., Ogawa, K., Ohmori, K., Paparella, M., Sharma, A. K., and Vasseur, P. (2020). Chemical carcinogen safety testing: OECD expert group international consensus on the development of an integrated approach for the testing and assessment of chemical non-genotoxic carcinogens. *Archives of Toxicology*, 94, 2899-2923. doi:10.1007/s00204-020-02784-5

Jacobs, M. N., Colacci, A., Louekari, K., Luijten, M., Hakkert, B. C., Paparella, M., and Vasseur, P. (2016). International regulatory needs for development ofan IATA for non-genotoxic carcinogenic chemical substances. *ALTEX*, 33, 359-392. doi:10.14573/altex.1601201

Jennings, P., Chandrasekaran, V., Hardy, B., Langemeijer, E., Doktorova, T., Madia, F. A. C., and Prieto Peraita, M. D. P. (2023). *Mechanistic Analysis of Repeated Dose Toxicity Studies*, EUR 31427 EN, Publications Office of the European Union, Luxembourg, 978-92-76-99823-5, doi:10.2760/824535, JRC132210

Krebs, A., Waldmann, T., Wilks, M. F., Van Vugt-Lussenburg, B. M. A., Van der Burg, B., Terron, A., Steger-Hartmann, T., Ruegg, J., Rovida, C., Pedersen, E., Pallocca, G., Luijten, M., Leite, S. B., Kustermann, S., Kamp, H., Hoeng, J., Hewitt, P., Herzler, M., Hengstler, J. G., Heinonen, T., Hartung, T., Hardy, B., Gantner, F., Fritsche, E., Fant, K., Ezendam, J., Exner, T., Dunkern, T., Dietrich, D. R., Coecke, S., Busquet, F., Braeuning, A., Bondarenko, O., Bennekou, S. H., Beilmann, M., and Leist, M. (2019). Template for the description of cell-based toxicological test methods to allow evaluation and regulatory use of the data. *Altex*, 36, 682-699. doi:10.14573/altex.1909271

Leite, S. B., Brooke, M., Carusi, A., Collings, A., Deceuninck, P., Dechamp, J., Dekker, B., De Ranieri, E., Ganley, E., Gastaldello, A., He, F., LaFlamme, M., Langezaal, I., Morris, J., Pamies, D., Piergiovanni, M., Pulverer, B., Sadler, D., Shamu, C., Siegel, V., Straccia, M., and Weissgerber, T. L. (2023). Promoting Reusable and Open Methods and Protocols (PRO-MaP): Draft recommendations to improve methodological clarity in life sciences publications. *OSF Preprints*. doi:10.31219/osf.io/x85gh

Lejal, V., Cerisier, N., Rouquié, D., and Taboureau, O. (2023). Assessment of Drug-Induced Liver Injury through Cell Morphology and Gene Expression Analysis. *Chemical Research in Toxicology*, 36, 1456-1470. doi:10.1021/acs.chemrestox.2c00381

Musuamba, F. T., Rusten, I. S., Lesage, R., Russo, G., Bursi, R., Emili, L., Wangorsch, G., Manolis, E., Karlsson, K. E., Kulesza, A., Courcelles, E., Boissel, J. P., Rousseau, C. F., Voisin, E. M., Alessandrello, R., Curado, N., Dall'ara, E., Rodriguez, B., Pappalardo, F., and Geris, L. (2021). Scientific and regulatory evaluation of mechanistic drug and disease models in drug development: Building model credibility. *Cpt-Pharmacometrics & Systems Pharmacology*, 10, 804-825. doi:10.1002/psp4.12669

OECD (2005), *Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment,* OECD Series on Testing and Assessment, No. 34, Environment, Health and Safety, Environment Directorate, OECD. Retrieved from https://one.oecd.org/document/ENV/JM/MONO(2005)14/en/pdf

OECD (2007), *Test No. 426: Developmental Neurotoxicity Study,* OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, doi:10.1787/9789264067394-en

OECD (2016a), *Test No. 474: Mammalian Erythrocyte Micronucleus Test*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, doi:10.1787/9789264264762-en

OECD (2016b), *Test No. 489: In Vivo Mammalian Alkaline Comet Assay*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, doi:10.1787/9789264264885-en

OECD (2018), *Test No. 443: Extended One-Generation Reproductive Toxicity Study*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, doi:10.1787/9789264185371-en

OECD (2019), *Saving Costs in Chemicals Management: How the OECD Ensures Benefits to Society*, OECD Publishing, Paris, doi:10.1787/9789264311718-en

OECD (2021), *Guidance document on the characterisation, validation and reporting of Physiologically Based Kinetic (PBK) models for regulatory purposes*, OECD Series on Testing and Assessment, No. 331, Environment, Health and Safety, Environment Directorate, OECD. Retrieved from https://one.oecd.org/document/ENV/JM/MONO(2018)12/en/pdf

OECD (2023a), *Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery*, OECD Series on Testing & Assessment No. 377, Environment, Health and Safety, Environment Directorate, OECD. Retrieved from https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf

OECD (2023b), *(Q)SAR Assessment Framework: Guidance for the regulatory assessment of (Quantitative) Structure - Activity Relationship models, predictions, and results based on multiple predictions*, OECD Series on Testing and Assessment, No. 386, Environment, Health and Safety, Environment Directorate, OECD. Retrieved from https://one.oecd.org/document/ENV/CBC/MONO(2023)32/en/pdf

OECD (2023c), *Report on considerations from case studies on Integrated Approaches for Testing and Assessment (IATA). Eighth Review Cycle (2022)*, OECD Series on Testing and Assessment, No. 385, Environment, Health and Safety, Environment Directorate, OECD. Retrieved from https://one.oecd.org/document/ENV/CBC/MONO(2023)31/en/pdf

OECD (2023d), *Report on the WNT Workshop how to prepare the Test Guidelines Programme for emerging technologies*, OECD Series on Testing and Assessment, No. 378, Environment, Health and Safety, Environment Directorate, OECD. Retrieved from https://one.oecd.org/document/ENV/CBC/MONO(2023)14/en/pdf

OECD (2023e), *Test No. 487: In Vitro Mammalian Cell Micronucleus Test*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, doi:10.1787/9789264264861-en

Ohmori, K., Kamei, A., Watanabe, Y., and Abe, K. (2022). Gene Expression over Time during Cell Transformation Due to Non-Genotoxic Carcinogen Treatment of Bhas 42 Cells. *International Journal of Molecular Sciences*, 23. doi:10.3390/ijms23063216

Oku, Y., Madia, F., Lau, P., Paparella, M., McGovern, T., Luijten, M., and Jacobs, M. N. (2022). Analyses of Transcriptomics Cell Signalling for Pre-Screening Applications in the Integrated Approach for Testing and Assessment of Non-Genotoxic Carcinogens. *International Journal of Molecular Sciences*, 23. doi:10.3390/ijms232112718

Pappalardo, F., Wilkinson, J., Busquet, F., Bril, A., Palmer, M., Walker, B., Curreli, C., Russo, G., Marchal, T., Toschi, E., Alessandrello, R., Costignola, V., Klingmann, I., Contin, M., Staumont, B., Woiczinski, M., Kaddick, C., Di Salvatore, V., Aldieri, A., Geris, L., and Viceconti, M. (2022). Toward A Regulatory Pathway for the Use of in Silico Trials in the CE Marking of Medical Devices. *Ieee Journal of Biomedical and Health Informatics*, 26, 5282-5286. doi:10.1109/Jbhi.2022.3198145

Parish, S. T., Aschner, M., Casey, W., Corvaro, M., Embry, M. R., Fitzpatrick, S., Kidd, D., Kleinstreuer, N. C., Lima, B. S., Settivari, R. S., Wolf, D. C., Yamazaki, D., and Boobis, A. (2020). An evaluation framework for new approach methodologies (NAMs) for human health safety assessment. *Regulatory Toxicology and Pharmacology*, 112. doi:10.1016/j.yrtph.2020.104592

Patterson, E. A., and Whelan, M. P. (2017). A framework to establish credibility of computational models in biology. *Progress in Biophysics & Molecular Biology*, 129, 13-19. doi:10.1016/j.pbiomolbio.2016.08.007

Patterson, E. A., Whelan, M. P., and Worth, A. P. (2021). The role of validation in establishing the scientific credibility of predictive

toxicology approaches intended for regulatory application. *Computational Toxicology*, 17. doi:10.1016/j.comtox.2020.100144

Petersen, E. J., Elliott, J. T., Gordon, J., Kleinstreuer, N. C., Reinke, E., Roesslein, M., and Toman, B. (2023). Technical Framework for Enabling High Quality Measurements in New Approach Methodologies (NAMs). *Altex-Alternatives to Animal Experimentation,* 40, 174-186. doi:10.14573/altex.2205081

Piergiovanni, M., Mennecozzi, M., Sampani, S. and Whelan, M. (2024). Heads on! Designing a qualification framework for organ-on-chip. *ALTEX-Alternatives to animal experimentation.* doi:10.14573/altex.2401231

Piersma, A. H., Burgdorf, T., Louekari, K., Desprez, B., Taalman, R., Landsiedel, R., Barroso, J., Rogiers, V., Eskes, C., Oelgeschläger, M., Whelan, M., Braeuning, A., Vinggaard, A. M., Kienhuis, A., van Benthem, J., and Ezendam, J. (2018a). Workshop on acceleration of the validation and regulatory acceptance of alternative methods and implementation of testing strategies. *Toxicology in Vitro*, 50, 62-74. doi:10.1016/j.tiv.2018.02.018

Piersma, A. H., van Benthem, J., Ezendam, J., and Kienhuis, A. S. (2018b). Validation redefined. *Toxicology in Vitro*, 46, 163-165. doi:10.1016/j.tiv.2017.10.013

Pillo, G., Mascolo, M. G., Zanzi, C., Rotondo, F., Serra, S., Bortone, F., Grilli, S., Vaccari, M., Jacobs, M. N., and Colacci, A. (2022). Mechanistic Interrogation of Cell Transformation In Vitro: The Transformics Assay as an Exemplar of Oncotransformation. *International Journal of Molecular Sciences*, 23. doi:10.3390/ijms23147603

Pistollato, F., Campia, I., Daskalopoulos, E. P., Bernasconi, C., Desaintes, C., Di Virgilio, S., Kyriakopoulou, C., Whelan, M., and Deceuninck, P. (2023). Gauging innovation and health impact from biomedical research: survey results and interviews with recipients of EU-funding in the fields of Alzheimer's disease, breast cancer and prostate cancer. *Health Research Policy and Systems*, 21. doi:10.1186/s12961-023-00981-z

Rodríguez-Belenguer, P., Mangas-Sanjuan, V., Soria-Olivas, E., and Pastor, M. (2023). Integrating Mechanistic and Toxicokinetic Information in Predictive Models of Cholestasis. *Journal of Chemical Information and Modeling*. doi:10.1021/acs.jcim.3c00945

Sewell, F., Doe, J., Gellatly, N., Ragan, I., and Burden, N. (2017). Steps towards the international regulatory acceptance of non-animal methodology in safety assessment. *Regulatory Toxicology and Pharmacology*, 89, 50-56. doi:10.1016/j.yrtph.2017.07.001

Sewell, F., Ragan, I., Horgan, G., Andrew, D., Holmes, T., Manou, I., Muller, B. P., Rowan, T., Schmitt, B. G., and Corvaro, M. (2023). New supporting data to guide the use of evident toxicity in acute oral toxicity studies (OECD TG 420). *Regulatory Toxicology and Pharmacology,* 146, 105517. doi:10.1016/j.yrtph.2023.105517

Shahbaz, M. A., De Bernardi, F., Alatalo, A., Sachana, M., Clerbaux, L. A., Muñoz, A., Parvatam, S., Landesmann, B., Kanninen, K. M., and Coecke, S. (2022). Mechanistic Understanding of the Olfactory Neuroepithelium Involvement Leading to Short-Term Anosmia in COVID-19 Using the Adverse Outcome Pathway Framework. *Cells,* 11. doi:10.3390/cells11193027

Sovadinová, I., Upham, B. L., Trosko, J. E., and Babica, P. (2021). Applicability of Scrape Loading-Dye Transfer Assay for Non-Genotoxic Carcinogen Testing. *International Journal of Molecular Sciences*, 22. doi:10.3390/ijms22168977

Suciu, I., Delp, J., Gutbier, S., Uckert, A. K., Spreng, A. S., Eberhard, P., Karreman, C., Schreiber, F., Madjar, K., Rahnenfuhrer, J., Celardo, I., Amelio, I., and Leist, M. (2023). Dynamic Metabolic and Transcriptional Responses of Proteasome-Inhibited Neurons. *Antioxidants (Basel)*, 12. doi:10.3390/antiox12010164

Thienpont, A., Verhulst, S., van Grunsven, L. A., Rogiers, V., Vanhaecke, T., and Mertens, B. (2023). Novel Prediction Models for Genotoxicity Based on Biomarker Genes in Human HepaRGTM Cells. *Altex-Alternatives to Animal Experimentation*, 40, 271-286. doi:10.14573/altex.2206201

US CPSC (2022), Guidance for industry and test method developers: CPSC staff evaluation of alternative test methods and integrated testing approaches and data generated from such methods to support FHSA labeling requirements. Bethesda, MD. Retrieved from https://www.cpsc.gov/s3fs-public/Guidance-for-Industry-and-Test-Method-Developers-CPSC-Staff-Evaluation-of-Alternative-Test-Methods-and-Integrated-Testing-Approaches.pdf?VersionId=6EJxcMXMu4PzZEQFQivF3AUZODrMRK5J

US EPA (2018), Strategic plan to promote the development and implementation of alternative test methods within the TSCA program. Retrieved from https://www.epa.gov/sites/production/files/2018-06/documents/epa_alt_strat_plan_6-20-18_clean_final.pdf

US FDA (2017), FDA's Predictive Toxicology Roadmap. Retrieved from https://www.fda.gov/science-research/about-science-research-fda/fdas-predictive-toxicology-roadmap

US FDA (2021), Advancing Alternative Methods at FDA. Retrieved from https://www.fda.gov/science-research/about-science-research-fda/advancing-alternative-methods-fda

Valls-Margarit, J., Piñero, J., Füzi, B., Cerisier, N., Taboureau, O., and Furlong, L. I. (2023). Assessing network-based methods in the context of system toxicology. *Frontiers in Pharmacology*, 14, 14:1225697. doi:10.3389/fphar.2023.1225697

van der Zalm, A. J., Barroso, J., Browne, P., Casey, W., Gordon, J., Henry, T. R., Kleinstreuer, N. C., Lowit, A. B., Perron, M., and Clippinger, A. J. (2022). A framework for establishing scientific confidence in new approach methodologies. *Archives of Toxicology*, 96, 2865-2879. doi:10.1007/s00204-022-03365-4

Viceconti, M., Emili, L., Afshari, P., Courcelles, E., Curreli, C., Famaey, N., Geris, L., Horner, M., Jori, M. C., Kulesza, A., Loewe, A., Neidlin, M., Reiterer, M., Rousseau, C. F., Russo, G., Sonntag, S. J., Voisin, E. M., and Pappalardo, F. (2021). Possible Contexts of Use for In Silico Trials Methodologies: A Consensus-Based Review. *Ieee Journal of Biomedical and Health Informatics*, 25, 3977-3982. doi:10.1109/Jbhi.2021.3090469

Vieira, D., Franco, A., De Medici, D., Martin Jimenez, J., Wojda, P., and Jones, A. (2023). *Pesticides residues in European agricultural soils - Results from LUCAS 2018 soil module*, Publications Office of the European Union, Luxembourg, doi:10.2760/86566, JRC133940

Westmoreland, C., Bender, H. J., Doe, J. E., Jacobs, M. N., Kass, G. E. N., Madia, F., Mahony, C., Manou, I., Maxwell, G., Prieto, P., Roggeband, R., Sobanski, T., Schütte, K., Worth, A. P., Zvonar, Z., and Cronin, M. T. D. (2022). Use of New Approach Methodologies (NAMs) in regulatory decisions for chemical safety: Report from an EPAA Deep Dive Workshop. *Regulatory Toxicology and Pharmacology*, 135. doi:10.1016/j.yrtph.2022.105261

List of abbreviations and definitions

3D	Three-dimensional
3Rs	Replacement, Reduction, Refinement
ADME	Absorption, distribution, metabolism and excretion
AI	Artificial intelligence
ANSES	French National Agency for Food, Environmental and Occupational Health and Safety
AOP	Adverse Outcome Pathway
AR2	Androgen receptor dimerization assay
ARM	Application Reporting Module
ASPA	ASPIS Safety Profiling Algorithm
ASPIS	Animal-free Safety assessment of chemicals: Project cluster for Implementation of novel Strategies (H2020)
ASTM	American Society for Testing and Materials
ATHENA	Assays for the identification of Thyroid Hormone axis-disrupting chemicals: Elaborating Novel Assessment Strategies (EURION cluster)
ATP	Adaptation to Technical Progress
CARACAL	Competent Authorities for REACH and CLP
CDPC	Common Data Platform on Chemicals
CEN	European Committee for Standardization
CENELEC	European Committee for Electrotechnical Standardization
CIAO	Modelling the Pathogenesis of COVID-19 using the Adverse Outcome Pathway Framework
CLP	Classification, Labelling and Packaging
COVID-19	Coronavirus disease
CPSC	United States Consumer Product Safety Commission
CRO	Contract Research Organisation
DA	Defined approach
DB	Database
DB-ALM	Database on Alternative Methods
DE	Germany
DG ENV	Directorate-General for Environment (EC)
DG GROW	Directorate-General for Internal Market, Industry, Entrepreneurship and SMEs (EC)
DG RTD	Directorate-General for Research and Innovation (EC)
DG SANTE	Directorate-General for Health and Food Safety (EC)
DILI	Drug-Induced Liver Injury
DNA	Deoxyribonucleic Acid
DNT	Developmental neurotoxicity
DRP	Detailed review paper
EARM	Enrichment Analysis Reporting Module
EC	European Commission (EU)
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECHA	European Chemicals Agency
ED	Endocrine disruptor
EDCMET	Metabolic effects of Endocrine Disrupting Chemicals: novel testing METhods and adverse outcome pathways (EURION cluster)
EDITH	European Virtual Human Twin
EEA	European Environment Agency (EU)
EEB	European Environmental Bureau (EU)
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ENDpoiNTS	Novel Testing Strategies for Endocrine Disruptors in the Context of Developmental NeuroToxicity (EURION cluster)
EPA	Environmental Protection Agency
EPAA	European Partnership for Alternatives to Animal Testing
ERGO	EndocRine Guideline Optimisation (EURION cluster)
ESAC	EURL ECVAM Scientific Advisory Committee
ESR	Early Stage Researchers (MSCA-ITN)

EU	European Union
EURION	European Cluster to Improve Identification of Endocrine Disruptors
EUROoCS	European Organ-on-Chip Society
EUROTOX	Federation of European Toxicologists & European Societies of Toxicology
EU-NETVAL	European Union Network of Laboratories for the Validation of Alternative Methods
EU-ToxRisk	An Integrated European 'Flagship' Programme Driving Mechanism-based Toxicity Testing and Risk Assessment for the 21st century
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
FAIR	Findability, accessibility, interoperability, and reusability (of data)
FDA	US Food and Drug Administration
FGOoC	Focus Group on OoC
FP	Framework programme
FREIA	Female Reproductive Toxicity of EDCs (EURION cluster)
GD	Guidance Document
GHS	Globally Harmonised System of Classification and Labelling of chemicals
GLP	Good Laboratory Practice
GOLIATH	Beating Goliath: Generation Of NoveL, Integrated and Internationally Harmonised Approaches for Testing Metabolism Disrupting Compounds (EURION cluster)
GR	Glucocorticoid receptor
H2020	Horizon 2020
HBM	Human biomonitoring
HH	Human health
HLRT	Human Liver Ring Trial
HTP	High-throughput
IATA	Integrated Approaches to Testing and Assessment
ICATM	International Cooperation on Alternative Test Methods
ICCA	International Council of Chemical Associations
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods (NIEHS)
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IPCHEM	Information platform for chemical monitoring
IPCS	International Programme on Chemical Safety
ISO/IEC	International Organization for Standardization/International Electrotechnical Commission
ISS	Istituto Superiore di Sanità
ISW	In Silico World
IUCLID	International Uniform Chemical Information Database
IVB	*In vitro* battery
IWG	Informal working group
JRC	Joint Research Centre (EC)
KC	Key characteristic
KE	Key event (AOP)
KER	Key event relationship (AOP)
KNDP	Key NeuroDevelopmental Processes
LC-MS	Liquid chromatography - mass spectrometry
LGBTQIA+	Lesbian, gay, bisexual, transgender, intersex, queer/questioning, asexual
MAD	Mutual acceptance of data
MCRA	Monte Carlo Risk Assessment
MIE	Molecular Initiating event
ML	Machine Learning
MPS	Microphysiological systems
MRF	Metabolomics Report Framework
MS	Member States

NAM	New Approach Methodology
NATM	Non-Animal Test Method
NGO	Non-governmental organisation
NGRA	Next generation risk assessment
NIEHS	National Institute of Environmental Health Sciences (US)
NL	Netherlands
NSF	National Science Foundation (US)
NTP	National Toxicology Programme (US)
NTS	Non-technical summaries
NWA-ORC	Dutch Research Agenda - Research along Routes by Consortia
NWO	Nederlandse Organisatie voor Wetenschappelijk Onderzoek
OBERON	An integrative strategy of testing systems for identification of EDs related to metabolic disorders (EURION cluster)
OECD	Organisation for Economic Co-operation and Development
OHTs	OECD Harmonised Templates
ONTOX	Ontology-driven and artificial intelligence-based repeated dose toxicity testing of chemicals for next-generation risk assessment (ASPIS cluster)
OoC	Organ-on-chip
OORF	OECD Omics Reporting Framework
PARC	European Partnership for the assessment of risks from chemicals
PARERE	Preliminary Assessment of Regulatory Relevance network
PBK	Physiologically based kinetic (also PBPK, PBBK, PBTK)
PBT	Persistent, Bio-accumulative and Toxic
PEPPER	Public-private platform for the validation of endocrine disruptors characterisation methods
PFAS	Perfluoroalkyl sulphonates
PHI	Potential hazard issues
PMT	Persistent, Mobile and Toxic
PrecisionTox	Toward Precision Toxicology: New Approach Methodologies for Chemical Safety (ASPIS cluster)
PRO-MaP	Promoting Reusable and Open Methods and Protocols
QAF	QSAR Assessment Framework
qAOP	Quantitative AOP
QIVIVE	Quantitative *in Vitro* to *in Vivo* Extrapolation
QSAR	Quantitative Structure Activity Relationship
QST	Quantitative systems toxicology
RAR	Retinoic acid receptor
RDT	Repeated dose toxicity
REACH	European Regulation (EC) No. 1907/2006 Registration, Evaluation, Authorisation and Restriction of Chemicals
RISK-HUNT3R	RISK assessment of chemicals integrating HUman centric Next generation Testing strategies promoting the 3Rs (ASPIS cluster)
RIVM	National Institute for Public Health and the Environment (NL)
RRI	Responsible Research & Innovation
RS	Reconstructed skin
RSMN	Reconstructed skin micronucleus
SCHEER	Scientific Committee on Health, Environmental and Emerging Risks (EC)
SCREENED	Screening for endocrine disruptors (EURION cluster)
SOP	Standard Operating Procedure
SOT	Society of Toxicology
SPSF	Standard Project Submission Form
SSbD	Safe and Sustainable by Design
STEM	Science, technology, engineering, and mathematics
TDM-EG	Thyroid Disruption Methods Expert Group
TG	Test Guideline (OECD)
TGP	Test guidelines programme

TRF	Transcriptomics Report Framework
TSAR	EURL ECVAM Tracking System on Alternative Methods towards Regulatory acceptance
UK	United Kingdom
UN	United Nations
US	United States (of America)
VHP	Virtual Human Platform
VHP4Safety	Virtual Human Platform for Safety Assessment
VHT	Virtual Human Twin
vPvB	Very Persistent and very Bio-accumulative
vPvM	Very Persistent and very Mobile
VR	Virtual Reality
VU	Vrije Universiteit
WC12	World Congress on Alternatives and Animal Use in the Life Sciences
WG	Working group
WGCNA	Weighted gene coexpression network analysis
WHO	World Health Organization
WNT	Working Party of the National Coordinators of the Test Guidelines Programme (OECD)
WP	Work Package
WPHA	Working Party on Hazard Assessment (OECD)

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