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Non-Animal Methods in Science and Regulation

EURL ECVAM Status Report 2024

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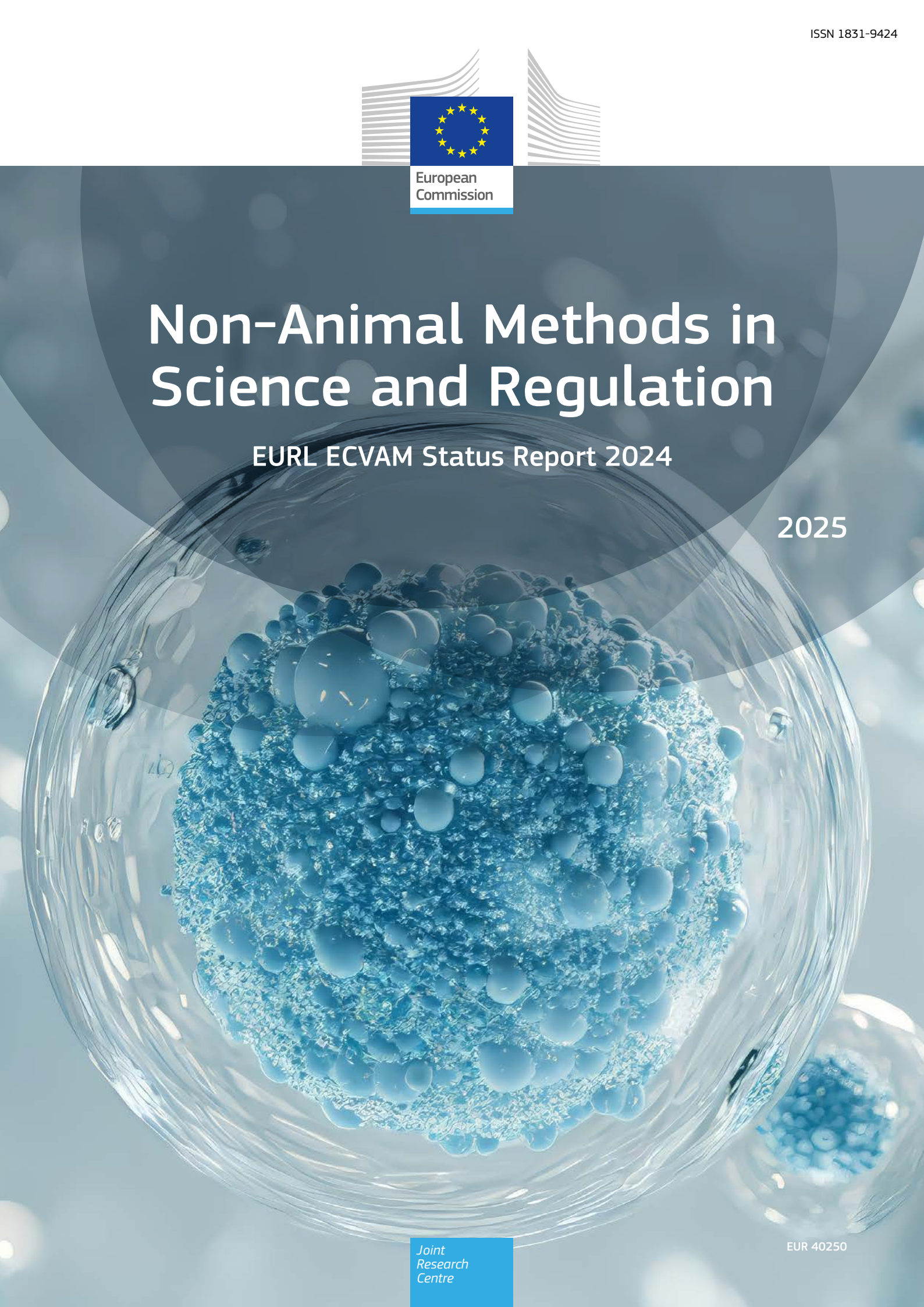

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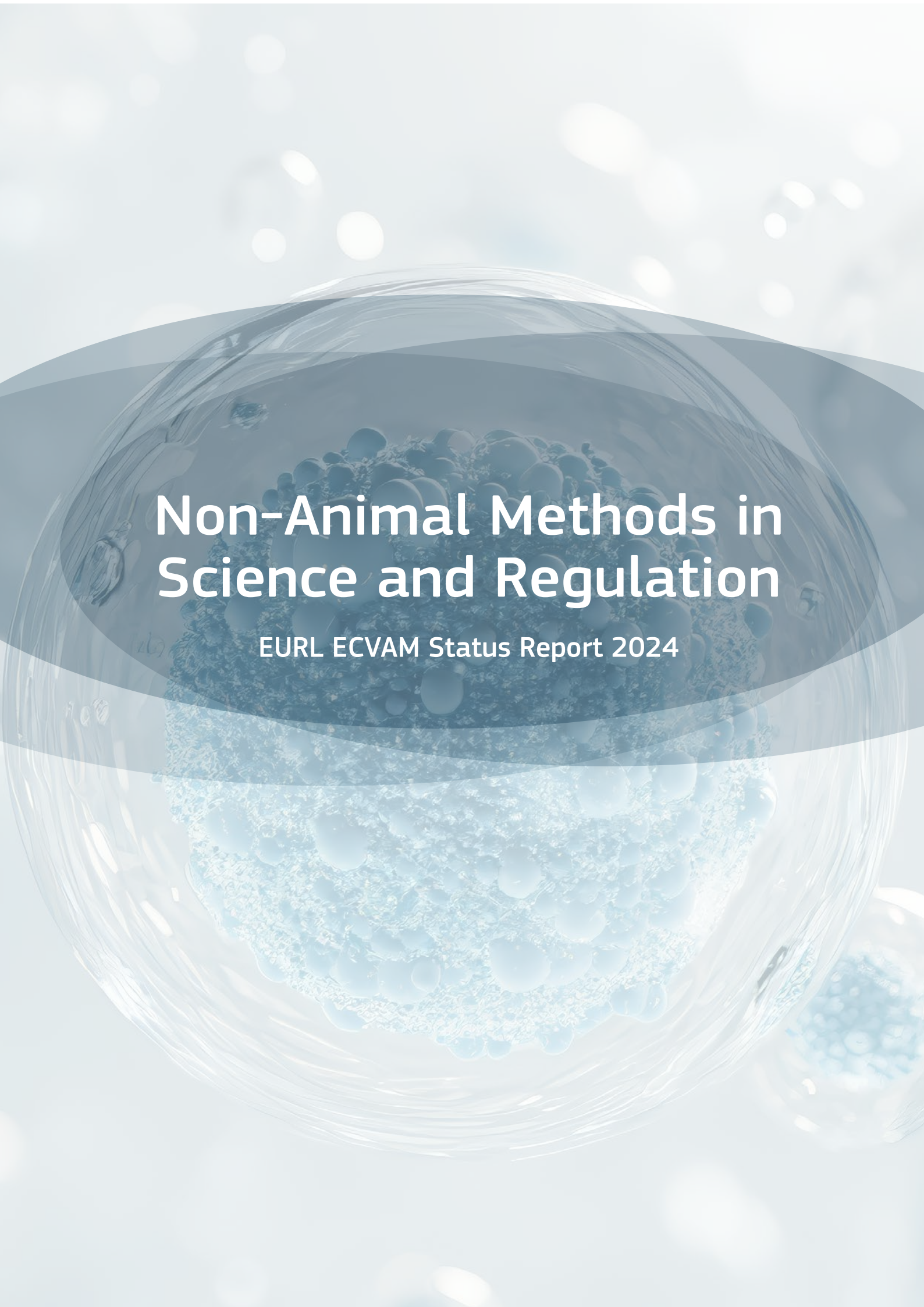

Non-Animal Methods in Science and Regulation

EURL ECVAM Status Report 2024

Contents

Abstract

The 2024 EURL ECVAM Status Report highlights progress in advancing non-animal methods for science and regulation. Driven by EU-funded collaborations, research focuses on chemical safety assessment, endocrine disruptor identification, and risk assessment, using genomics, *in vitro* and *in silico* tools, and AI.

EURL ECVAM supports new approach methodologies (NAMs) validation through initiatives such as revising OECD Guidance Document 34, standardising emerging technologies like organ-on-chip, and engaging the EU-NETVAL network. Peer reviews by EURL ECVAM's Scientific Advisory Committee (ESAC) assess the scientific validity of submitted test methods.

EURL ECVAM collaborates with international organisations and EU agencies to advance emerging non-animal sciences and technologies, with the goal of integrating them into regulatory practices for chemical hazard and risk assessment. The European Commission's roadmap towards phasing out animal testing is supported, as is the 3Rs (Replacement, Reduction, Refinement) approach in the pharmaceutical sector through the EMA 3Rs Working Party and the EMA Innovation Task Force. Education and training are key to expanded NAMs use. Ultimately, this report provides evidence-based scientific information, aiming to increase NAMs acceptance and implementation globally.

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Executive summary

The 2024 EURL ECVAM Status Report offers a comprehensive look at the ongoing efforts to develop, validate, and implement non-animal testing methods in research and regulatory contexts. It also underscores initiatives aimed at integrating these innovative approaches into education and training programs, fostering a more humane and effective landscape for scientific inquiry.

Development

The EURL ECVAM Status Report 2024 highlights significant advancements in developing non-animal testing methods and approaches for chemical safety assessments, spearheaded by EU funding and collaborative research.

Key research projects under Horizon 2020, such as RISK-HUNT3R, PrecisionTox, and ONTOX, grouped under the ASPIS cluster, enhance chemical safety testing without traditional animal use. These projects leverage advancements in genomics, metabolomics, *in vitro* and *in silico* methodologies, and artificial intelligence (AI) to improve the accuracy, speed, and cost-effectiveness of chemical risk assessments. Recent symposiums highlighted progress in implementing these methodologies, promoting collaboration among researchers, regulators, and industry stakeholders. Future goals include developing a central metadata registry and further integrating NAMs into EU chemical safety assessments.

The EURION cluster, consisting of eight projects funded by Horizon 2020, has focused on identifying endocrine disruptors (EDs) through innovative testing methods. Concluding six projects, with two extended until the end of 2024, EURION has engaged with regulators and contributed proposals to the OECD's Test Guidelines Programme work plan. The cluster published a Methods Table with over 100 new test methods and endpoints, transitioning its findings to the newly formed ENKORE cluster, which continues research on endocrine disruptors. ENKORE, a five-year Horizon Europe cluster, builds on EURION's success, focusing on endocrine-disrupting chemicals (EDCs) and bridging science-policy gaps. EURL ECVAM participates in ENKORE's Advisory Panel to enhance regulatory adoption of EDC identification methods.

The European Partnership for the Assessment of Risks from Chemicals (PARC), launched in May 2022, is a seven-year initiative with a 400 million Euro budget, aiming to improve chemical risk assessment and management. PARC focuses on developing new assessment methodologies, reducing animal testing reliance, and enhancing the regulatory framework for chemical mixtures. A collaborative knowledge platform, Parcopedia, is being built to foster stakeholder communication.

The Virtual Human Platform for Safety Assessment (VHP4Safety), funded by the Dutch Research Council, aims to replace animal testing with innovative safety assessments by integrating human physiology data. It incorporates safety assessment tools, evaluates *in vitro* models, and explores AI's potential in improving risk assessment.

In Silico World (ISW), a four-year EU-funded project, promotes In Silico Trials through computer modelling and simulation. Achievements include developing solutions for medical fields, publishing resources on good simulation practices, and conducting validation data collections. The project emphasises commercial exploitation of solutions and addresses legal-ethical issues.

The Virtual Physiological Human (VPH) Institute, an international non-profit organisation, advances virtual human technology in medicine, reducing animal testing and enhancing personalised healthcare. Recent activities include a clinician survey, publication of best practices, and the Human Digital Twin Summer School.

PANORAMIX, launched in 2021, develops a framework for assessing chemical mixtures' health impacts, analysing samples for reproductive and neurotoxicity risks. The project applies effect-directed analysis to identify hazardous chemicals and explores omics technologies for determining safe chemical doses.

Innovations in predictive toxicology include a bioinformatics protocol using the Universal Immune System Simulator (UISS) to differentiate allergens, highlighting advancements in non-animal testing methodologies.

The use of omics technologies, *in silico* trials, and virtual physiological human technology is becoming increasingly important in predictive toxicology, and collaborations between researchers, regulators, and industry stakeholders are essential for advancing these efforts. As these initiatives continue to evolve, they are likely to

have a significant impact on the field of chemical safety assessment and the development of new, non-animal testing methods.

Validation

EURL ECVAM is working to establish confidence in new non-animal test methods, including assessing scientific validity, supporting external validation studies, and standardising complex test systems and technologies. A key initiative for supporting advancements in toxicological science and promoting timely adoption of NAMs is the revision of OECD Guidance Document 34 on the validation and international acceptance of new or updated test methods for hazard assessment that EURL ECVAM co-leads with the United States and the Netherlands. During the project group's meetings in April and December 2024, discussions centered on crucial topics such as test method readiness criteria, validation considerations for individual methods and Defined Approaches (DAs), including transferability and reproducibility studies, and discussions around prevalidation.

Another important activity involves standardisation of emerging technologies, such as organ-on-chip, high-content imaging, and omics-based methods. In fact, the biotechnology sector is rapidly evolving, leading to greater accessibility of advanced systems and technologies across various fields. These innovations are being applied in industrial processes, regulatory frameworks, and biomedical research. To maximise the benefits of these developments, standardisation and harmonisation help establish a common language, develop consistent protocols, and create uniform criteria for design, characterisation, analysis, and data reporting. EURL ECVAM is actively facilitating the effective use of data generated by these technologies while promoting their integration into practical applications.

In 2024, EURL ECVAM also consulted its network of regulators PARERE on various topics presented at a PARERE meeting. Key findings highlighted the complexity of transitioning to animal-free assessments, the need for capacity building, and the importance of standardising emerging technologies in hazard and risk assessment.

EURL ECVAM's Scientific Advisory Committee (ESAC) has peer-reviewed the Reconstructed human Skin (RS) Comet and Micronucleus (MN) assays for genotoxicity, and has concluded that these methods have sufficient scientific validity.

EU-NETVAL, EURL ECVAM's network of 33 validation laboratories, has initiated a collaboration with the EU-funded PARC project to advance the validation of regulatory applicable methods.

EURL ECVAM is also promoting collaboration in scientific research by opening its laboratories to public and private users through the JRC Open Lab framework. EURL ECVAM will provide access to its High Throughput Testing laboratory starting in early 2025, aiming to enhance human cell-based toxicological methods while avoiding animal testing. The initiative fosters method evaluation/validation, knowledge sharing, and innovative approaches.

Training on validation and good cell culture practice is a significant part of EURL ECVAM's dissemination efforts. In 2024, training sessions for ECHA staff and regulators focused on various aspects of validation, aiming to improve confidence in evaluating *in vitro* data in regulatory contexts.

Regulatory application

EURL ECVAM is actively involved in EU and international collaborations with organisations like the OECD, UN GHS, and EU regulatory agencies to advance non-animal sciences and technologies. The goal is to integrate these advancements into regulatory practices for chemical hazard and risk assessment. Its efforts span, among others, the development of international guidelines and guidance documents and the promotion of innovative assessment frameworks.

In terms of policy and regulation, EURL ECVAM is supporting the European Commission's roadmap towards phasing out animal testing for chemical safety assessments, with publication planned for the first quarter of 2026. This plan involves creating milestones and actions to accelerate the transition towards non-animal testing methods, supported by three working groups focusing on human health, environmental safety, and change management. The European Commission has so far organised two conferences on the roadmap.

EURL ECVAM participates in the European Partnership for Alternative Approaches to Animal Testing (EPAA), promoting the replacement, reduction, and refinement (3Rs) of animal testing by using more effective and predictive scientific methods to meet regulatory standards. The EPAA has launched projects such as the EPAA NAM Designathon, which focuses on creating new classification systems for human systemic toxicity using non-animal methodologies, predicting the carcinogenic potential of agrochemicals, and exploring alternatives for environmental safety assessments. In 2024, the EPAA Project Platform strategically redirected several projects towards creating action plans in various toxicological fields to support the EC's roadmap towards phasing out animal testing for chemical safety assessments.

Additionally, EURL ECVAM has been involved in developing new guidance documents and implementing the new CLP criteria for hazard classes for endocrine disruptors. At the level of the United Nations Globally Harmonized System (UN GHS) for the classification and labelling of chemicals, EURL ECVAM is heavily involved in developing global criteria for hazards like endocrine disruptors and Persistent, Bioaccumulative and Toxic and very Persistent and very Bioaccumulative (PBT/vPvB) through the Potential Hazard Issues' Informal Working Group (PHI-IWG). EURL ECVAM also supported the Informal Working Group on the use of Non-animal Test Methods (IWG-NATM) for the update of GHS Chapter 3.4 on skin sensitisation to align with non-animal testing methods, approved in July 2024, and for revising Chapter 1.3 on general classification and non-animal testing methods. Finally, EURL ECVAM leads the germ cell mutagenicity group, updating classification criteria for all germ cell mutagenicity categories, updating the relevant chapter to reflect current scientific understanding and drafting amendments to facilitate hazard classification using non-animal methods.

To promote the 3Rs in the pharmaceutical sector, EURL ECVAM is collaborating with the European Medicines Agency (EMA) to develop new guidelines for the regulatory acceptance of 3Rs testing approaches and supports the EMA 3Rs Working Party and the EMA Innovation Task Force.

Furthermore, EURL ECVAM is involved in initiatives to assess the impact of chemicals on biodiversity, including developing new approach methods, issues recommendations for using epidemiological evidence to quantitatively assess health impacts in EU policy evaluations, and supports the development of indicators for chemical monitoring.

Alternatives in research and education

The EURL ECVAM Status Report 2024 highlights significant progress in promoting non-animal approaches in research and education. Key initiatives include the development of the BioMedical Models Hub (BimmoH) database, the Student Ambassador Project, and a Virtual Reality laboratory.

Analysis of animal use data for 2021 and 2022 reveals a complex picture. While 2021 saw an 18.5% increase in animal use due to large-scale projects, 2022 showed a 10.9% decrease, aligning with the long-term trend of reduced animal use for regulatory purposes.

EURL ECVAM initiated a pilot thematic review on 3Rs implementation in cardiovascular research, aiming to accelerate the adoption of non-animal methods. The review involves examining approved research projects, scientific literature, and gathering stakeholder information to assess the utility of non-animal methods.

In 2024, EURL ECVAM advanced BimmoH initiative by developing an automated database designed to collect and organise data on non-animal methodologies used in biomedical research. This system leverages automated techniques to extract and structure information from the extensive body of published scientific literature. In October 2024, an interactive workshop brought stakeholders together to unveil and explore the prototype database. The database, expected to be available in the second half of 2025, will serve as a valuable resource for project evaluators and research funding organisations. Furthermore, the BimmoH database is designed to assist researchers in identifying the most suitable model for addressing their specific research questions.

An analysis of EU-funded research projects in Alzheimer's disease, breast cancer, and prostate cancer revealed that non-animal methods were more frequently used in cancer research, while animal-based methodologies were more prevalent in Alzheimer's research. The study suggests that human-based approaches may be more conducive to societal impact.

The PRO-MaP initiative made progress in promoting open and reproducible methods and protocols in life sciences. A comprehensive report was published, providing recommendations for researchers, institutions, publishers, and funders to improve methodological clarity in publications.

In education and training, EURL ECVAM launched the Student Ambassador Project, targeting university students across Europe to raise awareness about non-animal approaches. The project aims to create a self-sustaining movement to spread knowledge across European universities.

EURL ECVAM continued its engagement with students through various activities, including hosting visits from the Karolinska Institute and conducting a survey on the impact of its biennial Summer School on Non-animal Approaches in Science. The survey results demonstrated the positive impact of the Summer School on participants' understanding and application of non-animal methodologies.

Finally, EURL ECVAM developed an open-access virtual reality application to educate students aged 14 to 18 about alternatives to traditional animal testing. This interactive tool immerses users in a laboratory environment, allowing them to explore non-animal testing methods through guided experiments.

These initiatives collectively demonstrate EURL ECVAM's commitment to advancing non-animal approaches in research and education, fostering a more ethical, sustainable, and scientifically sound alternative to animal testing.

1. Introduction

Since decades, the European Union (EU) is actively pursuing a comprehensive strategy to minimise the use of animals in scientific research and for regulatory purposes, through the implementation of the Three Rs principle: replacement, reduction, and refinement. This framework aims for the complete replacement of animal testing with innovative non-animal technologies whenever feasible. In instances where animal testing remains necessary, it must comply with the stringent provisions set forth in EU Directive 2010/63/EU (EU, 2010), which mandates that animal use is only permissible when no viable alternatives exist and when the anticipated benefits justify any potential harm to the animals involved.

The desire to eliminate animal testing has also gained significant momentum through two recent European Citizens' Initiatives (ECIs): "Stop Vivisection[1]" and "Save Cruelty Free Cosmetics – Commit to a Europe Without Animal Testing[2]." These initiatives collectively garnered over one million signatures and fulfilled the rigorous criteria set by the ECI framework. Additionally, a resolution[3] from the European Parliament has called for a transition away from animal testing, advocating for more humane and scientifically advanced alternatives. In response to the recent European Citizens' Initiative (ECI), the European Commission has initiated the development of a roadmap aimed at phasing out animal testing in chemical safety assessments. This roadmap will include a series of actions and a detailed plan for gradually replacing animal testing, engaging all relevant stakeholders in the process.

The commitment to reducing animal testing not only reflects ethical considerations but also aligns with the EU's broader objectives of scientific advancement and sustainable development. The ongoing development and validation of non-animal methods and approaches are crucial for enhancing research quality and relevance, particularly in basic, applied, and translational research domains. The 2024 EURL ECVAM Status Report highlights significant progress in this area, detailing efforts to promote non-animal approaches in both research and regulatory contexts, as well as educational frameworks.

EURL ECVAM, as the European Union Reference Laboratory for Alternatives to Animal Testing, plays a pivotal role in this initiative. Its responsibilities encompass a wide array of activities aimed at fostering alternative methodologies across various sectors, including industrial chemicals, plant protection products, biocidal products, medicinal products, cosmetic products, toys and medical devices. By facilitating collaboration among stakeholders—including legislators, regulators, industry representatives, researchers, test method developers and animal welfare advocates—EURL ECVAM is instrumental in advancing the development, validation and regulatory acceptance of innovative non-animal testing strategies. EURL ECVAM is an integral part of the European Commission's Joint Research Centre.

1 http://www.stopvivisection.eu/
2 https://europa.eu/citizens-initiative/initiatives/details/2021/000006_en
3 https://oeil.secure.europarl.europa.eu/oeil/popups/ficheprocedure.do?reference=2021/2784(RSP)&l=en

2. Development

Scientists are making notable strides in the development of non-animal testing methods, facilitated by funding from the European Union and collaborative efforts among researchers. EURL ECVAM is contributing its expertise in various ways, such as offering guidance, sharing best practices for method characterisation and standardisation, and helping to identify promising methods that could be implemented and recognised by regulatory authorities. The overarching aim is to discover and advance these innovative methods so they can be applied in practical settings and receive 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 Leiden University (RISK-HUNT3R), University of Birmingham (PrecisionTox), and the Vrije Universiteit Brussel (ONTOX). The three projects have joined forces in the collaborative group ASPIS ("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 use 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, ASPIS provides NAMs to rapidly accelerate and improve chemical risk assessment in the EU and beyond. The three, 5-year projects are complementary to each other and share common elements that form the basis of collaboration at a 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 contributes to activities in ASPIS.

Box 2.1. PrecisionTox

The overarching goal of PrecisionTox is to propose a new integrative assessment framework for the protection of human health and the environment 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 considering genetic variation in individual susceptibility. This is accomplished by using a suite of alternative biomedical model species that have already transformed our understanding of the human condition by obtaining knowledge of the root causes of disease. Moreover, PrecisionTox does not rely on the strength of science alone to engineer change; instead, it addresses the socio-technical and legal barriers to the uptake of NAMs. Work relating to the objectives is beginning to detect the degree to which genetic variation among individuals determines the level of susceptibility to toxicity by studying fruit fly populations and globally diverse human cell lines with known DNA variation, which may serve as a 'susceptibility model' for human populations. This concept of "quantitative susceptibility" stems from statistical genetic evidence that the heritable basis for individual differences is often found in genes and pathways shared among species. The anticipated impact is a new method to set regulatory limits on chemical exposure based on variations in people's genetic susceptibility to adverse outcomes.

Recent highlights include:

- Completed chemicals library of 200 substances with information about their chemical class, diversity in terms of structure, physical-chemical properties, toxicity modes of action and database/literature-derived associations with disease pathology, genes and putative metabolic biomarkers.
- Conducted harmonised toxicity testing experiments on over 100 substances with comparative toxicology results for a first "phylogenetic toxicity analysis" to allow cross-species extrapolation.
- Conducted a pilot project that informed an experimental design that produces a larger volume and diversity of omics data per species for each tested chemical.
- Validated a customised methodology for RNA sequencing for integration with procedures for high-throughput sample processing for metabolomics and transcriptomics data.
- Uploaded pilot project data and results to the Data Commons, which benefits from an additional data processing pipeline (for RNA sequencing data) and can be accessed by browsing the results using a highly modular and adaptable PrecisionTox Data Explorer application.
- Produced early-stage discoveries on modes of action shared between Daphnia and Drosophila
- Genome-wide screening of genetic variation for toxicity is also producing publishable results.
- Submitted the report on the socio-technological barriers to the uptake of NAMs in chemical regulation.
- Progress made on (i) the development of 2 to 3 case studies with Nordic regulators, (ii) possible steps towards solutions and governance reform for the uptake of NAMs in regulation, (iii) legislative mapping of key EU chemical-related legislation and case law, (iv) developing reporting templates and other informational support for the regulatory use of NAMs data and results.

As the new knowledge from this project emerges for demonstrating its concepts, it is on track to accelerate the pace of regulating chemicals as groups by promoting a more mechanistic and integrative approach to assessing chemical hazards.

Considering the interdisciplinary nature of PrecisionTox, which integrates science and law, the following are seen as desirable outcomes.

a) Legal, political, and regulatory frameworks are expected to be established that leverage NAMs in chemical safety assessments.
b) Defined pathways for the early commercial adoption of NAMs across various mechanisms are foreseen, contingent upon the reliability of biomarkers in establishing a causal link between chemicals and their adverse health effects.
c) The results generated are expected to be instrumental in shaping future legislative tools aimed at enhancing chemical safety.

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 include:

- WP1 reviewed and updated all physiological maps (PMs) for bile secretion and lipid metabolism (liver), nephron physiology (kidney), and neural tube closure and cognitive function development (brain). Updates included standardising interactions in Systems Biology Graphical Notation Process Description (SBGN-PD) language, designing a cell-cell interaction map and new submaps (brain case), modularising maps for reuse, and implementing a unique ID system for versioning. Curation guidelines were established covering design, annotation, documentation, quality control, and storage. All maps are accessible via permanent MINERVA links, with versions stored on BioStudies and GitHub. An introductory video and CellDesigner tutorial are publicly available.
- WP2 developed a model to predict the severity in drug-induced cholestasis patients (Moreno-Torres *et al.*, 2024). Through literature searches, enhanced by machine learning and AI, a new database was created containing clinical data from 259 patients with drug-induced steatosis. Additionally, WP2 collected human fresh-frozen samples for transcriptomics: 40 brain samples from fetuses with spina bifida and 48 paired liver/serum samples from patients with steatosis. Quantitative models for common Adverse Outcome Pathway (AOP) KEs/KERs and AOP networks are under development.
- WP3 developed computational models (QSAR and read-across) to predict the capability of chemicals to interact with endogenous protein 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 ONTOX Hub. DockTox[4], a new docking server was developed and implemented to simulate the binding of ligand with 23 proteins relevant for MIEs of the project's AOPs.
- WP4 developed a High-throughput (HTP) Physiologically Based Kinetic (PBK) model to predict *in vivo* simulations after oral uptake and is developing PBK models and kinetic read across strategies for specific classes of chemicals. 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 and three workshops discussing it in the context of the advent of AI-enabled methods; developed ~90 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. The first version of a toxicology co-pilot, i.e., an AI-based predictive tool comprehensively exploiting available information to predict toxic properties from what we know about similar chemicals, is currently being finalised.
- WP6 developed a protocol for probabilistic risk assessment (ProbRA) to initiate a case study on PFOA. WP6 maintained collaboration with a range of stakeholders to facilitate end-user acceptance of ONTOX ProbRA approach and facilitated discussions on identified challenges towards full replacement of animal testing with NAMs and AI, during an issue-oriented Hackathon in April 2024. A detailed strategy for achieving the overall ONTOX goal/desired end-state - a fit for purpose "AI supported ProbRA approach" was developed in 2024.
- WP7 developed two batteries of *in vitro* assays assessing the most relevant molecular initiating events and key events in the newly updated AOP networks on chemical-induced cholestasis and steatosis.
- WP8 developed an AOP network for toxicant-induced kidney failure and developed a test battery of *in vitro* assays to monitor this. The focus is on chemical-induced tubular necrosis and crystallopathy leading to kidney failure.
- WP9 focused on characterising the developed human cell-based *in vitro* assays to study the key neurodevelopmental processes crucial for brain development to increase human relevance and confidence in the *in vitro* battery. A computational model was developed to predict the probability of neural tube closure defects based on gene perturbations.
- WP10 continued to guide the ONTOX research and non-research activities, leading to a positive evaluation of the second periodic report by the European Commission. This effort also enhanced existing collaborations with external partners such as VHP4Safety and PARC, and a newly established one with the University of Seoul. WP10 also continued co-coordination of the ASPIS cluster, which includes ONTOX, PrecisionTox,

4 https://chemopredictionsuite.com/DockTox

and RISK-HUNT3R, and supported the creation of the ASPIS Academy. From September 2024, ONTOX took over the chairmanship of ASPIS, which will be coordinated by WP10.

- WP11 expanded the ONTOX data collection in BioStudies which now contains 36 private accessions and one publicly available one (S-ONTX24). In more detail, the accessions comprise 20 transcriptomics datasets from WP2, 3 ToxTemps from WP7-9, and 8 physiological maps from WP1.
- WP12 ONTOX released two tutorials on CellDesigner, a software for curating molecular interaction models, and another one on physiological maps, both accessible on the ONTOX YouTube channel. In addition, over 10 videos were produced during events such as SOT, ESTIV, the ONTOX Hackathon and the ONTOX PRA (probabilistic risk assessment) workshop. Video games created for the ONTOX Hackathon, aimed at the general public, are available at the ONTOX GameLab.
- WP13 further developed ONTOX Hub to establish the platform to offer tools and services from the partners developed in the consortium for exploitation, performed a thorough market analysis to define and analyse the target market and ecosystem of ONTOX Hub, participated in several intellectual property and exploitation courses provided by the EU, and began work on a business and a sustainability plan for ONTOX Hub.
- WP14 has been instrumental in advancing communication on the ONTOX project achievements. It managed the project website, introducing the ONTOX TV and IMPACT sections to highlight consortium successes. Engaging audiences on Facebook, LinkedIn, and YouTube, WP14 also created a trailer for the ONTOX Hackathon and led targeted event campaigns. Additionally, WP14 issued the ONTOX Insights, five newsletters, and helped plan events like the ONTOX Hackathon, the ONTOX-ESTIV workshop, and the ASPIS Academy sessions, also managing the ASPIS website.

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:

- Over 10 case studies have now commenced, and the data generation and interpretation are greatly advancing, guided by developing the ASPIS Safety Profiling Algorithm (ASPA) in a tiered fashion.
- To test if a chemical will enter the human body upon exposure, *in vitro* tests were implemented for uptake via the lung or gut. Moreover, the biokinetic models can predict concentrations in the developed tests while accounting for metabolism in the ADME pillar of ASPA, as well as for biological variability.
- To predict toxicological hazards that might occur when chemicals are taken up by the body, computational pipelines were established based on chemical structures of compounds to predict to which targets they may bind for various tissue types. A panel of pluripotent stem cells with stress response reporters for high-throughput hazard screening in different target organ lineages, like hepatocytes, renal proximal tubular cells and cardiomyocytes, have been generated. High-throughput data for over 100 compounds include phenotypic, MIE (molecular initiating event) and KE (key event) assay data.
- For transcriptomics, showing changes in all cellular processes at the same time, comprehensive toxicogenomics prediction platforms were set up for kidney and liver cells, including stem cell-based models for these cell types, as well as for mature peripheral neurons. State-of-the-art combined transcriptome and metabolome studies were also set up to determine cell fate. Kidney and liver organoids were further developed, used for transcriptome mapping, and combined in a two-organ chip. Challenging compounds were assembled and tested to show how to avoid false negatives.

- Network mapping was used to delineate gene and protein networks and compounds associated with kidney toxicity, as well as putative new kidney biomarkers. Transcriptomic and morphological data can be connected with clinical information, and a database for all project compounds based on existing human exposome knowledge has been constructed. AOPs can be quantified, all the way from MIE to late KEs, and a framework to evaluate such qAOPs has been developed.
- Implementation of the uncertainty framework for NGRA.
- RISK-HUNT3R put in place a data and knowledge infrastructure with a harmonised data template and compound database, respecting FAIR data storage criteria.
- For NAMs >25 innovations were identified including their readiness levels for relevant applications, required for their success and commercial prospects.
- With ASPIS partners, the ASPA workflow has been refined via interaction with stakeholders (regulators, industry, academia, NGOs) at multiple international events. A major innovation that has started to bring ASPA to the end-users is the NAMASTOX online dashboard to transparently operate on the workflow whilst integrating and documenting the safety assessment procedure.
- RISK-HUNT3R has continued to provide highly rated training on NGRA to the new generation of scientists on risk assessment, including the practical context of linking lab research and regulatory reality.

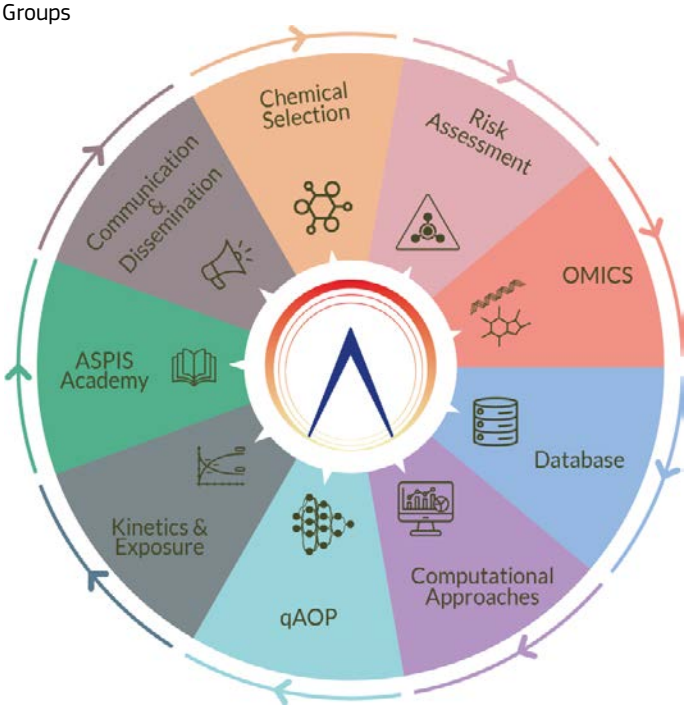
Website: https://www.risk-hunt3r.eu

Coordinator: Bob van de Water, Leiden University

2.1.2. ASPIS Working Groups

ASPIS functions through nine 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. Activities during the fourth 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**.

Figure 2.1. ASPIS Working Groups



Box 2.4. Highlights from the ASPIS Working Groups

Chemical Selection

The goal of this WG was to coordinate chemical selection among the three projects. Because chemical selection has been completed, other activities have taken precedence. This WG is responsible for the development of ASPIS-wide case studies and assisting other WGs by providing information about test chemicals.
Recent highlights include:

- Providing an assembled chemicals list and cognate physical-chemical and toxicological information from the three consortia. Provide this information to our partners including the JRC, US NTP, US EPA, and the EU-co-funded PARC projects.
- Providing two cross-cutting case studies pursued by all WGs: (a) steatosis and (b) conazoles in collaboration with PARC.
- Creating automated workflows to collect data from multiple databases including CompTox, AOP Wiki, DrugBank, ICE, T3Db, and the exposome explorer.
- Developing of a third case study on developmental neurotoxicants.
- Providing information to facilitate the development of the ASPA workflow and the ASPIS Compound Database.

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. Taking in consideration these target audience objectives.
Recent highlights include:

- ASPIS was actively reaching out stakeholders and regulators by operating a booth at the 2024 SOT ToxExpo.
- Participating in the EPAA designathon workshop hosted by the JRC.
- Presenting the work of the cluster at the 3Rs working party of the European Medicine Agency.
- Attending the Helsinki Chemical forum with a poster and presenting ASPA at a satellite event of the 2nd workshop of the EC roadmap to phase out animal testing and the EPAA NAM user forum in Helsinki at ECHA.
- Communication and dissemination material were produced such as video interviews of ASPIS WP leaders at the SOT in Nashville and at the ASPIS Open Symposium in Copenhagen to be released in social networks as well as reorganisation of the ASPIS website.
- The Communication and Dissemination working group continuously supported the activities of ASPIS Academy.

ASPIS Academy

The ASPIS Academy is a network of Early-Stage Researchers (ESRs) focused on the development and use of NAMs in the safety assessment of chemicals. 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.
Recent highlights include:

- Adding an ASPIS Academy subtab on the ASPIS Website for updates on news and activities of the group.
- Organising training courses and career development sessions on science communication, grant writing, policy in science and start-up business in science.
- Organising and promoting ESR involvement in the different ASPIS cluster activities.
- Establishing a mentoring activity across the three consortia and organisation of mobility facilitation for collaboration and exchange between the scientific projects.
- Communicating and disseminating activities including the organisation of sessions at conferences (ESTIV Prague 2024), poster sessions (ASPIS OS), workshops, networking opportunities, interviewing of scientists, and social media management.
- Initiating broader collaboration with other ESR communities (e.g. PARC, SETAC, Transition Programme for Innovation without the use of animals [TPI][5]).

5 https://www.animalfreeinnovationtpi.nl/

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. It is focused on the 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.

Recent highlights include:

- Discussing the integration of models within combined schemes. This resulted in a common paper with authors from ONTOX and RISK-HUNT3R related to steatosis.
- Collaborating with other WGs (in particular, Risk Assessment) by providing predictions (for conazoles).
- Discussing the integration of *in silico* tools developed within the different projects within the ASPA scheme.
- Discussing a repository of *in silico* tools from the different projects.

Database

This WG is responsible for the F.A.I.R. management and sharing of ASPIS 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.

Recent highlights include:

- A beta-version release of the ASPIS database.
- Database resources include biology repositories, omics data and workflows, computational data, methodologies and Knowledge Graphs.
- Standardising processes for data acquisition including cutting-edge technologies using AI to mine the literature for toxicological and risk assessment information.
- Establishing the above knowledge resources supporting ASPIS case study work including documenting compound selection information, evaluating computational predictions and experimental information, and updating models represented by knowledge graphs, including collaboration with other WGs.

Kinetics and Exposure

This WG integrates NAM-based tools to define chemical exposures at a cell, tissue, organism and population level. 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 (PBK) and quantitative *in vitro-in vivo* extrapolation (QIVIVE) models.

Recent highlights include:

- Comparative analysis of tools available to the consortium to assess the exposure and biokinetics of chemicals and how they can be integrated into the ASPA NGRA workflow.
- Evaluating *in vitro* distribution kinetics and physiologically based kinetic (PBK) models to characterise the steatotic hazard of azole fungicides in humans.
- Developing a tiered testing strategy beginning with *in silico* predictions, then progressing to *in vitro* experiments to address chemical absorption, metabolism and excretion – including ecotoxicity exposure and bioaccumulation testing strategies.

Omics

This WG promotes the use of omics data for Next Generation Risk Assessment by exploring the utility of gene expression and metabolomics data by providing information about chemical modes of action for regulatory purposes. It has assembled experts in bioinformatics to share methodologies used across the three consortia, with a special focus on early career researchers.

Recent highlights include:

- Establishing best practice of omics data analysis and reporting by promoting the use of the OECD Reporting Framework while accounting for the data management systems in place by the three individual projects.
- Comparing different approaches to classifying the hazards of substances based on bioactivity associated with steatosis (case study).

- Developing an interactive platform to improve transcriptomic use and interpretation, to be used in ASPA.
- Developing gene co-expression models for several test systems including liver, kidney and neuronal test systems.
- Progress at leveraging opportunities to harmonise omics datasets generated by the three consortia.

Quantitative AOP

This WG investigates models that quantify molecular initiating events (MIE) 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.
Recent highlights include:

- Developing a steatosis qAOP through data and model sharing, including modelling approaches to simulate steatotic Key Events.
- Updating the AOP network for liver steatosis (Verhoeven *et al.*, 2024) is used as a starting point for quantification. Key events and relationships with abundant data and high reliability are selected for quantification as proof of concept. In addition to liver steatosis, the same approach will be used for the Developmental Neurotoxicity (DNT) case.
- Developing a framework for validating qAOPs and providing qAOP input into regulatory decisions.
- An ASPIS qAOP Validation sub-WG was established to focus on this task. A paper titled "A Framework to Evaluate, Verify and Assess the Validity of quantitative Adverse Outcome Pathways (qAOPs)" has been drafted.
- Developing a prototype of a model to integrate steatosis qAOP and (toxico)kinetics.
- Multiple model strategies were discussed for the steatosis and DNT cases. Two sub-groups have been established: Steatosis qAOP sub-WG and DNT qAOP sub-WG to initiate this task in practice. These sub-groups meet twice a month to discuss progress and further work.

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.
Recent highlights include:

- Mapping data from the steatosis and conazoles case studies to include objectives of the other WGs.
- Integrating activities conducted by EU-ToxRisk and by the OECD with those of ASPIS.
- Maturing the ASPA by incorporating elements from all three consortia into a NAMs chemical safety assessment workflow.

2.1.3. EURION

EURION
European Cluster to Improve Identification of Endocrine Disruptors

EURION, the cluster of eight European research projects funded by Horizon 2020 (call SC1BHC-27-2018), aimed at developing new test methods for identification of endocrine disruptors, has been finalised for six out of the eight projects. Two projects (ATHENA and GOLIATH) were granted an extension until the end of 2024. The EURION cluster was launched on 1st January 2019. The final event of the EURION cluster meeting took place in Brussels in June 2024, in which the most prominent findings and highlights of all the projects were showcased to the relevant stakeholders. These included, among others:

- the zebrafish obesogenic test (OBERON).
- the further validation of the CYP induction assay with additional industrial chemicals, focussing on EDs (originally validated for pharmaceuticals by EURL ECVAM).
- the expert elicitation for WoE approaches to form DAs and IATAs (GOLIATH).
- QSARs for female fertility related mechanisms (FREIA).
- the enhancement of TG456 with 19 steroids which is

under validation in PEPPER (FREIA).
- the importance of changes in thyroxine (T4) levels without concomitant changes in Thyroid-Stimulating Hormone (TSH) leading to adverse outcomes (ATHENA), acknowledging decreased T4 levels as a hub Key Event (KE).
- the importance of cross-vertebrate class approach for ED assessments (ERGO).

Some projects have been active in connecting with regulators at national level, with OECD WNT and the OECD AOP community, and have brought forward project proposals on certain methods to the OECD. The methods that are on the OECD workplan are the following:
- 2.64 Fish early-life stage toxicity test and Fish embryo toxicity test with four added thyroid hormone system sensitive endpoints (ERGO).
- 4.159 Updated TG 456 for LC-MS based steroidogenesis assay (FREIA).
- Mammary Gland Whole Mount (MGWM) in rat (FREIA).
- Augmentation of CYP induction assay (GOLIATH).

A few methods have been taken up by PEPPER[6] 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) as well as the Peroxisome Proliferator-Activated Receptor γ (PPARγ) transactivation assay (GOLIATH).

EURL ECVAM was 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 June, EURION published their Methods Table in the publicly available repository Zenodo[7] and in the Horizon Results Platform[8]. The table includes information for more than 100 new test methods and endpoints. It includes, among others, a short description of each assay, the type of assay (e.g. *in vivo*, *in vitro*, *in silico*), which ED modality it tackles, the readiness of the method in terms of extent of validation, the availability of the protocol, the potential link to AOPs and the point of contact for further information.

The EURION cluster "passed the baton" to the ENKORE cluster (see **Section 2.1.4**), which is the new EU-funded cluster on endocrine disruptors research launched in January 2024.

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

2.1.4. ENKORE

ENKORE

ENKORE is a new Horizon Europe cluster which began its work in January 2024 and it will have a duration of five years. It is composed of five research projects funded under the call HORIZON-HLTH-2023-ENVHLTH-02-03, focusing on the "Health impacts of EDCs: bridging science-policy gaps by addressing persistent scientific uncertainties". Given the positive experience from EURION, the planning of the cluster activities for ENKORE had been already pre-planned since the beginning so that the projects can identify synergies between them early in the process. The main fields the new projects deal with are how endocrine disruptors and/or their mixtures affect steatotic liver disease (EDC MASLD), immune system toxicity (ENDOMIX), and the hypothalamus-pituitary axis (HYPIEND). Novel effect biomarkers of metabolic disruption (NEMESIS) are also under investigation. There is also a focus on how to merge scientific evidence with regulatory practices, leveraging identification of EDs using NAMs (MERLON).

JRC is part of the International Advisory Panel (IAP) of ENKORE which will provide advice particularly in relation to regulatory needs to facilitate uptake of the methods for regulatory purposes of ED identification.

The kick-off meeting of the ENKORE cluster took place in June in Brussels (following the final event meeting of the EURION cluster).

6 Public-private platform for the validation of endocrine disruptors characterization methods

7 https://zenodo.org/records/13643083

8 https://ec.europa.eu/info/funding-tenders/opportunities/portal/screen/opportunities/horizon-results-platform/77820?keywords=endocrine%20disrupting%20chemical&isExactMatch=false&order=DESC&pageNumber=1&pageSize=50&sortBy=publicationDate

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

Coordinator 2024 (Merlon): Terje Svingen, DTU National Food Institute, Denmark

Figure 2.2. Kick-off meeting of the ENKORE cluster (Brussels, June 2024)

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. Formally starting on 1 May 2022, 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 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 Chemical 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, 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.

One important measure of the success of PARC will consist of tangible positive impact on the regulatory process and decision-making contexts. Many projects within PARC are aimed at making new approaches fit for use in current regulatory practice.

PARC is working (amongst other topics) towards implementation of a NAM-based Next Generation Risk

Assessment (NGRA). In this context, PARC WP2 ("a common science-policy agenda") is working towards improving the interface between the scientific and regulatory domain by:

- prioritising PARC projects regarding the development of NAMs and tools, as well as NGRA concepts and frameworks, with the regulatory needs as formulated by EU and Member State authorities
- supporting the European Commission's roadmap towards phasing out animal testing in chemical safety assessments under the roadmap activity "NGRAroute"[9];
- building the knowledge management and community platform, Parcopedia, for capacity building knowledge exchange and collaboration between all subcommunities involved in chemical risk assessment, i.e. academia, authorities, industry, NGOs etc., within and beyond PARC.

One major obstacle that has been identified in the field of NAM based NGRA is the lack of validated methods and limited validation capacities in both the European Commission and the Member States.

In 2024, the management board of PARC released a "scoping paper on validation-related activities within PARC to progress new methods and approaches for hazard and risk assessment of chemicals". The scoping document delineates the scope of PARC regarding validation. To support this goal, WP 5 initiated a project in Year 3. This project in collaboration with many partners within PARC, including the JRC, has the following objectives:

- Expand the ReadEDTest to self-assess methods for some endpoints prioritised within WP5;
- Selection of NAMs addressing gaps in regulatory needs;
- Organise independent peer-review of readiness of NAMs developed in WP5;
- Support validation activities of these selected NAMs for willing partners;
- As an optional objective, writing a manuscript based on the scoping document.

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

Parcopedia: https://parcopedia.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 integration of tools for safety assessment on the platform, chemometrics, models for kinetic and bioactivity prediction, and qAOPs.

9 https://www.parcopedia.eu/wp-content/uploads/2024/10/PARC_AD2.1_submitted_approval_pending.pdf

- The assessment of the applicability domains of *in vitro* models for modelling of chemical adsorption, distribution, metabolism and elimination (ADME) (i.e. intestinal, lung, skin, blood-brain barrier, kidney models).
- The development of AOPs and safety assessment workflows for each of the three case studies (chronic kidney disease, neurodegenerative disease and life course exposure, and thyroid mediated developmental neurotoxicity).
- The identification of detailed user profiles for the platform and stakeholder perspectives.
- The design of a multi-stakeholder process, focusing on integration of stakeholders into work packages, communication and constructive technology assessment activities.
- The exploration of the transformative potential of artificial intelligence, particularly Large Language Models (LLMs), in enhancing our data integration, analysis, and risk assessment capabilities.

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 EU-funded project under the H2020 programme that started in January 2021 to lower the barriers to 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 eight identified barriers: development, validation, accreditation, optimisation, information, training, exploitation, and legal-ethical.

As the ISW project ends in December 2024, some of its main achievements are summarised below. All the resources developed to facilitate In Silico Trials were presented by the ISW consortium at a final public event[10] during the VPH 2024 conference (Stuttgart, 2024)[11]. Many of the outputs are publicly available on Zenodo:

- Development: the project developed or further developed 11 solutions for *in silico* trials (five for developing medical devices, four for medicinal products, one for vaccine development, and one for regenerative medicine products. Two are already commercially available as software-as-a-service for preclinical use through InSilicoTrials[12], three are sold as part of the consulting services provided by Mimesis[13], and one will be made commercially available by Materialise Motion[14]. For the remaining five, a clear exploitation plan has been defined and will be pursued by the developers using other resources.
- Validation: six validation data collections (TBValid[15], StentValid[16], HFValid[17], ValveValid[18], DPValid[19] and MSValid[20]) are publicly available.).

10 https://www.vph-institute.org/events/in-silico-world-final-meeting-resources-for-in-silico-trials.html
11 https://vph-conference.org/
12 https://insilicotrials.com/
13 https://www.mimesis.srl/
14 https://www.materialise.com/en/healthcare/hcps/pressure-measurements-orthotics
15 https://zenodo.org/record/8307759
16 https://zenodo.org/record/7752991
17 https://zenodo.org/record/7555270
18 https://zenodo.org/records/11616538
19 https://zenodo.org/records/11221707
20 https://zenodo.org/records/10875606

- Accreditation: the consortium worked closely with ASME and ISO/IEC to support the formation of a new ISO/IEC workgroup[21], which will develop an EU-harmonised version of the ASME VV40 technical standard. Through the ISW Community of Practice, the Toward Good Simulation Practice (Viceconti *et al.*, 2024) was developed as an open-access book published by Springer Nature. In addition, the consortium completed two qualification advice procedures for new *in silico* methodologies with EMA. For both methodologies, a credibility analysis was published (Curreli *et al.*, 2023; Aldieri *et al.*, 2023) and one of them received a letter of support[22] from EMA. The experience gained with two EMA submissions was also published in a report entitled "Regulatory barriers to the adoption of In Silico Trials"[23].
- Communication: substantial work on Responsible Research & Innovation (RRI) was carried out. There have been more than 200 communication activities, including papers, conferences, scientific events, workshops, and press releases. Among the available open-access resources is a mapping of clinical uses. More stakeholder information resources will be made available through the Zenodo ISW community soon after the end of the project.
- Training: the consortium has produced various resources for the training and re-training of the workforce on developing and using In Silico Trials. These were informed by a complete analysis of the Intended Learning Outcomes[24] necessary to train students in biomedical engineering, medicine, life science, and allied health professions and re-train professionals in research, biomedical Industry, and governmental agencies. From the learning outcomes, complete curricula[25] were developed. Additional teaching materials will be shared through the Zenodo ISW community soon after the end of the project.
- Exploitation: in addition to commercialising some of the *in silico* solutions and defining commercial exploitation (including regulatory) pathways for all the others, a report is being prepared on the exploitation the ISW results, which should be available in December 2024 via the Zenodo ISW community. In addition, an up-to-date Strength-Weakness-Opportunities-Threats (SWOT) analysis is being prepared with a view to its use by the technical management of biomedical companies to convince their senior management to invest in In Silico Trials. The final version will be available in December 2024 via the Zenodo ISW community.
- Legal and Ethical: an in-depth analysis of legal and ethical requirements[26] offers a complete inventory of all the legislation relevant to In Silico Trials. This is accompanied by ethical and legal implementation guidance and guidelines[27], which help to overcome such legal and ethical barriers. Recommendations for policymakers should also be available in December 2024 via the Zenodo ISW community.

Coordinator: Marco Viceconti, University of Bologna

Website: https://insilico.world/

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

Zenodo ISW Community: https://zenodo.org/communities/insilicoworld-openaccess/

21 IEC TC62/ PWI 62-5 "Establishing the credibility of computational modelling in the field of medical devices through verification, validation, and uncertainty quantification https://www.iec.ch/dyn/www/f?p=103:38:309679269497564::::FSP_ORG_ID,FSP_APEX_PAGE,FSP_PROJECT_ID:1245,23,123752)

22 https://www.ema.europa.eu/en/documents/other/letter-support-universal-immune-system-simulator-tuberculosis-disease-model-uiss-tb-dr_en.pdf

23 https://zenodo.org/records/10948478

24 https://zenodo.org/records/8380239

25 https://zenodo.org/records/10400657

26 https://zenodo.org/records/7991233

27 https://zenodo.org/records/11173758

Figure 2.3. Final meeting of the In Silico World Project, September 2024

"With over 180 participants joining us – both in person and remotely – the event was a fantastic opportunity to reflect on the incredible progress we've made together. Your engagement and insights were invaluable, and we couldn't be more grateful for your support.

A huge thank you to everyone who attended and contributed to making this event memorable, but even more to all the extraordinary people who have poured their effort and talent into this project over the years. Your dedication has truly driven the success of In Silico World, and we couldn't have reached this milestone without you.

In Silico World is ending but we must continue driving innovation in In Silico Medicine!"

Marco Viceconti
at the In Silico World Final Meeting (September 2024, Stuttgart, Germany)

2.1.8. Virtual Physiological Human Institute

The Virtual Physiological Human (VPH) Institute is an international non-profit organisation, based in Belgium, whose mission is to promote the realisation and adoption of virtual physiological human technology in biomedical research and in the clinic. The virtual physiological human is an application of *in silico* medicine, consisting of the use of computer models of human pathophysiology to carry out medical research and improve healthcare and personalised medicine. This technology offers numerous advantages from pre-clinical applications, such as reducing, refining and replacing animal tests, to *in silico* clinical trials, and to clinical applications allowing better disease diagnosis and prognosis, and supporting (personalised) treatment planning. The VPH Institute, supported by the European Commission, works on initiatives to inform, connect and give voice to stakeholders in computational medicine, such as researchers, clinical organisations, industry, policy makers, and patient associations. Highlights of recent activities of the VPH Institute are:

- Launching a survey addressed to clinicians[28] to collect their awareness, opinions and experience on the use of computer modelling and simulation in the clinical practice. The current survey expands on one conducted in 2021, whose results were published in 2023 (Lesage *et al.*, 2023).
- Publication in February 2024 of an open access book titled "Toward Good Simulation Practice - Best Practices for the Use of Computational Modelling and

28 https://www.vph-institute.org/news/are-you-a-clinician-fill-out-the-survey-to-share-your-familiarity-level-of-adoption-of-in-silico-med.html

Simulation in the Regulatory Process of Biomedical Products" (Viceconti *et al.*, 2024). The book is the result of the Community of Practice, led by the VPH Institute, the Avicenna Alliance[29] and the InSilicoWorld (see **Section 2.1.7**) consortium, collecting the opinions of more than 100 experts in *in silico* trials from academia, industry, hospitals, regulatory bodies and consulting firms. Discussions among these experts led to the drafting of a preliminary definition of a Good Simulation Practice, i.e. a consensus-based standard for biomedical modelling favouring the acceptance and adoption of *in silico* trial technology as a regulatory decision support tool for new drugs and medical devices.

- Every year, the VPH Institute organises the Human Digital Twin Summer School in Barcelona, Spain, addressed to junior engineers, young researchers and medical doctors, to provide them with an overview of the state-of-the-art in *in silico* medicine and hands-on training sessions. The 2024 event, held in June, was focused on "Models and Simulation in Translational Research"[30] and covered topics such as health data processing, organ, cell and tissue modelling, and model validation.
- In September 2024, the biennial VPH Conference[31] took place in Stuttgart, Germany. The VPH Conference is one of the most important events in Europe for the *in silico* medicine community, highlighting the latest scientific advancements in the field, and fostering connections between academic researchers, companies, clinicians and policy makers. Counting more than 400 participants, the main theme of the 2024 Conference edition was "Data-driven Simulation Technologies for Clinical Decision Making", with sessions covering all the applications of computational medicine. The next edition of the VPH Conference will take place in Milan, Italy, in September 2026.

Coordinator: Liesbet Geris,Universities of Liège and KUL – Leuven (Belgium)

Website: https://www.vph-institute.org/

2.1.9. Panoramix

The overarching goal of PANORAMIX, a four-year project which started in November 2021, is to propose a new assessment framework for health protection from exposure to chemical mixtures. Emphasis is placed on characterising and comparing the chemical exposome in various environmental, food and human samples, and on estimating reproductive and developmental neurotoxicity of the samples by using whole mixture assessments. Specifically, the focus is on chemical and toxicity profiling of 750 human cord blood samples, which is coupled with a large amount of information on reproductive and neuropsychological function in the children. The aim is to identify chemical mixture drivers of adverse health outcomes in the children. This is accomplished by making use of a suite of technologies ranging from whole mixture studies using bioassays that are linked to adverse outcomes, suspect screening for chemical profiling, and effect-directed analysis. Moreover, PANORAMIX relies on theoretical mixture risk assessments based on the chemical profiling, and on development of a web-based tool for estimation of mixture effects.

Recent highlights include:

- Determination of chemical mixtures in the environment (wastewater, effluent, surface water), foods (fish, milk, drinking water) and human tissue (adult and cord blood, milk), representing an 'average' European situation.
- Confirmation of the concentration-additivity principle for a large number of chemical mixtures tested at low concentrations in a large panel of bioassays, which are linked to adverse outcomes for reproductive toxicity and developmental neurotoxicity. Compositions of the chemical mixtures were based on chemicals identified in environmental, food, and human samples.
- Effect-directed analysis on environmental, food, and human blood samples which includes a combination of micro-fractionation, bioassay testing and chemical

29 https://www.avicenna-alliance.com/

30 https://eventum.upf.edu/112371/detail/%E2%80%A6

31 https://www.vph-institute.org/news/vph2024-a-resounding-success-for-the-in-silico-medicine-community.html

profiling of the fractions. The complexity of the chemical analysis is reduced by reducing the number of chemicals in the fractions and by focusing only on chemicals that may contribute to a hazard related to the bioassay outcome.
- Creation of a database on 750 children from the Odense Child Cohort on their health outcomes related to reproductive toxicity and neuropsychological function together with detailed analysis of their cord blood. The analysis covered profiling of all 750 cord blood samples in five bioassays and chemical profiling by suspect screening of selected samples of 'low' or 'high' adverse outcomes. Data analysis is in progress.
- A mixture risk assessment of chemicals identified in human breast milk has been conducted. The starting point was the exposure identified in a pooled breast milk sample, and secondly, grouping for hazard assessment was performed. Next step is to perform a mixture risk assessment based on prenatal human exposures.
- Policy briefs on NAMs and chemical profiling approaches.

Coordinator: Anne Marie Vinggaard, Technical University of Denmark, National Food Institute

Website: https://panoramix-h2020.eu/

2.1.10. In vitro points of departure

To determine the maximum dose of a chemical without an appreciable risk to an individual, it is necessary to establish its point of departure (POD) (EFSA, 2021). Traditionally, this has been obtained using animal studies based on clinical or pathological endpoints, but in recent years focus has been drawn to using omics technologies (e.g., transcriptomics or metabolomics) to comprehensively characterise molecular responses to toxicants in biological test systems, including *in vitro* models (Meier *et al.*, 2025).

In collaboration with the University of Birmingham, EURL ECVAM investigated derivation of PODs using metabolomics (i.e., the measurement of low molecular weight biochemicals) in the HepaRG cell line (Malinowska *et al.*, 2023). Using a high-throughput workflow for sample exposure and subsequent metabolomics measurements, the responses of the HepaRG cell line were examined to four chemicals at seven concentrations of each chemical and five sampling time points, which ultimately enabled derivation of metabolomics PODs for these chemicals. As applications of metabolomics in this area have been scarce, the study first focused on investigating the most appropriate methodology to derive metabolomics PODs, followed by their determination across experimental conditions. The results revealed that changes in metabolomics-based PODs over sampling time points were chemical-specific, with some having a clear temporal trend (resulting in decreasing PODs over time); whilst for others that trend was less apparent. Although the study highlighted certain technical challenges related to the use of high-throughput *in vitro* metabolomics, it demonstrated that technological advances now enable its application for derivation of PODs. This work was presented at the 63rd Annual Meeting and ToxExpo of the Society of Toxicology in Salt Lake City in March 2024.

To further investigate the critical influence of exposure duration on biological responses, EURL ECVAM conducted a study examining transcriptomic responses of the HepaRG cell line to five known toxicants. Transcriptomics provides insights into molecular mechanisms of toxicity by assessing changes in the expression of thousands of genes. In this study, five different concentrations and five exposure time points were tested enabling comprehensive characterisation of gene expression using TempO-Seq, a targeted transcriptomics analysis. Non-parametric factor modelling was applied to analyse the data, allowing interdependencies among differentially expressed genes to be accounted for. Notably, for two of the five chemicals, PODs were significantly influenced by exposure duration. These results highlight the importance of considering both concentration and exposure time in the design of *in vitro* studies aimed at establishing PODs (Carpi *et al.*, 2024).

EURL ECVAM is advancing its work on omics-based PoD methodologies by participating in a working group focused on the application of transcriptomics PoDs for chemical risk assessment. This initiative is led by the Emerging Systems Toxicology for the Assessment of Risk (eSTAR) under the Health and Environmental Sciences Institute (HESI), aiming to enhance the integration of these methodologies into future toxicological assessments.

2.1.11. A bioinformatics protocol to identify skin or respiratory allergic reactions to chemical sensitisers

Whilst progress has been made in the use of NAMs to identify skin sensitisers, detecting respiratory allergens remains challenging due to limited understanding of the immunobiological processes involved and the lack of standardised predictive methods. EURL ECVAM collaborated with the Department of Drug and Health Sciences of the University of Catania on a project to explore the performance of a bioinformatics workflow involving the use of the Universal Immune System Simulator (UISS). The UISS model was adapted to discriminate between skin and respiratory sensitisers by predicting their distinct T-helper response and different cytokine profiles. It is generally aknowledged that skin sensitisers typically induce a Th1-type immune responses, whereas respiratory sensitisers are associated with Th2-type responses. The protocol was tested by applying it to 2,4-dinitrochlorobenzene and to trimellitic anhydride, typical skin and respiratory allergens. By predicting the immune responses of Th1, Th2, Th17 and immunoglobulins alongside a comprehensive panel of cytokines, it was shown that the system could distinguish and accurately predict whether a chemical under investigation is more likely to act as a contact sensitiser or respiratory sensitiser (Russo *et al.*, 2024). The workflow represents a substantial step forward in predictive toxicology, offering a reliable and efficient alternative to traditional animal-based testing. Future plans include expanding the range of chemicals tested to enhance the predictive capacity and applicability of the model.

3. Validation

Validation plays a crucial role in the scientific process, serving as a cornerstone to establish trust in cutting-edge methods and techniques. Over the years, well-established principles have guided the validation of non-animal testing methods that have gained regulatory approval. However, as the landscape continues to evolve, the validation process must adapt to accommodate the increasing number, diversity, and complexity of non-animal methodologies.

This chapter focuses on EURL ECVAM's efforts to modernise and simplify the validation process, taking into account the distinct needs and demands across various industries, regulatory bodies, and academic communities. Furthermore, it highlights key initiatives aimed at establishing and fostering confidence in new non-animal test methods, including assessing the scientific validity through the EURL ECVAM validation process, support from EURL ECVAM networks, and standardising complex test systems and technologies. Throughout this report, various aspects of scientific validation and standardisation are presented across multiple projects, encompassing the development and optimisation of new approach methodologies (as discussed in **Section 2**) to international standards (as discussed in **Section 4**).

3.1. Validation studies

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

EURL ECVAM is currently validating a high-throughput androgen receptor dimerization (AR2) assay that complements international activities in better protecting public health and environment from effects of endocrine disrupting chemicals. To move away from animal-based methods (e.g. Hershberger assay screening for (anti) androgenic properties of chemicals in rats), computational models predicting bioactivity of the androgen receptor are also urgently needed. The AR2 assay, originally developed by US EPA (Brown *et al.*, 2023), identifies agonists and antagonists of the androgen receptor in a reporter cell line (HepG2-AR2) and is envisioned to feature a revised computational model of androgen receptor bioactivity. A central part of this validation study is a transfer of the assay across automated liquid handling platforms to comprehensively characterise its performance for 45 reference chemicals. It is anticipated that this successful validation will accelerate the uptake of new *in vitro* methods to screen for effects of endocrine disrupting chemicals.

3.1.2. EURL ECVAM's support to external validation studies

EURL ECVAM is contributing to the validation management teams of the public-private platform for the pre-validation of endocrine disruptors methods (PEPPER) and the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) that organised validation studies to progress *in vitro* methods for endocrine disruption to regulatory acceptance.

So far, PEPPER focussed its discussions on the SOP and chemical selection for the validation of the *in vitro* method designed to measure Deiodinase 1 inhibition, which originated from the EURL ECVAM coordinated EU-NETVAL thyroid validation study (see TM2019-10[32] in TSAR). To support this effort, EURL ECVAM shared with PEPPER the data obtained during the EURL ECVAM

32 https://tsar.jrc.ec.europa.eu/index.php/test-method/tm2019-10

thyroid validation study and suggested chemicals to be tested. Data were generated by two laboratories, which will be presented to the validation management group in 2025.

The validation of the US-EPA's *in vitro* thyroid microtissue model (Deisenroth *et al.*, 2020; Foley *et al.*, 2024), coordinated by NICEATM, started in 2023 and includes four laboratories that will generate data in different phases of the validation study.

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

The EU Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) was established by the Commission in response to the provision of Directive 2010/63/EU (Article 47). This directive requires EU Member States to contribute to the validation of alternative methods by nominating suitable laboratories to conduct validation tasks. The network, which is coordinated by EURL ECVAM, comprises 33 test facilities that have been endorsed by the Member State National Contact Points for the Directive.

In 2024, EURL ECVAM established a collaboration with the EU funded Partnership for the Assessment of Risks from Chemicals (PARC) (see **Section 2.1.5**). Given the ongoing development and evaluation of numerous *in vitro* methods, as well as the design of various IATAs and case studies within PARC, EURL ECVAM will support the partnership to advance the more technically refined and regulatory applicable methods through validation activities. Over the next few years, EU-NETVAL member laboratories may undertake transferability and reproducibility studies of selected methods. These efforts aim to establish the reliability and practical applicability of these methods in regulatory contexts.

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

3.3.1. Reconstructed human Skin (RS) Comet and Micronucleus (MN) assays

After receiving full submissions of the RS Comet and RSMN *in vitro* test methods for genotoxicity between May and September 2021, EURL ECVAM assessed the two submissions and deemed that both methods could enter the ESAC peer review process. An ESAC Sub-Group (SG) was established early in 2023 and met for the first time in June 2023 at the JRC. Subsequent virtual meetings took place in October, November and December 2023, and in January, February, March and May 2024 to advance the peer review and draft an Opinion and SG Reports on each method. The ESAC SG Reports and draft Opinion on the RS Comet and RSMN test methods were endorsed by the ESAC SG on 23 May 2024 but were slightly revised and finally endorsed by the ESAC by written procedure on 24 January 2025. During the peer review, EURL ECVAM facilitated interactions between the ESAC SG and the test submitters to clarify aspects of the submissions and to provide additional information requested by the ESAC SG to conduct a proper assessment of the two methods.

The ESAC concluded that the evidence provided on RS Comet and RSMN is sufficient to support their scientific validity. The RS Comet and RSMN are separate *in vitro* assays that use human reconstructed skin models. The ESAC considered these to be better models for predicting human response to dermal chemical exposure because of their improved biological fidelity to human skin tissue compared to traditional submerged monoculture models. These assays are proposed as a follow up to positive results in the traditional genotoxicity *in vitro* test battery to confirm or reject potential for genotoxicity following skin exposure without the need for confirmatory animal tests. The ESAC recommends starting with the RS assay covering the mode of action that tested positive in the standard *in vitro* test battery and, if a negative result is obtained, performing the second RS assay for confirmation. A positive result in either the RS Comet or RSMN assay would be sufficient to conclude that the test item is genotoxic in the skin. The ESAC also considers that these tests could add value to a WoE-based evaluation of systemic genotoxicity provided liver metabolism and/or systemic bioavailability are considered.

The ESAC noted that the approach taken in the design and execution of the validation studies, which spanned a period of more than 10 years and multiple laboratories, brings inherent challenges and led to some uncertainty in the conclusions of the studies. Nevertheless, the ESAC concluded that the transferability, reproducibility and, when the assays are used in combination as proposed by the ESAC, the predictive capacity of the RS Comet and RSMN are sufficient.

The ESAC Opinion and SG reports are available at: https://publications.jrc.ec.europa.eu/repository/handle/JRC140306

Box 3.1. EURL ECVAM Scientific Advisory Committee (ESAC)

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 hazard and 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

Box 3.2. 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/88753/download.

Experts who are interested in participating in ESAC peer reviews are invited to apply to this continuously open call. The members of a specific sub-group who are not members of the ESAC shall be selected by the JRC 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.

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

The 13th meeting of the Preliminary Assessment of Regulatory Relevance (PARERE) network was held online on 29 May 2024. The meeting began with voluntary updates on activities from the Member States, including Austria, Germany, Italy, Finland, Belgium, Luxembourg and the Netherlands. These updates covered various topics, such as innovative approaches in toxicology and risk assessment, and the development of non-animal methods and approaches.

Presentations from EURL ECVAM included updates on the European Partnership for the Assessment of Risks from Chemicals (PARC; Horizon Europe project; see **Section 2.1.5**), the Animal-free safety assessment of chemicals: Project cluster for implementation of novel Strategies (ASPIS; Horizon 2020 project; see **Section 2.1.1**), the EC roadmap towards phasing out animal testing for chemical safety assessments (see **Section 4.2**), the European Partnership on Alternative Approaches to Animal Testing (EPAA) Designathon (see **Section 4.3.2**), emerging technologies (see **Section 3.7**), qualification framework for organ-on-chip (see **Section 3.7.1**) 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 3.5**).

Most of the presentations from EURL ECVAM included some questions to be addressed by the PARERE network through written consultation after the meeting.

In a subsequent consultation, PARERE members were surveyed on these topics. Eleven EU Member States, one Commission Service, one EU Agency, and one Scientific Committee responded.

The key findings were the following:

- PARC Involvement: Many respondents are actively engaged in PARC work packages, contributing to innovation in regulatory risk assessment, human biomonitoring, and method development.
- PARERE-ASPIS webinar interest: There is widespread interest in a webinar to discuss updates and future directions of the ASPIS project and in particular the ASPIS Safety Profiling Algorithm (ASPA).
- Transition to animal-free safety assessment: Respondents recognise the long and complex nature of this transition, emphasising the need for scientific advancements, policy changes, and a stepwise approach to build confidence in non-animal methods.
- Change management in the framework of the EC roadmap towards phasing out animal testing for

chemical safety assessments: Various strategies are being implemented to facilitate change, including capacity building, participation in research projects, and active engagement in key bodies. Removing barriers to the uptake of NAMs and NGRAs in regulatory practice is a priority.

- Emerging technologies in risk assessment: For 'omics technologies, standardisation of experimental design, data processing, and analysis is crucial. Expert judgment is preferred over binary outcomes due to the complexity of 'omics data. While expertise in complex *in vitro* systems like iPSC-based models and organ-on-chip models exists, their use in regulatory assessments is not yet widespread.

Box 3.3. Preliminary Assessment of Regulatory Relevance (PARERE) network

The Preliminary Assessment of Regulatory Relevance (PARERE) network is a key player in promoting alternative approaches to animal testing. Established by EURL ECVAM under Directive 2010/63/EU, this trans-sectoral network brings together regulators from EU Member States, representatives from EU agencies, and relevant policy services of the EC.

PARERE members are consulted on the regulatory relevance and suitability of various alternative approaches proposed for validation. These consultations can involve individual methods or entire approaches submitted to EURL ECVAM for validation, peer review, or evaluation. Additionally, their expertise is sought on broader topics including EURL ECVAM Recommendations, and methods and approaches developed within EU Framework Programme for Research and Innovation funded projects.

Ultimately, the PARERE network plays an important role in ensuring that alternative approaches to animal testing are not only scientifically sound but also relevant to the needs of regulators, paving the way for their acceptance 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

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

OECD Guidance Document 34 (OECD, 2005), published in 2005, has been crucial for validating and accepting test methods for hazard and risk assessment. However, toxicological science has advanced significantly since then, necessitating an update to reflect current methodologies and practices.

In April 2023, the Working Party of National Coordinators of the OECD Test Guidelines Programme (WNT) approved a project to revise GD 34 (see Zuang *et al.*, 2024). This update aims to:

1. Encourage timely adoption of NAMs.
2. Provide practical guidance for validating various method types.
3. Address key considerations identified by the WNT and recent publications.

The project is co-led by the EU (JRC/EURL ECVAM), the United States, and the Netherlands. A project group was established and held its first virtual meeting in December 2023. Two face-to-face meetings of the project group took place in 2024.

At the project group meeting of 22 to 23 April 2024, held in conjunction with the WNT 36 meeting at the OECD, discussions focused on key topics such as:

- Method Readiness and Validation.
- Defined Approaches (DAs) Validation.
- Validation considerations including technical validation of mechanistic methods, transferability, and between-laboratory reproducibility studies.

The group agreed to establish focused subgroups to continue working on DAs, readiness criteria, and transferability.

The third project group meeting took place at the RIVM, the Netherlands on 9 to 10 December 2024. This meeting aimed to increase consensus on various aspects of the validation process, including:

- Templates for readiness criteria for individual methods and defined approaches.
- Training and transferability issues.
- The role of prevalidation in the process.
- Reference to protected elements in methods that are candidates for validation or validated.

- Evaluation of reproducibility in validation studies.
- Clarification of terms used interchangeably to refer to reproducibility.

Two online meetings and one face-to-face meeting of the project group are scheduled for 2025, reflecting the ongoing commitment to updating and improving the validation process for new and updated test methods in hazard assessment.

3.6. Readiness criteria

Over the years, the demand for training on test readiness assessment and the development of test readiness criteria has grown among all stakeholders involved in the development, validation and regulatory application of *in vitro* methods. The (self)-assessment of test readiness for validation has emerged as a crucial component of the deliverables expected from European projects such as EU-ToxRisk, EURION and PARC. The EURL ECVAM's test submission process requires relevant information from test submitters to assess test readiness for validation; however, the criteria utilised have not been widely disseminated to the public. To address this gap and standardise the assessment of test readiness at an international level, criteria have been developed to evaluate the readiness of *in vitro* methods for three key areas: 1) entering validation after completion of definition and description, 2) being transferred to other laboratories, and 3) undergoing peer review for test guideline development. The initial draft of these criteria has been positively received by the OECD GD 34 project group and is currently under review (see **Section 3.5**).

3.7. Standardisation of complex test systems and technologies

The biotechnology sector is experiencing rapid growth and innovation, making sophisticated systems and technologies increasingly accessible across various domains. These advancements are finding applications in industrial processes, regulatory frameworks, and biomedical research. To fully harness the potential of these cutting-edge developments, standardisation and harmonisation have become critical strategies, which are essential for establishing a universal terminology, developing consistent protocols, and creating uniform criteria for design, characterisation, analysis, and data reporting. EURL ECVAM is taking a proactive approach to these technological advancements facilitating the effective utilisation of data generated by these technologies, and promoting their seamless integration into various practical applications (**Figure 3.1**).

Figure 3.1. EURL ECVAM is proactively investigating the standardisation of complex systems and emerging technologies, such as organ-on-chip, stem cell-based models, high-content imaging, and omics-based methods. The goal is to enable the efficient use of the data generated by these technologies and to promote their seamless integration into various applications.

3.7.1. Organ-on-chip

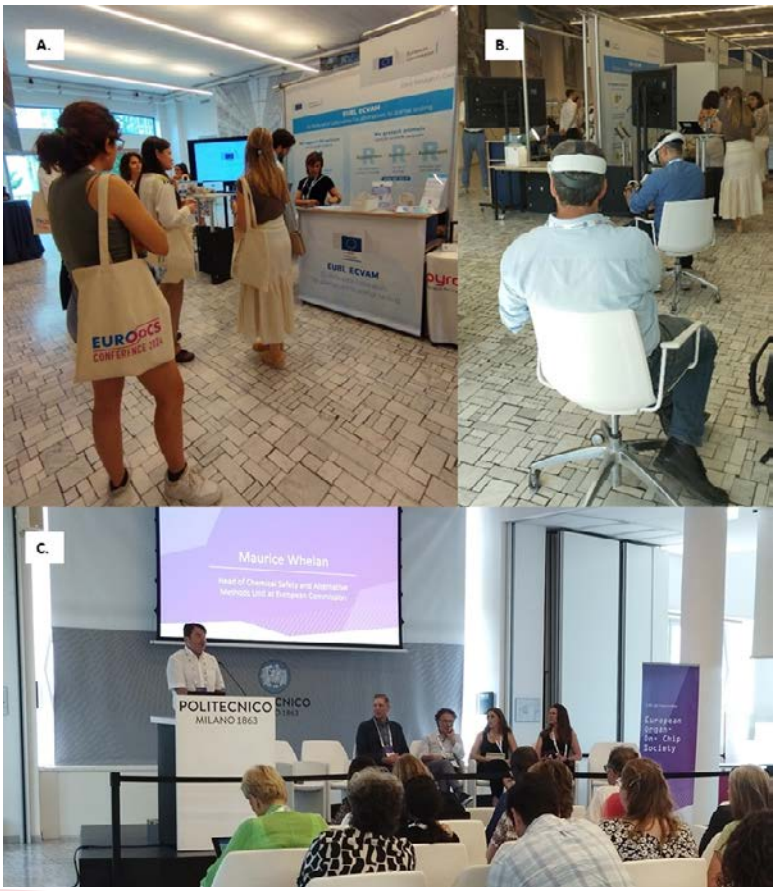
Standards for Organ-on-Chip (OoC) are crucial to ensure reliability, effectiveness, and data quality for advancing biomedical research and drug safety and efficacy assessment. Standardisation will also foster a robust biotech ecosystem and facilitate technology transfer from research to industry.

The CEN-CENELEC Focus Group on OoC (FGOoC) was established to address the growing need for standards. After two years of collaborative work involving approximately 120 European experts, including EURL ECVAM, the FGOoC published a roadmap outlining key recommendations for future standardisation activities (CEN/CELEC, 2024). This document emphasises the importance of a global effort in developing standards for OoC devices. Internationally, standardisation activities are progressing, with collaboration being key to harmonising technical solutions and policies (Reyes *et al.*, 2024; Parvatam *et al.*, 2024). The International Organization for Standardization (ISO) has recently established a new Subcommittee on 'Microphysiological systems and Organ-on-Chip' under the Technical Committee (TC) 276 – Biotechnology[33] to facilitate global engagement and agreement.

The OoC community is working together to define quality management criteria and share best practices throughout the experimental process, from the system set-up to the actual performance and the data analysis. A published manuscript addresses technical aspects of quality control in OoC device production, covering issues such as bubble absence, leak tightness, and dimensional tolerance. Taken together, these considerations provide a basis to assess the reproducibility of data generated with this emerging technology (Pamies *et al.*, 2024).

ECVAM's ongoing collaboration with the European Society of OoC (EUROoCS[34]) and the International MPS Society (IMPSS[35]) led to the publication of a workshop report titled "Heads on! Designing a Qualification Framework for Organ-on-Chip" (Piergiovanni *et al.*, 2024). In 2024, EURL ECVAM actively participated in the EUROoCS conference in Milan, organising an exhibition booth and contributing to the regulatory-industrial round table on "Organ-on-Chip based test methods for the authorization of medicinal products" (**Figure 3.2**).

Figure 3.2. Snapshot from EUROoCS 2024 Annual Conference that took place at the Politecnico di Milano. A. EURL ECVAM booth organised at the exhibition B. Conference participants experiencing the virtual reality. C. Maurice Whelan participating to the Regulatory-Industrial Round Table.



33 https://www.iso.org/committee/10713488.html
34 https://euroocs.eu/
35 https://impss.org/

3.7.2. High-content imaging

Imaging technologies are increasingly used to gather morphological and functional information from cellular models in biomedical research and experimental toxicology. However, these imaging-based *in vitro* methods are still under-represented in international guidelines like OECD test guidelines, limiting their use in regulatory decision-making. To address this gap, an international workshop titled "Facilitating the uptake of imaging-based *in vitro* methods in regulatory toxicology" was held in Ispra, Italy, on 7 to 8 March 2024. The workshop brought together experts from various sectors, including academia, CROs, industry, regulatory bodies, and GLP inspectors.

The workshop examined case studies based on the developmental neurotoxicity *in vitro* battery (DNT) and phenotypic profiling assays (cell painting). Key challenges identified included lack of standardisation, transferability, and validation. Other issues discussed were GLP implementation, performance assessment, reference materials, instrument calibration and maintenance, SOP development, data reporting, and metadata generation. As a result of the workshop, a manuscript titled "Bridging imaging-based *in vitro* methods from biomedical research to regulatory toxicology" was drafted. This paper addresses the main challenges associated with image acquisition and analysis and provides considerations on using AI to support the experimental process of data generation (Piergiovanni *et al.*, 2025). To successfully translate imaging test methods from R&D to international regulatory acceptance, the scientific community must implement imaging test methods under quality management systems, assess between-laboratory transferability, and demonstrate data reliability and robustness. The manuscript serves as a valuable resource for experts and regulators engaged in research based on imaging methods.

3.7.3. Omics-based methods

EURL ECVAM is actively working to standardise omics-based methods (transcriptomics, metabolomics) to facilitate their adoption in regulatory toxicology. At the OECD level, EURL ECVAM is contributing to the development of a new module for reporting omics data, which can be used to identify chemical groups for read-across purposes (OECD, 2024a). Additionally, EURL ECVAM is involved in drafting new guidance on best practices for sample collection for omics analysis.

The increasing interest in applying omics-based methods in regulatory toxicology led to a three-week visit by EURL ECVAM to the European Chemicals Agency (ECHA). This visit provided insights into how omics data could be integrated into REACH and CLP regulations, enhancing EURL ECVAM's understanding of regulatory processes that could be leveraged to develop NAMs, including omics-based methods.

EURL ECVAM also participated in the 63rd Annual Meeting and ToxExpo of the Society of Toxicology, presenting on deriving points of departure using high-throughput *in vitro* metabolomics and engaging in panel discussions.

Lastly, in collaboration with Professor Dario Greco's group from the Finnish Hub for Development and Validation of Integrated Approaches (FHAIVE), EURL ECVAM is launching the "Omics2AOPs" project. This initiative aims to establish methodologies for linking molecular data to key events and Adverse Outcome Pathways addressing the urgent need for interpreting omics measurements. The project will involve creating an OECD working group to build upon previously published work in this area.

3.7.4. Stem cells

Stem cell-based models have become increasingly prominent in biomedical research and toxicology over recent decades. These models offer significant value in pre-clinical studies and chemical safety assessments due to their ability to mimic human physiology and model diseases accurately. However, challenges persist, particularly in terms of variability and reproducibility. Standards play a crucial role in balancing the inherent biological variability of stem cells with the need for reproducible test results. While several guidance documents on best practices exist (Pamies *et al.*, 2022; OECD, 2018a; Ludwig *et al.*, 2023), the extent of their adoption remains unclear.

To address this issue, EURL ECVAM organised a panel discussion during the International Society for Stem

Cell Research (ISSCR) Annual Conference 2024 in Hamburg. The session brought together experts from stem cell research, industry and regulatory agencies to discuss about the current adoption level of standards and best practices in non-clinical research and strategies to facilitate their implementation. The panel and audience interaction highlighted several important aspects:

- The need for collaborative efforts from funding organisations, journal editors and researchers.
- The importance of training early career researchers.
- The role of supporting core facilities.

The discussion emphasised the necessity of a comprehensive approach to achieve effective standardisation. This approach would significantly enhance the quality and reproducibility of stem cell-based models and methods, promoting their wider adoption in industrial and regulatory testing environments. A manuscript detailing the perspectives presented during the panel discussion, along with the challenges and proposed solutions, is currently in preparation.

3.8. EURL ECVAM Open Lab

With the overarching goal of promoting collaboration, innovation, and transparency in scientific research, the European Commission's Joint Research Centre (JRC) opens some of its laboratories to users from both the public and private sectors. The Open Lab framework[36] that details the necessary documentation, rules for access, safety protocols, and eligibility criteria for users, governs this initiative. The access is free of charge under certain conditions. The selection process involves open calls for competitive applications, followed by a rigorous peer review conducted by a User Selection Committee composed of field experts.

As part of this initiative, EURL ECVAM, an integral part of the JRC, offers open access to its High Throughput Testing (HTT) laboratory for users aiming to scale up human cell-based toxicological methods. The first call for proposals for this facility has been launched on 5 February 2025[37]. Successful proposals must be suitable for execution in the facility, and be relevant for enhancing effective chemical risk assessment without relying on animal testing.

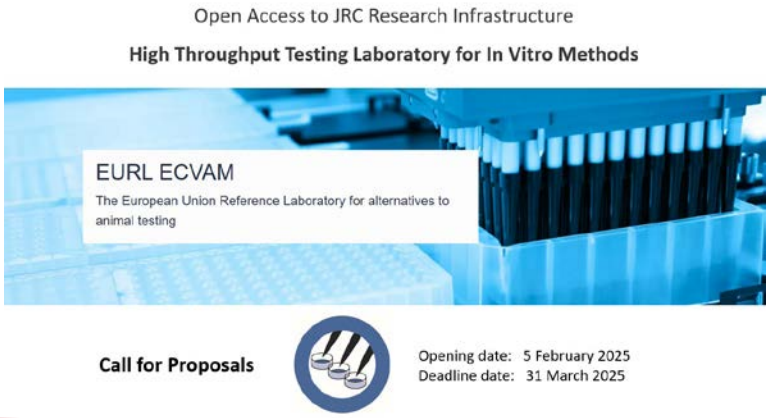
The HTT laboratory is equipped with cutting-edge technologies that enable precise and rapid testing of large chemical libraries. By providing access to this facility, EURL ECVAM aims to:

1. Allow developers to rigorously evaluate the robustness of their methods using consistent reference chemicals.
2. Facilitate scientific and technical knowledge sharing.
3. Offer hands-on training in non-animal based chemical hazard assessment methodologies.
4. Scout innovative approaches.
5. Assess and promote the transferability of regulatory relevant *in vitro* methods.

By leveraging automated technologies and high-throughput screening capabilities, we can significantly streamline and enhance the efficiency of scientific method validation procedures.

More information about the JRC Open Lab System can be found here: https://joint-research-centre.ec.europa.eu/tools-and-laboratories/open-access-jrc-research-infrastructures_en

Figure 3.3. The image reports dates related to the first Call for Proposals and the icon representing the EURL ECVAM HTT laboratory in the framework of the JRC Open Labs.



36 https://joint-research-centre.ec.europa.eu/tools-and-laboratories/open-access-jrc-research-infrastructures/framework-access-infrastructures_en

37 https://joint-research-centre.ec.europa.eu/calls-proposals/european-union-reference-laboratory-alternatives-animal-testing-2025-1-rd-eurl-ecvam_en

3.9. Training on validation and good cell culture practice

EURL ECVAM actively disseminates information on NAMs and their validation processes.

From 26 to 27 November 2024, EURL ECVAM conducted a two half-day training session for ECHA staff and Member State Committee (MSC) delegates. The training covered critical aspects such as the scope of validation, readiness evaluation of methods for validation, and designing effective validation studies. It also addressed assessing methods under optimisation or standardisation, referencing OECD guidance documents like GD 286 on Good in Vitro Method Practices and GD 34 on test method validation. Real-life examples of research data were used to illustrate these principles, aiming to enhance participants' confidence in evaluating *in vitro* data.

On 4 September 2024, EURL ECVAM contributed to the second Organ-on-Chip Summer School organised by the 3R-Centre Tübingen and EUROoC's 'Teaching & Training' Working Group. The session focused on Good Cell Culture Practices (GCCP), emphasising their importance for early-career scientists to improve research quality and impact.

"It is important for regulators to understand how validation is done, to gain trust in methods that have passed validation for a particular purpose. Understanding the process will also help in critical review of information obtained from methods that have not been validated e.g. what could be the potential pitfalls."

Laura Rossi
Scientific Officer
European Chemicals Agency, Helsinki, Finland

4. Regulatory application

EURL ECVAM plays a significant role in EU and international initiatives, collaborating with organisations like the OECD, UN GHS, and EU regulatory agencies. By collaborating with diverse stakeholders, EURL ECVAM works to advance emerging non-animal sciences and technologies, with the goal of integrating them into regulatory practices for chemical hazard and risk assessment. This chapter provides an overview of the organisation's ongoing efforts across multiple platforms in 2024.

4.1. Test methods and integrated approaches to testing and assessment

4.1.1. Developmental neurotoxicity

Developmental neurotoxicity (DNT) has emerged as a pressing concern due to its potential to cause a wide range of cognitive disabilities, including impairments in learning, memory, and behavioural development, ultimately leading to negative public health and socioeconomic consequences. There is a growing interest in developing innovative and reliable non-animal methods for evaluating developmental neurotoxicity (DNT) also considering the limitations and shortcomings of traditional animal tests.

Collaborative efforts have led to the development of DNT *in vitro* assays based on animal and human cell culture models, assembled into a battery (DNT-IVB) (OECD, 2023). The OECD has converged most of this work and it is now progressing towards addressing the current challenges associated with the employment of the DNT-IVB in the regulatory setting. One key aspect to consider is the transferability of the methods from the test developers' laboratories to naïve laboratories and the assessment of their level of reproducibility. The European Food Safety Authority (EFSA) is engaged in this effort by funding the DNT RAP2 project conducted by the Leibniz Institute for Environmental Medicine (IUF) with the overall objective of optimising the standard operating procedures (SOPs) for all the DNT-IVB test methods and assessing their transferability and initial reproducibility.

EURL ECVAM is supporting this project by providing expert advice on the design and management of key aspects of the transferability study in view of minimising potential sources of variability. The project started in 2024 with a forecast to be finalised in 2027.

"A multiyear effort led by EFSA and US EPA under the OECD resulted in a document on developmental neurotoxicity in vitro battery (DNT-IVB) assays. This document provides criteria for evaluating data relevance and determining result certainty for regulatory hazard determinations [ENV/CBC/MONO(2023)13]. The work is ongoing with two parallel projects under the OECD Test Guidelines and Hazard Assessment Programmes. These projects aim to provide critical elements and guidance to further support the use of NAMs in addressing DNT in chemical assessments. The DNT-IVB exemplifies developing solutions for regulatory needs using cutting-edge science. It also demonstrates the integration of NAMs to address complex endpoints with a flexible workflow, reducing variability in expert judgment. Lessons from this example can be applied to other NAM activities, paving the way for a new paradigm. Finally, the DNT work is a model of successful international collaboration and an example of co-designing NAMs with multiple stakeholders."

Magdalini Sachana
Policy Analyst to the Test Guidelines, Hazard Assessment and Pesticides Programmes
Organisation for Economic Cooperation and Development, Paris, France

4.1.2. Developmental and reproductive toxicity

An area that heavily relies on animal testing is the regulatory assessment of developmental and reproductive toxicity (DART) for industrial chemicals (Rovida *et al.*, 2023), pesticides, cosmetic products, food additives, and pharmaceuticals. Thus, there is a need to find alternative approaches that are human relevant and allow testing of thousands of chemicals in a cost- and time-effective way.

Following the example of the International STakeholder NETwork (ISTNET) meeting on Developmental Neurotoxicity (DNT) in 2014 (Crofton *et al.*, 2014; Bal-Price *et al.*, 2015), which relied on the early involvement of regulatory, academic and industrial scientists and resulted in the roadmap for the development of the DNT *in vitro* battery (DNT-IVB), the first ISTNET on Developmental and Reproductive Toxicity (DART) meeting was held in Zurich, Switzerland on September 12 to 13, 2024. The aim of the meeting was to develop a roadmap for animal-free DART regulatory testing. To set the stage for discussion, the biology of the reproductive and developmental cycles was presented together with insights on currently available NAMs for DART. The perspectives of different regulatory agencies on the use of NAMs for DART, the implementation of DART in next generation risk assessment frameworks (NGRA) and the overall strategy of the European Commission's roadmap towards the phasing out of animal testing were also presented. EURL ECVAM was invited to provide insights on readiness criteria and validation of *in vitro* test methods. The second half of the meeting consisted in breakout group discussions for female and male reproductive toxicity, developmental toxicity and physiology-based kinetic (PBK) modelling. This allowed an active exchange of perspectives and views between different stakeholder representatives. The meeting not only showcased groundbreaking scientific advancements, but also highlighted the needs of regulators and industry stakeholders, ensuring their perspectives were considered. As a follow up, the outcomes of the meeting are being shared through two publications: a flash meeting report (Fritsche *et al.*, 2024) and an upcoming detailed manuscript that outlines a roadmap for developing tiered testing strategies for regulatory adoption in DART.

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

The assessment of non-genotoxic carcinogens has been considered a regulatory gap. In this context, EURL ECVAM is contributing to the EPAA carcinogenicity project (see **Section 4.3.1**) and is supporting the OECD expert group on the development of an IATA for non-genotoxic carcinogenicity. Both activities focus on approaches based on mechanistic understanding of carcinogenicity. The OECD expert group has so far developed an overarching IATA for non-genotoxic carcinogenicity (Jacobs *et al.*, 2020). Moreover, a number of assays have been evaluated resulting in the publication of two special issues on advances in mechanism-based toxicity and hazard assessment of non-genotoxic chemicals[38,39]. In 2024, the expert group met in Paris to take stock of the progress made and identify next concrete actions. The main outcomes of the meeting included the discussion of a proposed modular framework for evaluating non-genotoxic carcinogens in light of different regulatory frameworks (Louekari and Jacobs, 2024). To facilitate the visualisation of the approach in line with an already accepted strategy, the expert group adapted the OECD IATA scheme to the recently adopted ICH carcinogenicity waiving strategy (ICH, 2022). Additionally, the meeting focused on developing a list of prioritised validation needs and designing experimental validation work. This included a new proposal for the validation of cell proliferation *in vitro* and *in vivo* assays (BrdU and Ki67 methods), support to the new project proposal on the Bhas42 cell transformation assay from Japan, the creation of working groups on chemical selection and exploring GHS classification criteria for non-genotoxic carcinogens.

To better understand the role that *in vitro* mechanistic studies already play in regulatory decision-making for carcinogenicity assessment, EURL ECVAM collected and analysed carcinogenicity data from harmonised classification and labelling (CLH) dossiers, examined by the Risk Assessment Committee (RAC) in view of preparing ECHA opinions on the proposed harmonised classification of substances. The analysis included agrochemicals and industrial chemicals and focused on the constitutive androstane receptor (CAR) mode of action, one of the most common mode of action involved in rodent liver carcinogenicity. The findings indicated that the lack of clear and detailed regulatory guidance for mechanistic studies related to carcinogenicity mode of action evaluation results in significant variability in how experimental data are presented across different submissions. Despite these inconsistencies, a considerable number of *in vitro* mechanistic studies are already contributing meaningfully to the weight of evidence assessments that clarify

38 https://www.mdpi.com/journal/ijms/special_issues/NGTxC
39 https://www.mdpi.com/journal/ijms/special_issues/348DJPZT0M

the human relevance of the CAR MoA in RAC opinions. Consequently, this information can offer valuable insights for harmonising *in vitro* mechanistic studies within this regulatory domain. Developing such guidance would enhance confidence in the use of *in vitro* data for CLH proposals, reduce resources (such as animals, funds, and time), facilitate the RAC's assessment of submissions, guide data generation by applicants, and ensure that these data are effectively integrated into the evaluation process. The project results and accompanying discussions can be found in a technical report published by the JRC (Capeloa *et al.*, 2024).

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

Between 2020 and 2024, the European Commission, through EURL ECVAM, led a project to develop an OECD TG for determining the relative release of metals from various inorganic metal-containing materials in powder or massive forms using a simple simulated gastric fluid at pH 1.5. Throughout the OECD process, the project underwent multiple rounds of commenting and bilateral meetings to address concerns raised by several member countries, including issues related to pH and fluid composition, biological relevance, loadings, saturation, reproducibility, and particle size.

Since August 2022, the project leads worked to address all questions and concerns through bilateral discussions. Despite collective efforts to resolve the issues over the past two years, a consensus could not be reached, and the project was stopped at the April 2024 WNT meeting. The project was moved to Annex 1 of the OECD TG Programme Work Plan (OECD, 2024c).

Further insights into the reasons for the project's discontinuation are detailed in two recent articles published in *Regulatory Toxicology and Pharmacology* (RTP). In their Letter to the Editor, Rasmussen et al. (Rasmussen *et al.*, 2024) expressed concerns regarding the study by Henderson et al. (Henderson *et al.*, 2014), which discussed a round robin on the method, while primarily addressing the OECD initiative aimed at developing the draft TG. Oller et al. (Oller *et al.*, 2024) subsequently addressed[40] all the issues raised by Rasmussen et al.

Additionally, two manuscripts are currently in development. One is undergoing peer review and examines how particle size affects metal release. The other manuscript, which is in preparation, will chronicle the progression of the metal release protocol from the initial round robin testing (Henderson *et al.*, 2014) to its submission to EURL ECVAM and subsequent peer review by ESAC (ESAC, 2020), along with its further revisions during the OECD project. The latest iteration of this protocol can be accessed through the EURL ECVAM Tracking System on Alternative Methods towards Regulatory Acceptance (TSAR)[41].

Figure 4.1. Leaders of the OECD project and experts supporting the project held a working meeting on 10 to 11 September 2024 at JRC. From left to right: João Barroso, Pilar Prieto, Adriana Oller (Oller Consulting LLC, USA), Valérie Zuang (EU National Coordinator), Violaine Verougstraete (Eurometaux, Belgium), and Katherine Heim (NiPERA, USA).

40 The RTP Editors-in-Chief published the Letter to the Editor by Rasmussen et al. (2024) without notifying the authors of Henderson et al. (2014) or providing them an opportunity to respond. The authors of Henderson et al. (2014) learned of the LTE only after its publication and subsequently requested permission from RTP to submit a rebuttal. As a result, the response by Oller et al. (November 2024) was not published alongside the LTE by Rasmussen et al. (August 2024).

41 https://tsar.jrc.ec.europa.eu/test-method/tm2016-02

4.1.5. Progress made for methods relevant to the thyroid hormone system

On behalf of the OECD WNT, the Thyroid Disruption Methods Expert Group (TDM-EG) has advanced its evaluation of data produced by EU-NETVAL laboratories, focusing on methods pertinent to thyroid hormone system disruption. The assessment of an additional method for activation of the Thyroid Stimulating Hormone receptor has been finalised, with the findings reported to the full TDM-EG assembly. New criteria have been established for evaluating proposed methods within the TDM-EG framework. While all identified mechanisms could be significant for disrupting thyroid hormone systems (THSD), current emphasis is placed on methods that assess inhibition of TPO-, NIS-, dehalogenase-, Deiodinase 1, 2 or 3 or MCT-8, as well as binding interactions with TTR or TBG.

The TDM-EG has completed assessments for eleven methods that examine deiodinase-1 activity, thyroperoxidase (TPO) activity, tyrosine iodination by TPO, thyroid hormone transport in serum, inhibition of thyroid hormone glucuronidation, thyroid hormone receptor (in)activation and a method incorporating several modes of action utilising zebrafish eleutheroembryos. The results of these assessments are available on the OECD website (OECD, 2024b). Based on the TDM-EG recommendations, validation efforts continue for two methods assessing deiodinase 1 inhibition and binding to serum protein TTR through PEPPER, aimed at demonstrating their transferability and reproducibility across laboratories. Additional work is required to identify and validate methods specifically targeting NIS, MCT-8 and dehalogenase inhibition. The OECD encourages member countries to submit Standard Project Submission Forms to facilitate the validation process for these mechanisms.

4.1.6. Proposal for a defined approach for estimating human hepatic clearance and plasma protein binding

The properties of Absorption, Distribution, Metabolism and Excretion (ADME) for chemicals have typically been assessed through in vivo studies such as those outlined in OECD TG 417 (OECD, 2010). Recently, there has been a shift towards utilising *in vitro* methods to experimentally assess critical ADME characteristics and to translate these findings into relevant in vivo data via *in vitro*-to-*in vivo* extrapolation (IVIVE) techniques.

In October 2024, EURL ECVAM on behalf of the European Commission, alongside the US EPA, proposed a new project to the OECD WNT. This initiative aims to create a defined approach (DA) for estimating the plasma clearance of orally absorbed chemicals. Specifically, the proposal includes developing test guidelines for evaluating human intrinsic hepatic clearance and plasma protein binding through *in vitro* methods. These parameters will be integrated into a defined approach (DA) that delivers essential human toxicokinetic summaries, such as chemical half-life, steady-state blood concentration, and area-under-the-curve (AUC) concentration profiles.

Data generated from these TGs and the DA could be directly applicable in various regulatory frameworks. Potential applications include prioritisation processes, screening-level assessments for human health, evaluations of persistent, bioaccumulative and toxic / very persistent and very bioaccumulative (PBT/vPvB) properties indicative of human bioaccumulation risks, and weight of evidence analyses. Furthermore, the information produced by these methodologies will contribute valuable insights for advancing Integrated Approaches to Testing and Assessment (IATA), including Next Generation Risk Assessments.

4.2. EC roadmap towards phasing out animal testing for chemical safety assessments

The European Commission is preparing a "Roadmap towards phasing out animal testing for chemical safety assessments" that will serve as a guiding plan for accelerating the path towards replacing, reducing and refining animal testing for the safety assessments of chemicals.

The roadmap was announced in Communication C(2023)5041 (EC, 2023b), which was published as a response to the European Citizens' Initiative (ECI) "Save cruelty-free cosmetics – Commit to a Europe without animal testing". In the Communication, the Commission expressed the commitment to develop a roadmap that "will outline milestones and specific actions, to be implemented in the short to longer term, to reduce animal testing and that would be pre-requisites for a transition towards an animal-free regulatory system". The roadmap is planned to be published in the form of a Communication, in the first quarter of 2026.

The organisation of the roadmap development mainly relies on Working Groups (WGs) with representatives from the Commission and Agencies and with experts from all pieces of chemicals legislation. The three WGs report to a Commission Interservice Group co-led

by DGs ENV and GROW. The following WGs have been established: a) Human Health (led by DG ENV); b) Environmental Safety Assessment (led by DG GROW); and c) Change Management (co-led by JRC and DG GROW).

The Commission has so far organised two conferences on the roadmap towards phasing out animal testing for chemical safety assessments. The first event[42] was held on 11 to 12 December 2023, with three presentations from EURL ECVAM. All presentations were recorded[43], and a meeting report[44] is available.

The second event was held on 25 October 2024, again with active EURL ECVAM participation. The conference webpage[45] provides access to a series of pre-reads, recordings of all sessions, posters and a flash report.

4.2.1. Change Management Working Group

The Change Management WG, co-led by JRC (EURL ECVAM) and DG GROW, complements the WGs on Human Health and Environmental Safety Assessment. The tasks of this WG are to:

- Develop the concept and operational details of transitional initiatives, as a means of promoting transformational efforts and giving them directionality.
- Conduct bilateral stakeholder discussions on aspects of change management as an input to the overall consultation strategy.
- Develop indicators to monitor progress to the goals of the roadmap.
- Propose collaboration models to promote trust among stakeholders and develop confidence in non-animal assessment strategies.

In 2024, the emphasis of the WG has been on developing the concept and operational details of transitional initiatives, and on conducting a series of bilateral stakeholder discussions. These bilaterals are designed to create a safe space to discuss aspects of change management from different stakeholder perspectives. They are not minuted, but the Commission will prepare a report summarising the main themes and messages from the discussions, taken as a whole and without attribution to individuals. This aim is to inspire the roadmap construction and to inform on how to create trust and better collaboration models during the development and implementation of the roadmap.

4.2.2. Transitional initiatives

As an action under the Commission's roadmap towards phasing out animal testing for chemical safety assessments (see **Section 4.2**), the Change Management Working Group developed the concept and operational details of transitional initiatives. A Transitional Initiative is defined as "any initiative contributing directly or indirectly to the replacement or reduction of animal use in regulatory assessments." At the second Commission Conference on the roadmap, EURL ECVAM launched the process of notifying transitional initiatives, which all stakeholders are invited to do via an online survey using the EU Survey Tool[46]. EURL ECVAM will carry out completeness and clarity checks before publishing notified initiatives.

The intention is to build up a dynamic online catalogue of transitional initiatives. The value of such a catalogue will be that it: a) reflects the complexity of the challenge of phasing out animal testing (**Figure 4.2**); b) provides a means of learning from others (how to generate impactful outcomes); c) provides a basis for dialogue and collaboration; d) supports monitoring and forward planning; and e) will become an increasingly valuable resource for large language modelling, which in turn will support the implementation of the roadmap. Publication of a transitional initiative in the JRC Catalogue provides visibility but does not imply endorsement by the European Commission.

42 https://single-market-economy.ec.europa.eu/events/commission-roadmap-phasing-out-animal-testing-chemical-safety-assessments-2023-12-11_en

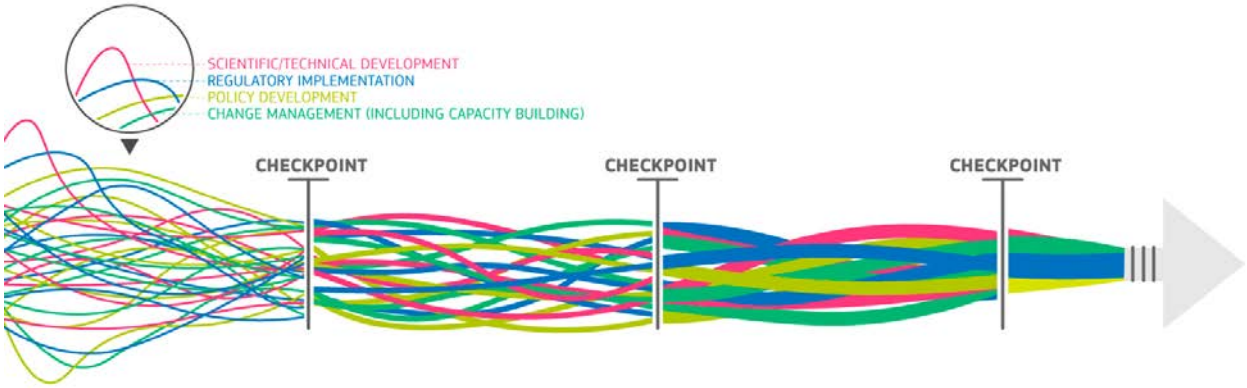
43 https://single-market-economy.ec.europa.eu/presentations-workshop-commission-roadmap-towards-phasing-out-animal-testing-chemical-safety_en

44 https://op.europa.eu/en/publication-detail/-/publication/e350d987-3820-11ef-b441-01aa75ed71a1/language-en

45 https://single-market-economy.ec.europa.eu/events/roadmap-phasing-out-animal-testing-chemical-safety-assessments-second-workshop-2024-10-25_en

46 https://joint-research-centre.ec.europa.eu/reference-measurement/european-union-reference-laboratories/eu-reference-laboratory-alternatives-animal-testing-eurl-ecvam/transitional-initiatives_en

Figure 4.2. Progress toward phasing out of animal testing in chemical safety assessments depends on a complex interplay of different activities. The Commission will take stock of progress of these activities at periodic checkpoints.



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

As part of its agenda-setting efforts in chemicals policy, EURL ECVAM has published several discussion papers aimed at envisioning a future regulatory framework (Berggren and Worth, 2023; Worth and Berggren, 2025). The primary goal is to increase the efficiency and effectiveness of chemical safety assessments, while also aligning with political commitments to phase out animal testing in these assessments. At the core of this proposal is a framework grounded in biological reasoning, where biological inquiries can be addressed through a variety of methods, gradually favouring non-animal approaches. This framework incorporates a tiered testing and assessment strategy that promotes efficiency and effectiveness, while also considering proportionality and cost-effectiveness. Testing strategies, and their respective methods, will be developed concurrently, evaluated based on their outcomes and the protection levels they provide, rather than their capacity to replicate animal test results (Worth *et al.*, 2025). The principle of equal protection was also emphasised in the EPAA Designathon, as detailed in **Section 4.3.2**.

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

The European Partnership for Alternative Approaches to Animal Testing (EPAA) is a collaborative initiative involving eight industry sectors, various Commission services and EU Agencies. Its primary objective is to advance the replacement, reduction and refinement (3Rs) of animal testing by employing more effective and predictive scientific methods to meet regulatory standards.

The EPAA projects, managed by the Project Platform focus on developing NAMs that address significant information gaps and demonstrate their relevance in regulatory decision-making, often supported by case studies. These efforts include exploring future strategies for hazard classification and fostering communication with stakeholders both within the EU and globally. EURL ECVAM co-chairs the Project Platform and participates in several individual projects that are detailed in **Section 4.3.1**, **Section 4.3.2** and **Section 4.3.3.** The status of all five ongoing projects is summarised in the EPAA Annual Report. This year, the Project Platform has begun to strategically redirect several projects towards creating action plans in various toxicology fields to support the EC's roadmap towards phasing out animal testing for chemical safety assessments.

Additionally, the EPAA emphasises knowledge sharing through its annual conference and stakeholder dialogues, benefitting from valuable feedback from its mirror group. The 2024 annual conference aimed to provide insights into the EPAA's achievements in 2024, announce the winner of the EPAA 3Rs Science Prize, and discuss strategies to enhance the adoption of NAMs under current regulations, particularly within EU chemical and pharmaceutical frameworks.

Read more:

EPAA Annual report[47]:
https://single-market-economy.ec.europa.eu/document/download/2612b269-bff4-4060-b19b-d93d2f25f7d2_en?filename=Annual%20report-2023-WEB.pdf

EPAA annual conference[48]:
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. Prediction of the carcinogenic potential of agrochemicals

This project aimed at enhancing the scientific foundation for regulatory testing of agrochemicals and reducing animal usage in carcinogenicity assessments, is nearing completion. The primary goals include (i) consolidating mechanisms of action (MoAs) related to agrochemical carcinogenicity, and (ii) developing a weight-of-evidence approach to predict the carcinogenic potential of agrochemicals without relying on two-year rodent studies, effectively creating a virtual waiver for such assays.

Led by RIVM, this initiative involved an extensive review of data from cancer studies in rodents, revealing that numerous substances caused various tumour types across different organs, often through unknown or known MoAs. The project team engaged in several dedicated meetings to leverage expertise from industry and regulatory members. The findings will be compiled into a scientific manuscript, which will also include the database developed during the project. This research will support the development by a dedicated EPAA team of a new framework for carcinogenicity safety assessment to ultimately contribute to the EC roadmap aimed at phasing out animal testing for chemical safety assessments (see **Section 4.2**).

4.3.2. EPAA New Approach Methodologies Designathon for human systemic toxicity

The EPAA NAM Designathon challenge[49] is focused on an innovative approach to the classification of human systemic toxicity, utilising exclusively non-animal methodologies. The primary objective is to establish a new classification system that ensures the same level of protection as current standards, facilitating equivalent risk management decisions for classified chemicals (Berggren and Worth, 2023; Worth *et al.*, 2025). Details on the concept of equivalent protection can be found in **Section 4.2.3**.

At the core of the Designathon is a classification matrix that categorises chemicals into three levels of concern: high, medium and low. This classification is based on bioactivity and systemic bioavailability data derived solely from non-animal sources. In 2023, 23 teams presented prototype NAM-based solutions during the pilot phase of the challenge, culminating in a workshop held in March 2024 at the JRC (**Figure 4.3**), where participants compared and discussed their initial findings.

The next phase of co-creation started in November 2024, focusing on the areas of chemical space, biological space, and classification strategies identified during the workshop, building on prior work. Three specialised working groups have been formed to tackle each area, supported by a steering team overseeing the entire initiative[50].

This Designathon project aligns with the EC roadmap towards phasing out animal testing for chemical safety assessments outlined in **Section 4.2**.

47 https://ec.europa.eu/docsroom/documents/62494/attachments/1/translations/en/renditions/native
48 https://single-market-economy.ec.europa.eu/events/2024-epaa-annual-conference-maximising-nam-uptake-under-existing-eu-regulations-2024-11-13_en
49 EPAA NAM Designathon for human systemic toxicity: https://single-market-economy.ec.europa.eu/calls-expression-interest/epaa-designathon-human-systemic-toxicity_en
50 Designathon co-creation phase: https://ec.europa.eu/docsroom/documents/61054

Figure 4.3. Designathon workshop held on 20 to 22 March 2024. Representatives of the 23 teams who submitted prototype NAM-based solutions to the Designathon pilot phase and the EPAA organising team attended the workshop.

4.3.3. Environmental safety assessment

The integration of alternative methods to animal testing in environmental safety assessment (ESA) is a priority for JRC. To facilitate this, EURL ECVAM collaborates with research institutions, EU agencies, and international partners, including the OECD, to progress towards a next-generation ESA. A significant part of this initiative involves leveraging NAMs not just to minimise animal testing but also to enhance environmental protection. EURL ECVAM actively participated in the EPAA Partners' Forum held in Brussels in November 2023, which focused on alternatives to animal testing for ESA. The outcomes of this forum are compiled in a report (Tarazona *et al.*, 2025) that reviews NAMs' applications in ESA and highlights collaborative insights among attendees.

Following these discussions, the EPAA established an ESA project team comprising experts who are assessing the current state of non-animal methodologies related to areas such as acute and chronic fish toxicity, bioaccumulation testing, endocrine disruptors, assessments related to birds and mammals, and new approaches for validation. For each of these areas, this team aims to provide expert recommendations for short-, medium-, and long-term strategies that align with the EC roadmap towards phasing out animal testing for chemical safety assessments.

Additionally, the EPAA ESA team is envisioning a new paradigm for ESA that emphasises a shift from traditional testing methods, often limited to single endpoints and species, to a more holistic and integrative approach. This transformation is essential for facilitating the replacement of animal testing with innovative methodologies that can better assess environmental safety.

4.4. Classification and Labelling

4.4.1. CLP Guidance update on new ED hazard classes

The Classification, Labelling and Packaging (CLP) Regulation (EC, 2008) mandates that companies disclose the hazards associated with chemical substances and mixtures they market. This regulation aims to safeguard workers, consumers and the environment from these hazards while facilitating the free movement of substances, mixtures and articles across the EU.

In December 2022, the CLP regulation was revised to introduce new hazard classes specifically for classifying, labelling and packaging (CLP) endocrine disruptors (EDs). These changes took effect on 20 April 2023 (EC, 2023a).

The newly established hazard classes for EDs include:

- Endocrine disruption for human health:
  - → ED HH Category 1: Known or presumed endocrine disruptors.
  - → ED HH Category 2: Suspected endocrine disruptors.
- Endocrine disruption for the environment:
  - → ED ENV Category 1: Known or presumed endocrine disruptors.
  - → ED ENV Category 2: Suspected endocrine disruptors.

Guidance on implementing the new CLP criteria for these ED hazard classes has been developed by ECHA

throughout 2023 and 2024. This process involved contributions from Member States and stakeholders, including EURL ECVAM, through a designated 'Partner Expert Group'. The guidance underwent consultations with ECHA's Committees and concluded with a final review by the European Commission and Member State Competent Authorities. The finalised guidance was published in mid-November 2024 (ECHA, 2024a; ECHA, 2024b).

4.4.2. Inclusion of new hazard classes in UN GHS

Since the CLP Regulation is based on the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS), the EU proposed new hazard classes to the UN GHS Sub-Committee in December 2022, which was accepted. The EU now leads the 'potential hazard issues' informal working group (PHI-IWG) to develop global criteria for various hazard classes.

The PHI-IWG's 2023-24 work plan focuses on hazard classes recently added to the CLP regulation, including, endocrine disruptors for human health and the environment, PBT/vPvB, and PMT/vPvM. It also considers neurotoxicity, immunotoxicity, and terrestrial environment hazards, approved at the 44th UN GHS sub-committee session in July 2023.

The OECD accepted a mandate to review the science needed for classifying and labelling endocrine disruptors based on the IPCS/WHO definition. An ad hoc expert group, co-chaired by JRC until June 2024, began work in September 2023, aiming to respond to the UN GHS's request by late 2024.

OECD submitted reports of the ad hoc group on Human Health, Environment, and the IPCS WHO definition's appropriateness for GHS criteria, considered at the UN Sub-Committee for GHS at their meeting in December 2024[51].

The PHI-IWG[52] is also developing an OECD mandate for 'persistence and mobility' within the new PMT/vPvM criteria and starting work on PBTs/vPvBs and terrestrial environment hazards. Co-leads include:

- Germany - EU for persistence and mobility
- USA - International Council of Chemical Associations (ICCA) for immunotoxicity.

Neurotoxicity and immunotoxicity work may begin in the 2025-2026 biennium, resources permitting.

4.4.3. Revision of the UN GHS chapter on skin sensitization

During the 2023-2024 biennium, the informal working group on the use of non-animal test methods (NATM) concentrated on updating Chapter 3.4 of the GHS. This revision specifically addressed the classification of skin sensitisation for mixtures, following the previous adoption of revised guidelines for substances.

The group aimed to maintain consistency with the recently updated Chapters 3.2 (skin corrosion/irritation) and 3.3 (serious eye damage/eye irritation), which now incorporate non-animal testing methods.

Key revisions include i) comprehensive new text for classifying mixtures with complete data sets and ii) amendments to the background guidance section.

The revised Chapter 3.4 was approved by the Committee of Experts on the Transport of Dangerous Goods and on the Globally Harmonized System of Classification and Labelling of Chemicals in July 2024 and it is expected to be included in the eleventh revision of the GHS, scheduled for publication in 2025.

51 Report of the Sub-Committee of Experts on the Globally Harmonized System of Classification and Labelling of Chemicals on its forty-seventh session, paragraphs 25-27: https://unece.org/sites/default/files/2025-01/ST-SG-AC10-C4-94e.pdf

52 Report of the Sub-Committee of Experts on the Globally Harmonized System of Classification and Labelling of Chemicals on its forty-seventh session, paragraphs 28: https://unece.org/sites/default/files/2025-01/ST-SG-AC10-C4-94e.pdf

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

The informal working group on germ cell mutagenicity, led by EURL ECVAM on behalf of the EU, began its work in 2021. Their primary objectives were to review classification criteria for all categories, update the relevant chapter to reflect current scientific understanding and draft amendments to facilitate hazard classification using non-animal methods (*in vitro* and non-testing methods). In late 2023, the Netherlands proposed changing the hazard class from "germ cell mutagenicity" to "mutagenicity". This proposal aimed to broaden the scope, covering mutation-inducing mechanisms in both germ cells and somatic cells, which could lead to heritable genetic damage and carcinogenicity. The informal working group explored various options for introducing mutagenicity classification into the GHS. They ultimately decided to cover these tasks in a sequential manner:

1. Finalise the current revision on the germ cell mutagenicity chapter.
2. Re-start discussions on introducing mutagenicity classification into the GHS. This introduction could be achieved by either extending the germ cell mutagenicity class to include somatic cell mutagenicity or by creating a separate class for mutagenicity.

The informal working group has made significant updates to the current chapter. Their focus now is on concluding discussions on germ cell mutagenicity criteria, incorporating information from *in vitro* and non-testing methods and developing the guidance section of the chapter.

4.4.5. Work of the Informal Working Group on non-animal testing methods

EURL ECVAM actively supports the UN GHS informal working group NATM, led by the UK and NL. Following the completion of GHS chapter 3.4 on mixtures, the NATM group has begun revising chapter 1.3. This revision focuses on general considerations for classifying hazardous chemicals across all hazard classes. The group aims to discuss the potential inclusion of non-endpoint specific text on non-animal methods in chapter 1.3, as well as the incorporation of non-testing methods.

4.5. Data and knowledge management

4.5.1. AOP Knowledge Base

The AOP Knowledge Base (left part of **Figure 4.4**) is the primary central repository for all AOPs developed either as part of the OECD AOP Development Programme, or by the larger scientific community. The AOP Knowledge Base Tools (right part of **Figure 4.4**) are a collection of web-based resources that are constantly undergoing development and refinement. Together, the AOP Knowledge Base and accompanying tools aim to bring together knowledge and evidence pertaining to how chemicals and other stressors (e.g. radiation, nanomaterials, and viruses) induce adverse effects on humans and ecosystems, shedding light on the step-wise mechanisms of adversity, which opens the door for the identification and application of NAMs to individual events of a particular mechanism.

The AOP-Wiki serves as the primary authoring tool and user interface for submitting AOPs and their building blocks (Key Events and Key Event Relationships) to the AOP Knowledge Base. AOP contributions to the AOP Knowledge Base are being crowd sourced from the international AOP community, which represents many research organisations, including government agencies and academic institutions. Financial support for ongoing maintenance and evolution of the AOP-KB and AOP-Wiki framework has come from EURL ECVAM, the US Environmental Protection Agency (EPA), and Environment and Climate Change Canada (ECCC) and the OECD.

As shown in **Figure 4.5**, the AOP-Wiki is governed by the AOP-KB Coordination Group (AOP-KB CG), which oversees the work auf various working groups dealing with specific topics. EURL ECVAM is active in the AOP-KB CG and all specialty groups:

- The AOP-KB CG group consists of members from AOP-KB funding organisations and is facilitated by EURL ECVAM. The group meets once per month to discuss the latest AOP-KB development and maintenance issues, both short-term and strategic. It ensures that the goals and results from other groups are not conflicting or overlapping. The group liaises with the OECD Advisory Group on Emerging Science and Chemical Assessment (ESCA), and EURL ECVAM presents and reports AOP-KB issues at all ESCA meetings.
- The Society for the Advancement of AOPs (SAAOP) is an international organisation hosting the AOP-KB and discussing issues around the further development of the AOP framework. EURL ECVAM is an active member in this society and represents European interests. SAAOP recently affiliated itself with ASCCT (American

Society for Cellular and Computational Toxicity) and ESTIV (European Society of Toxicology In-Vitro), which increases its visibility and influence.

- The SAAOP Knowledgebase Interest Group (SKIG), closely associated with SAAOP and facilitated by EURL ECVAM, has more than 40 members from research and government organisations around the world. The group meets once per month to discuss AOP-Wiki-related issues. Each meeting features two to three presentations from volunteers on topics relevant to the AOP-Wiki. These presentations help members learn about issues they were previously unaware of, making SKIG an important forum for cross-fertilization and idea exchange.
- The FAIR AOP Cluster is facilitated by EURL ECVAM and brings together various international parties (EU PARC project, US EPA, and many others) interested in making the knowledge stored in the AOP-Wiki more FAIR (findable, accessible, interoperable, and reusable). A first paper published in 2024 (Wittwehr *et al.*, 2024) shows the way forward, and a follow-up publication is in preparation. In collaboration with PARC, a specific FAIR Implementation Profile (FIP) for the AOP domain is currently being developed.
- The Methods2AOP initiative, which was instigated and facilitated by EURL ECVAM, seeks to enhance the integration of test methods, particularly NAMs, into the AOP framework, which is crucial for organising mechanistic knowledge linked to regulatory-relevant adverse outcomes. This effort addresses the current underutilisation of test method information in the AOP-Wiki, hindering its regulatory utility. By involving international stakeholders from various sectors, the initiative aims to improve the quality and regulatory confidence in AOPs by linking detailed test method information to key events (KEs) using standardised ontologies. The initiative proposes a tiered data structure with controlled vocabularies to compile and structure comprehensive data, enhancing regulatory decision-making, visibility, and adoption of NAMs. Ultimately, Methods2AOP strives to create a more robust and transparent system that benefits the scientific and regulatory communities. A journal paper will be published in 2025.
- The AI4AOP effort, which was initiated by EURL ECVAM by the end of 2024, will deal with AI-related issues to explore the possibilities of using Artificial Intelligence to support the key processes in the AOP domain, namely AOP development, assessment and review (as laid out in (OECD, 2018b)), AOP usage and AOP community building. A collective of individuals sharing similar goals will collaborate to harness the capabilities of AI in addressing the challenge of limited specialised expertise necessary for developing a substantial number of AOPs. This initiative began in 2024 with the release of a foundational document (Wittwehr, 2024). The formation of the working group is scheduled for 2025, during which members will identify relevant topics and establish clear objectives and deliverables.
- Omics2AOP is a project that will connect omics and genes to AOPs. The initiative, launched by EURL ECVAM and the Finnish Hub for Development and Validation of Integrated Approaches, seeks to integrate omics data (such as transcriptomics, proteomics, and metabolomics) into the AOP framework. This project addresses the challenge of interpreting omics data in regulatory toxicology by linking genes, proteins, and metabolites to Key Events (KEs) and AOPs using established ontologies. Inspired by previous research that used Natural Language Processing to connect gene sets to KEs and AOPs, the effort aims to enhance data interpretation, support the integration of data streams, and facilitate the development of new *in vitro* assays. The initiative is being prepared for submission to the OECD's Working Party on Hazard Assessment, with feedback and involvement sought from the AOP community.

Figure 4.4. Adverse Outcome Pathway Knowledge Base (AOP-KB) and affiliated tools.

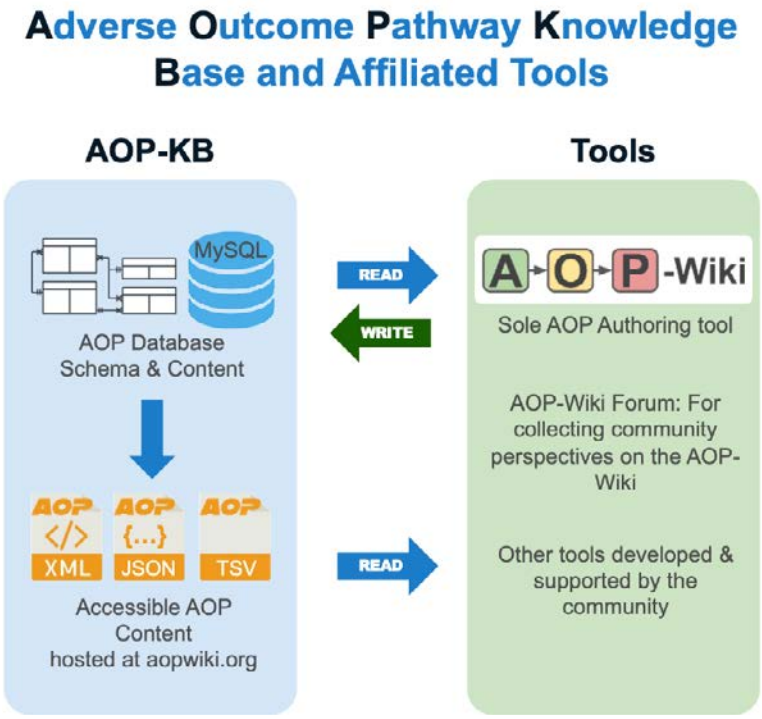
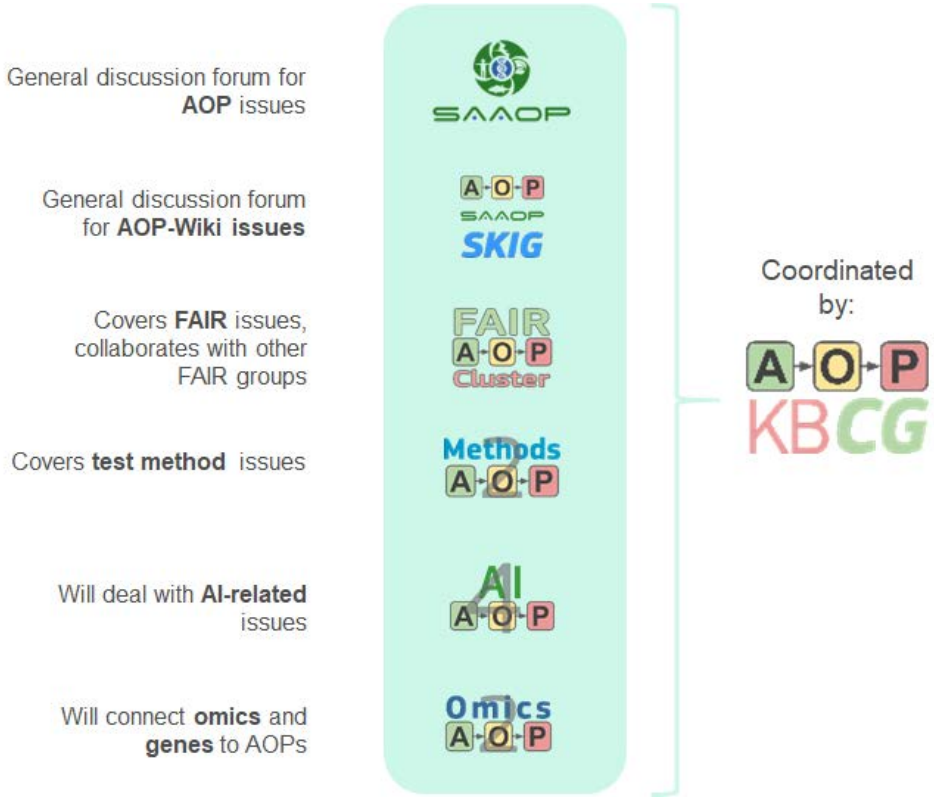


Figure 4.5. Governance of the AOP-Wiki and coordination of related activities by the AOP-KB Coordination Group.



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

Chemical monitoring data can support EU policies in many ways, for example to underpin exposure assessments, monitoring compliance with legal thresholds, evaluating the effectiveness of chemical management measures and to explore temporal and spatial trends in chemical exposure and related risks. The Information Platform for Chemical Monitoring[53] (IPCHEM) contains over 600 million concentration measurements across various media, including water, soil, air, food and humans.

53 https://ipchem.jrc.ec.europa.eu/

In 2024, EURL ECVAM/JRC has developed two important indicators based on IPCHEM data, which are now part of the EU indicator framework for chemicals (EEA, 2024). One indicator reports trends in risks from pesticide residue mixtures in EU soils[54] (Franco *et al.*, 2024), the other one reports trends in potential risks to human health based on mixtures of priority chemicals found in human biomonitoring[55].

To facilitate complex searches and data retrieval across multiple datasets and chemicals, the IPCHEM team has released open source software libraries for programmatic handling of IPCHEM data[56].

4.5.3. Generation, reporting and use of research data for regulatory assessment

The OECD Working Party on Hazard Assessment (WPHA) Expert Group on Research Data, led by EURL ECVAM/JRC, is completing the Guidance Document on the Generation, Reporting and Use of Research Data for Regulatory Assessments. The Guidance aims to bridge the gap between the increasing amount of non-standard research data and the need to consider all relevant and reliable scientific evidence in regulatory assessments.

A webinar hosted by the JRC in January 2024[57], brought together experts from EU funded projects to discuss best practices in generating and reporting research data for regulatory use.

The draft guidance document has undergone extensive review and revision, with input from the WPHA. The document is structured into four main sections:

1. An introduction to the policy challenge and general principles of data quality, scientific and regulatory relevance, and reliability.
2. Existing resources and good practices to increase the utility of research data in regulatory contexts.
3. Structured approaches for assessors to identify, screen, evaluate, and integrate research data, including systematic review methodologies, and tools to evaluate relevance and reliability.
4. Specific recommendations to various stakeholder groups involved in the life cycle of research data.

The document is accompanied by a detailed list of available resources and case studies.

The implementation of the Guidance is expected to enhance regulatory efficiency and coherence across policy domains and jurisdictions, benefiting all OECD Member Countries. Additionally, by optimising the use of existing research data, the Guidance supports the reduction of unnecessary animal testing.

The project team is now finalising the document for publication under the responsibility of the OECD Chemicals and Biotechnology Committee. The final guidance document is anticipated to be published in the first half of 2025.

During 2024, the US EPA in collaboration with the JRC have started a follow-up project under the WPHA work program. The objective of this new project is to develop OECD Harmonised Templates (OHTs) for reporting research data.

4.6. Support of the 3Rs in the pharmaceutical sector – EMA 3Rs Working Party

The European Medicines Agency (EMA) 3Rs Working Party (3RsWP)[58] is a joint working party of the Committee for Medicinal Products for Human Use and the Committee for Veterinary Medicinal Products, advising these committees on all matters concerning the use of animals in the regulatory testing of medicines, with particular focus on the application of the 3Rs principles - replace, reduce and refine[59]. EURL ECVAM acts as observer member of the EMA 3RsWP and has an active role in some strategic initiatives related to the qualification of complex *in vitro* methods for drug development purposes.

The 3RsWP started the revision process of the "Guideline on the principles of regulatory acceptance of 3Rs (replacement, reduction, refinement) testing approaches", aiming at updating it with the latest scientific, technological and regulatory knowledge on 3R testing approaches (EMA, 2023). The guideline will also provide new sections on specific guidance to define the regulatory acceptance criteria for microphsiological systems (MPS), including OoC technologies, for specific contexts of use to be applied in the pharmaceutical area. Two annexes will be added on 1) Liver-on-chip for predicting drug-induced liver injury

54 https://www.eea.europa.eu/en/european-zero-pollution-dashboards/indicators/ecological-risk-of-pesticides

55 https://www.eea.europa.eu/en/european-zero-pollution-dashboards/indicators/risk-of-chemical-mixtures-in-humans

56 https://code.europa.eu/ipchem-toolbox

57 https://joint-research-centre.ec.europa.eu/events/online-webinar-good-practices-and-resources-improve-utility-research-data-regulatory-assessments-2024-01-31_en

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

59 https://www.ema.europa.eu/en/human-regulatory-overview/research-development/ethical-use-animals-medicine-testing#ema-role-11486

and 2) Heart-on-chip for safety pharmacology. Moreover, the text of the guideline will be expanded to include a new section on 3R-related terminology. EURL ECVAM was initially invited to the kick-off multi-stakeholder workshop held in January 2024 to provide expertise on qualification of OoC-based methods (see **Section 3.7.1**) and is now part of the EMA steering committee on the guideline revision as well as the related drafting groups.

EMA offers Innovation Task Force (ITF) briefing meetings to facilitate early dialogue between regulators and developers of new technologies, such as e.g. OoC and stem cells. These meetings provide developers with guidance on their development programs and help them navigate the regulatory landscape. ITF briefing meetings are informal, 90-minute brainstorming sessions where applicants can ask questions and receive initial guidance from European regulators and experts. This service is free of charge and aims to assist developers in determining the next steps for their innovative products, particularly in relation to regulatory decision-making.

"The EMA 3Rs Working Party is committed to fostering the integration of 3Rs testing approaches in the development and evaluation of medicines within the European pharmaceutical regulatory framework. The ongoing revision of our Guideline on the principles of regulatory acceptance of 3Rs testing approaches aims to facilitate the development, qualification, and regulatory acceptance of innovative complex in vitro models, such as microphysiological systems, through the provision of context-of use qualification criteria and clear terminologies. Interaction and collaboration with key stakeholders are seen as key to prioritising contexts of use and harmonising views on qualification requirements, ultimately leading to wider adoption and implementation of these innovative methods. The importance of the global context cannot be underestimated here, and as such the setup of the International Medicines Regulators Working Group on 3Rs constitutes an important step forward."

https://www.ema.europa.eu/en/regulatory-acceptance-3r-replacement-reduction-refinement-testing-approaches-scientific-guideline

https://www.ema.europa.eu/en/documents/other/terms-reference-tor-international-medicines-regulators-working-group-3rs_en.pdf

Sonja Beken

Coordinator Non-Clinical Assessors, Division Assessors - Unit Non-Clinical Assessors

Federal Agency for Medicines and Health Products, Brussels, Belgium

4.7. Use of epidemiological studies in policy evaluations

EURL ECVAM has developed recommendations on the use of epidemiological evidence to support the quantitative characterisation of health impacts in EU policy evaluations (Chinchio *et al.*, 2024). The goal is to promote best practices and more standardised and transparent approaches for collecting, evaluating, and reporting epidemiological data in this context.

Policy evaluations and impact assessments are instrumental tools in the EU policy cycle. They enable policymakers to assess the performance of existing policies and proposed interventions and to identify areas for improvement, in line with the European Commission's ambition to promote evidence-based, transparent, and effective EU law-making[60]. It is crucial that these evaluations are grounded in robust and reliable evidence.

In the context of chemicals, epidemiological evidence provides valuable insights into the potential health impacts of environmental exposures. Its effective use, however, poses several challenges, including the intrinsic difficulties in characterising exposures occurring at very low levels from multiple environmental sources, establishing clear links with diseases, and quantification of the intangible impacts affecting population well-being. EURL ECVAM analysed recent case studies of policy evaluations and impact assessments in the EU chemical policy area, where epidemiological evidence has been used to quantify health impacts. The analysis highlights the importance of methodological quality and reporting

60 https://commission.europa.eu/law/law-making-process/planning-and-proposing-law/better-regulation_en#objectives-of-the-better-regulation-agenda

requirements, including the need for increased transparency and clarity when reporting assumptions, limitations and uncertainties, and to incorporate considerations on equity and vulnerable populations in future assessments.

The work will support policy officers in charge of considering health impacts, as well as contractors in charge of executing studies supporting policy evaluations.

4.8. Assessing the impact of chemicals on biodiversity

Chemical pollution stands as one of the five primary drivers of biodiversity loss. However, establishing a clear, causal link between environmental xenobiotic concentrations and their impact on biodiversity remains challenging. This gap in our understanding stems from key factors:

1. Extrapolation challenge: Most chemical toxicity tests are conducted at the individual organism level, requiring multiple layers of extrapolation to draw meaningful conclusions about biodiversity impacts.
2. Diverse biodiversity metrics: Biodiversity can be assessed at various levels (e.g. species abundance, genetic diversity), making it difficult to directly correlate ecotoxicological endpoints with biodiversity measurements.
3. Environmental risk assessment limitations: Current methods need improvement to better address biodiversity concerns and strengthen the connection between ecotoxicology and biodiversity protection.

To address these challenges, EURL ECVAM is spearheading several initiatives:

- Evidence mapping: Developing a comprehensive summary of ecotoxicological studies to identify chemical exposure effects on biodiversity across different biological organisation levels.
- Data integration: Exploring the combined use of chemical and biodiversity monitoring data to quantify chemical pollution's impact on biodiversity through case studies and potential indicator development.
- New approach methods: In line with "Key Areas of Regulatory Challenge" (ECHA, 2023), monitoring the development of NAMs that offer increased ecological relevance by extrapolating toxicity results to ecosystem levels (or other levels of biological organisation).

Amongst different external initiatives, EURL ECVAM is involved in a task force formed by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC). Its focus is on better understanding biodiversity initiatives and their alignment with current chemical regulations; connecting environmental measurements with laboratory assessments; identifying overarching principles for biodiversity protection and; developing actionable definitions and suitable metrics for various areas of the chemical sector. A workshop is scheduled for 2025 to further advance these efforts and consolidate findings from ongoing research and collaborations.

5. Alternatives in research and education

In 2024, EURL ECVAM has made significant advances in promoting non-animal approaches in research and education. Key initiatives include the development of the BioMedical Models Hub (BimmoH) database, the Student Ambassador Project, and the Virtual Reality laboratory.

5.1. Statistics on the use of animals for scientific purposes for EU and Norway, including re-uses, in 2022

Figure 5.1. Statistics on the use of animals for scientific purposes for EU and Norway, including re-uses, in 2022..

To support the implementation of Directive 2010/63/EU (EU, 2010), EURL ECVAM is responsible for compiling and analysing the data on the use of animals for scientific purposes reported on a yearly basis by the EU Member States and Norway. In 2024, EURL ECVAM prepared two reports covering the years 2021 and 2022, respectively, taking into account the new reporting format introduced by the Commission Implementing Decision 2020/5697/EU.

EURL ECVAM's analysis of the 2021 and 2022 data reveals a complex picture, with both positive and negative trends. In 2021, the total number of animals used for the first time increased significantly to 9.41 million, representing an 18.5% increase compared to 2020. This increase was primarily due to three large-scale projects in Norway and Spain, which contributed to a significant increase in the translational and applied research category. However, the 2022 data shows a reversal of this trend, with a 10.9% decrease in animal use compared to 2021, bringing the total number of animals used for the first time down to 8.39 million. This decrease is consistent with the long-term trend, which indicates a reduction in animal use for regulatory purposes.

Since 2018, the use of animals for regulatory procedures has decreased by 32%, with a notable reduction of over half a million uses. This decline is largely attributed to the continuous efforts to implement alternative methods and reduce animal use in various areas, such as medicinal products for human use, food legislation, and batch potency testing.

The data presented in this report can be consulted at both the Union and Member State levels using the open-access public ALURES Statistical EU[61].

5.2. Biomedical research

5.2.1. Updates on the pilot thematic review on the state of 3Rs implementation in cardiovascular diseases

In 2023, EURL ECVAM initiated a pilot thematic review on the state of 3Rs implementation in cardiovascular research by successfully performing a feasibility study (Zuang *et al.*, 2024). The goal of this review was to accelerate the adoption of non-animal methods, reducing animal use and enhancing research outcomes. In 2024, EURL ECVAM outlined key objectives to achieve this aim. Firstly, EURL ECVAM will review approved cardiovascular research projects using animals, examining non-technical project summaries (NTS) in the ALURES NTS EU database. This work has already started and will be completed by the end of 2024.

EURL ECVAM will also review scientific literature on advanced non-animal models and methods for cardiovascular research. Additionally, it will gather information from stakeholders to understand their experience with non-animal methods. The collected evidence will be integrated and synthesised to assess the utility of non-animal methods and their potential for wider application.

Next, EURL ECVAM will identify prominent investigators, academic groups, and scientific societies in the cardiovascular research community to inform them about the project and gather feedback. It will devise a process for stakeholder consultation and identify experts to conduct the thematic review. By working together, EURL ECVAM aims to increase awareness of animal use in cardiovascular research, identify areas for non-animal method development, and provide reliable scientific knowledge for project proposers and evaluators.

Ultimately, EURL ECVAM's goal is to promote dialogue and collaboration on the 3Rs in the cardiovascular research community. This project will help reduce animal use, enhance research outcomes, and foster a more sustainable and humane approach to cardiovascular research. By sharing knowledge and expertise, EURL ECVAM can drive progress towards more effective and efficient research practices.

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

In 2024, EURL ECVAM continued the development of the BioMedical Models Hub (BimmoH) project to develop an automated database that collects and structures information on non-animal approaches in use for biomedical research, using automated approaches to mine the vast body of published literature.

61 https://circabc.europa.eu/ui/group/8ee3c69a-bccb-4f22-89ca-277e35de7c63/library/051e5787-7746-46cf-8a0d-310f84fd1900/details?download=true

On 7 October 2024, EURL ECVAM organised a workshop to present the current objectives, analysis, and a prototype of the database to a representative panel of stakeholders. Attendees were invited to engage in group discussions to represent different user perspectives (i.e. project evaluators, researchers, Three Rs promoters and industry), providing valuable feedback on the functionality and user experience of the prototype. These discussions focused on identifying user needs, potential enhancements, and challenges in using the database.

The workshop helped to finalise the objectives of the database, allowing the identification of the main components of the user interface as well as the metadata that will be used to search for relevant human-biology based models in specific sectors of the biomedical research. The main search criteria will be the disease category, the human anatomy and the model type. Refinement will be allowed looking for additional information such as keywords from title and abstract, Mesh terms, authors and potentially cell lines.

The database is expected to become available during the second half of 2025 and will 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. Participants of the workshop on the BioMedical Models Hub (BimmoH) project, JRC, Ispra, Italy.

5.2.3. Assessing the relationship between the impact of EU-funded research in Alzheimer's disease, breast and prostate cancer and the methodological approaches used

The EURL ECVAM Status Report 2023 reported on the publication of a technical report on the assessment of the impact of EU-funded biomedical research in the areas of Alzheimer's disease (AD), breast cancer and prostate cancer using fourteen indicators (Zuang *et al.*, 2024). In 2024, this work has been expanded by assessing the relationship between the impact of EU-funded research in AD, breast and prostate cancer and the methodological approaches used. This analysis brought to the publication of a new technical report (Deceuninck *et al.*, 2024b) and a peer-reviewed manuscript (Deceuninck *et al.*, 2024a).

The two documents report on a retrospective analysis of EU-funded research projects on Alzheimer's disease (AD),

breast cancer, and prostate cancer from 1999 to 2019. The analysis aimed to assess the relationship between the societal impact of the research and the use of animal and non-animal- based approaches. The results show that while animal-based methodologies were more prevalent in AD research, non-animal methods were more frequently used in breast cancer and prostate cancer research. Projects focusing on drug development, testing, or repurposing heavily relied on animal models, whereas research on clinical trial design, patient stratification, diagnosis, and diagnostic tool development, lifestyle interventions, and prevention more frequently utilised non-animal methods. The analysis also highlights the importance of human-relevant research strategies, such as human cohorts and population studies, in generating results that are relevant to the original research question and hence translatable. The study suggests that the use of animals in basic and applied biomedical research may be associated with translational failures, and that human-based approaches may be more conducive to societal impact. The findings emphasise the need for a reconsideration of research strategy planning in future framework programs.

5.2.4. Promoting Reusable and Open Methods and Protocols (PRO-MaP)

PRO-MaP
PROMOTING REUSABLE
OPEN METHODS & PROTOCOLS

In 2024, EURL-ECVAM made significant progress in promoting open and reproducible methods and protocols in the life sciences through the PRO-MaP initiative. This effort aimed to increase and improve the reporting of detailed, reusable, and open methods and step-by-step protocols, supporting the EU's open science and valorisation policies.

A comprehensive report outlining recommendations for researchers, research institutions and departments, publishers and editors, and funders was published, providing a roadmap for improving methodological clarity in life sciences publications. The report emphasised the need for a cultural shift to reward and incentivise methods development and sharing of reusable open methods and protocols. It also highlighted the importance of sharing reusable step-by-step protocols, citing them in publications, and promoting responsible use of methodological shortcut citations.

The PRO-MaP initiative has the potential to enhance trust in scientific outputs, improve reproducibility, and increase the uptake of new methods, ultimately contributing to better research outcomes and more effective policy-making. EURL ECVAM will continue to promote the adoption of these recommendations and support the development of better tools for sharing, publishing, and discovering protocols, ultimately advancing the EU's open science agenda.

5.3. Education and training

5.3.1. Introducing the Student Ambassador Project

In 2024, EURL ECVAM launched the Student Ambassador Project, a new initiative targeting university students across Europe. This project aims to disseminate knowledge and raise awareness about non-animal approaches in research and regulatory fields among university students, fostering a network of student ambassadors who can share information with their peers.

The project was introduced to potential student ambassadors as a pilot, building on the results of a previous workshop with Karolinska Institute students to refine its implementation. During the workshop, students were divided into groups to discuss and address questions about the project's objectives, implementation, and potential challenges.

Student ambassadors will receive materials, guidelines, and mentorship to support their work, addressing key aspects identified during the workshop. The project seeks to harness the potential of students to positively influence research and education by promoting non-animal approaches as a more ethical, sustainable, and scientifically sound alternative to animal testing.

With the support of EURL ECVAM and local champions such as professors, lecturers, and researchers, student

ambassadors will be encouraged to organise events, presentations, and discussions to raise awareness at their university. The project also aims to provide students with opportunities for personal and professional growth through connections with peers and mentors at special gatherings. Ultimately, EURL ECVAM's goal is to create a self-sustaining movement that will spread across European universities, increasing awareness about non-animal approaches and inspiring new generations to develop, use, and teach about them.

5.3.2. Engaging with university students: visit from the Karolinska Institute

The second-year students from the Global Master´s Programme in Toxicology of the Karolinska Institute in Stockholm, Sweden, made their traditional study visit to EURL ECVAM accompanied by their professors on 16 May 2024. All students made a two-minute pitch presenting their master thesis, and the colleagues from EURL ECVAM informed about some of their current activities supporting the progress of non-animal approaches both in biomedical research and in regulatory toxicology. The students also visited EURL ECVAM's *in vitro* facility including four main experimental setups used for NAMs including High Throughput Screening, High Content Imaging, Micro-Electrode Array and Organ-on-Chip. They also got the opportunity to experience the newly developed virtual reality, and EURL ECVAM took the opportunity to collect their comments for the finalisation of the project.

5.3.3. Learning from students – The impact of the JRC Summer School

Since 2017, EURL ECVAM organises a biennial Summer School on Non-animal Approaches in Science, aimed at post-graduate students and early-career scientists. The Summer School focuses on sharing knowledge about non-animal methods and technologies in science. It also provides insights into the current and future roles of the Three Rs and explores career paths in non-animal approaches in science. Since its inception, four Summer Schools have trained over 400 post-graduate students worldwide.

To gauge its impact, EURL ECVAM conducted a survey among alumni. The recent report, "Learning from the students - The impact of the JRC Summer School Non-animal Approaches in Science - Survey results" (Ahs Lopez *et al.*, 2024), shows how the Summer Schools have impacted the participants' professional career and choices.

The survey results show the positive impact of the Summer School on the participants' understanding of non-animal methodologies and their relevance in scientific research, and how the Summer School significantly increased their knowledge and skills in the field. Moreover, a greater part of participants felt more confident in discussing non-animal approaches and advocating for their use in their respective fields. Many of them are now contributing to non-animal approaches in their current professional activities by actively developing Non-Animal Methodologies (NAMs) and encouraging others to use NAMs. An overwhelming majority answered that they would recommend young students and researchers to attend future JRC Summer Schools.

In conclusion, the JRC Summer School on Non-animal Approaches in Science is an effective platform for educating and inspiring the next generation of scientists to embrace and promote non-animal methods in their research. The report's findings demonstrate a significant positive impact on the students' knowledge, confidence, and intentions to apply non-animal approaches in their future work.

Figure 5.3. Representation of some of the answers to question 11 of the survey: Would you like to share any success story of achievement that can be attributed to attending the JRC Summer School(s)?

5.3.4. Finalisation of the Virtual Reality laboratory

EURL ECVAM has developed an open-access virtual reality (VR) application to educate students aged 14 to 18 about alternative to traditional animal testing in scientific research. This interactive tool immerses users in a real-life laboratory environment, where they explore cutting-edge non-animal testing methods.

Guided by a virtual professor, users step into the role of researchers and conduct a series of experiments that faithfully replicate typical laboratory procedures. This engaging 20-minute experience provides a fun and effective way to learn core laboratory tasks such as cell culture, microscopy, automation, and chemical assessment and gain basic knowledge of non-animal testing practices.

Accessible to everyone for free, the VR runs on both Oculus Quest 2 and web browsers. Download links can be found on the EURL ECVAM website[62]. This resource complements EURL ECVAM's existing educational resources on the 3Rs for primary and secondary students, seamlessly integrating into classroom lessons.

Figure 5.4. A view of the virtual EURL ECVAM laboratory. Representation of the virtual reality experience as developed by EURL ECVAM.

62 https://joint-research-centre.ec.europa.eu/reference-measurement/european-union-reference-laboratories/eu-reference-laboratory-alternatives-animal-testing-eurl-ecvam/education-and-training/eurl-ecvam-lab-virtual-reality_en).

6. Conclusions

The EURL ECVAM Status Report 2024 outlines key activities necessary to advance the adoption of animal-free methodologies in scientific research and regulatory testing. This shift is motivated by both ethical considerations and scientific needs, addressing the limitations of traditional animal models while leveraging innovative technologies.

Policy initiatives such as the forthcoming European Life Science Strategy (EU, 2024), which includes the European Biotech Act (EC, 2024) alongside the European Commission's roadmap to phase out animal testing in chemical safety assessments, support this transition. The European Life Science Strategy focuses on supporting the EU's green and digital transitions, fostering high-value technologies, and creating a competitive ecosystem for innovation. The European Biotech Act aims to simplify regulatory frameworks and accelerate the commercialisation of biotechnology innovations.

Recent advancements in animal-free methods present opportunities to improve the accuracy, efficiency, and human relevance of toxicological assessments. These approaches account for significant differences between human and animal biology, which often lead to poor translation of results from animal studies to humans.

The development and validation of NAMs are essential for addressing complex toxicological challenges, such as identifying endocrine disruptors and assessing the safety of mixtures and advanced materials. These challenges require mechanism-based approaches that are better suited to advanced *in vitro* and computational methods.

Collaboration among academia, industry, regulatory bodies, and contract testing laboratories is critical for overcoming implementation barriers. Such partnerships promote standardisation of protocols, harmonisation of data interpretation, and increased confidence in these methods among regulators and stakeholders.

To enable widespread adoption of non-animal methods, continued investment in education, training, and infrastructure is necessary. This transition not only reduces reliance on animal testing but also introduces more predictive and efficient approaches to safety assessments and biomedical research.

The growing momentum behind NAMs reflects a consensus on their scientific, ethical, and economic benefits. This progress aligns with global efforts to implement the 3Rs principles and advance relevant science. Bibliometric analyses indicate that publications using non-animal approaches have surpassed those relying on animal models in various fields (Taylor *et al.*, 2024). Additionally, the non-animal testing market is projected to grow significantly faster than traditional methods[63].

Despite these advancements, challenges remain in fully implementing NAMs. These include regulatory hurdles and the need for further confidence-building measures. Initiatives like the World Health Organization's guidelines for phasing out animal tests in biological product quality control address these barriers while emphasising the importance of transitioning away from animal use (WHO, 2024). Coordinated efforts are required to improve validation processes, update regulatory frameworks, invest in training programs, and demonstrate the benefits of NAMs through case studies.

In summary, the EURL ECVAM Status Report 2024 highlights significant progress in advancing non-animal methodologies while acknowledging remaining challenges. Its recommendations align with broader trends in science and policy aimed at fostering innovation in biotechnology while ensuring human health and environmental protection.

63 https://www.understandinganimalresearch.org.uk/fact-checking/factsheets/non-animal-methodologies

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List of abbreviations and definitions

3Rs	Replacement, Reduction, Refinement
3RsWP	3Rs Working Party
AD	Alzheimer's disease
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
AOP-KB	Adverse Outcome Pathway Knowledge Base
AR2	Androgen receptor dimerization assay
ASCCT	American Society for Cellular and Computational Toxicity
ASME	American Society of Mechanical Engineers
ASPA	ASPIS Safety Profiling Algorithm
ASPIS	Animal-free Safety assessment of chemicals: Project cluster for Implementation of novel Strategies (H2020)
ATHENA	Assays for the identification of Thyroid Hormone axis-disrupting chemicals: Elaborating Novel Assessment Strategies (EURION cluster)
AUC	Area-under-the-curve
BimmoH	BioMedical Models Hub
CAR	Constitutive androstane receptor
CEN	European Committee for Standardization
CENELEC	European Committee for Electrotechnical Standardization
CG	Coordination group
CLH	Harmonised Classification and Labelling
CLP	Classification, Labelling and Packaging
CROs	Contract Research Organisations
CYP	Cytochrome P450
DA	Defined approach
DART	Developmental and reproductive toxicity
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)
DNA	Deoxyribonucleic acid
DNT	Developmental neurotoxicity
EC	European Commission
ECCC	Environment and Climate Change Canada
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECHA	European Chemicals Agency
ECI	European Citizens' Initiative
ED	Endocrine disruptor
EDC	Endocrine-disrupting chemicals
EDC MASLD	Investigation of Endocrine-Disrupting Chemicals as contributors to progression of Metabolic Dysfunction-Associated Steatotic Liver Disease (ENKORE cluster)
EEA	European Environment Agency
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ENDOMIX	Understanding how ENDOcrine disruptors and chemical MIXtures of concern target the immune system to trigger or perpetuate diseas (ENKORE cluster)
ENDpoiNTS	Novel Testing Strategies for Endocrine Disruptors in the Context of Developmental NeuroToxicity (EURION cluster)

ENKORE	ENdocrine disrupting chemicals and Knowledge On health-Related Effects' cluster of five research projects from the call HORIZON-HLTH-2023-ENVHLTH-02-03 'Health impacts of endocrine-disrupting chemicals: bridging science-policy gaps by addressing persistent scientific uncertainties'
ENV	Environment
EPA	Environmental Protection Agency
EPAA	European Partnership for Alternative to Animal Testing
ERGO	EndocRine Guideline Optimisation (EURION cluster)
ESA	Environmental safety assessment
ESAC	EURL ECVAM Scientific Advisory Committee
ESCA	Advisory Group on Emerging Science in Chemicals Assessment
ESR	Early Stage Researchers (MSCA-ITN)
eSTAR	Emerging Systems Toxicology for the Assessment of Risk
ESTIV	European Society of Toxicology In-Vitro
EU	European Union
EU-NETVAL	European Union Network of Laboratories for the Validation of Alternative Methods
EURION	European Cluster to Improve Identification of Endocrine Disruptors
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
EUROoCS	European Society of OoC
EU-ToxRisk	An Integrated European 'Flagship' Programme Driving Mechanism-based Toxicity Testing and Risk Assessment for the 21st century
FAIR	Findability, accessibility, interoperability, and reusability (of data)
FGOoC	Focus Group on OoC
FHAIVE	Finnish Hub for Development and Validation of Integrated Approaches
FIP	FAIR Implementation Profile
FREIA	Female Reproductive Toxicity of EDCs (EURION cluster)
GCCP	Good Cell Culture Practices
GHS	Globally Harmonized 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
HESI	Health and Environmental Sciences Institute
HH	Human health
HTP	High-throughput
HTT	High Throughput Testing
HYPIEND	Understanding and preventing the impact of ENDocrine disruptors on the HYpothalamus-PItuitary axis in sensitive populations (ENKORE cluster)
IAP	International Advisory Panel
IATA	Integrated Approach to Testing and Assessment
ICCA	International Council of Chemical Associations
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IMPSS	International MPS Society
IPCHEM	Information platform for chemical monitoring
IPCS	International Programme on Chemical Safety
ISO	International Organization for Standardization
ISO/IEC	International Organization for Standardization/International Electrotechnical Commission
ISSCR	International Society for Stem Cell Research
ISTNET	International STakeholder NETwork
ISW	In Silico World
ITF	Innovation Task Force

IUF	Leibniz Institute for Environmental Medicine
IVB	In vitro battery
IVIVE	In vitro-to-in vivo extrapolation
IWG	Informal working group
JRC	Joint Research Centre (EC)
KE	Key event (AOP)
KER	Key event relationship (AOP)
LC-MS	Liquid chromatography - mass spectrometry
LLMs	Large Language Models
MCT-8	Monocarboxylate 8
MERLON	Merging scientific Evidence with Regulatory practices and Leveraging identification Of endocrine disruptors using New approach methodologies (ENKORE cluster)
MGWM	Mammary Gland Whole Mount
MIE	Molecular Initiating Event
MN	Micronucleus
MoA	Mode of action
MOAs	Mechanisms of action
MPS	Microphysiological systems
MSC	Member State Committee
NAM	New Approach Methodology
NATM	Non-Animal Test Method
NEMESIS	Novel Effect biomarkers for MEtabolic disruptorS: Evidence on health Impacts to answer science and policy needS (ENKORE cluster)
NGO	Non-governmental organisation
NGRA	Next generation risk assessment
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences (US)
NIS	Sodium/Iodide Symporter
NL	the 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
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 characterization methods
PFOA	Perfluorooctanoic acid
PHI	Potential hazard issues
PMs	Physiological maps
PMT	Persistent, Mobile and Toxic
POD	Point of departure

PPARγ	Peroxisome Proliferator-Activated Receptory
PRA	Probabilistic risk assessment
PrecisionTox	Toward Precision Toxicology: New Approach Methodologies for Chemical Safety (ASPIS cluster)
ProbRA	Protocol for probabilistic risk assessment
PRO-MaP	Promoting Reusable and Open Methods and Protocols
qAOP	Quantitative AOP
QIVIVE	Quantitative in Vitro to in Vivo Extrapolation
QSAR	Quantitative Structure Activity Relationship
R&D	Research and development
RAC	Risk Assessment Committee
RAR	Retinoic acid receptor
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)
RNA	Ribonucleic acid
RRI	Responsible Research & Innovation
RS	Reconstructed skin
RSMN	Reconstructed skin micronucleus
RTP	Regulatory Toxicology and Pharmacology
SAAOP	Society for the Advancement of AOPs
SBGN-PD	Systems Biology Graphical Notation Process Description
SETAC	Society of Environmental Toxicology and Chemistry
SG	Sub-Group
SKIG	Society for the Advancement of AOPs Knowledgebase Interest Group
SOP	Standard Operating Procedure
SOT	Society of Toxicology
SWOT	Strength-Weakness-Opportunities-Threats
T4	Thyroxine
TC	Technical Committee
TDM-EG	Thyroid Disruption Methods Expert Group
TG	Test Guideline (OECD)
THSD	Thyroid Hormone System Disruption
TPI	Transition Programme for Innovation without the use of animals
TPO	Thyroperoxidase
TSAR	EURL ECVAM Tracking System on Alternative Methods towards Regulatory acceptance
TSH	Thyroid Stimulating Hormone
TTR	Transthyretin
UISS	Universal Immune System Simulator
UK	United Kingdom
UN	United Nations
US	United States (of America)
VHP4Safety	Virtual Human Platform for Safety Assessment
VPH	Virtual Physiological Human
vPvB	very Persistent and very Bio-accumulative
VR	Virtual Reality
WG	Working group
WHO	World Health Organization
WNT	Working Party of the National Coordinators of the Test Guidelines Programme (OECD)
WoE	Weight of Evidence
WPHA	Working Party on Hazard Assessment (OECD)

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