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EURL ECVAM STATUS REPORT 2021

Non-animal methods in science and regulation

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EURL ECVAM STATUS REPORT 2021

Non-animal methods in science and regulation

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Executive summary

The 2021 EURL ECVAM status report describes research, dvelopment and validation activities, as well as initiatives that promote the uptake and use of non-animal methods and approaches in science and regulation.

In the field of regulatory toxicity testing, EU-funded research projects and research partnerships focus on the development of new approach methodologies (NAMs) for chemical risk assessment and propose new assessment frameworks that do not rely on animal data. The EU-ToxRisk project, funded under Horizon 2020, has shown how NAMs can be used to support chemical grouping and read-across to avoid the generation of new animal data. The EURION cluster of eight individual projects is focusing much of its effort on the development of NAMs for identifying endocrine disruptors. Recently, the ASPIS cluster started its broad range of research activities. It comprises the Ontox, PrecisionTox and RiskHunt3R projects and benefits from about EUR 60 million of EU funds. APCRA, a government-to-government initiative, promotes collaboration and dialogue on the scientific and regulatory needs for the application and acceptance of NAMs in regulatory decision making. Finally, the upcoming European Partnership for the Assessment of Risks from Chemicals (PARC) funded under Horizon Europe, will support the development and implementation of a research and innovation programme to address current and future needs in chemical risk assessment.

In the area of quality control of vaccines, research projects such as VAC2VAC funded under the Innovative Medicines Initiative[1], explore ways to move from the current *in vivo* based quality control strategy to a consistency approach that leads to replacement, reduction or refinement of animal use and shorten the overall release time of vaccine batches.

1 The Innovative Medicines Initiative is a joint undertaking of the EU Horizon 2020 Research and Innovation Programme and the European Federation of Pharmaceutical Industries and Associations (EFPIA).

EURL ECVAM contributes to such activities in a variety of ways, such as by offering expertise and advice, sharing best practices on method characterisation, validation and standardisation.

In line with the European Commission's Chemicals Strategy for Sustainability, part of the EU Green Deal, EURL ECVAM continues to invest efforts to promote the development and international uptake of standardised new approach methodologies and comprehensive knowledge bases on chemicals that support chemicals policies at EU and global level.

Regarding validation and standardisation of methods, EURL ECVAM has evaluated two test submissions based on reconstructed skin models for genotoxicity testing. If promising, the proposed test methods will progress to peer review by the ECVAM Scientific Advisory Committee (ESAC) and subsequently to Organisation for Economic Cooperation and Development (OECD) test guidelines development.

EURL ECVAM consulted its network of regulators PARERE on a method in the field of respiratory sensitisation and sought PARERE input on an overall validation framework for non-animal methods in this critical area. It furthermore explored with PARERE the potential usefulness of organ-on-chip devices for regulatory testing. In order to deploy this new and complex technology efficiently in various areas, standardisation needs have been identified in a "Putting Science into Standards" workshop on standards for organ-on-chip, organised by EURL ECVAM and CEN-CENELEC in April 2021.

EURL ECVAM and its European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) continue the validation of methods targeting different modes of actions relevant to the thyroid signalling pathway. For eight methods, standard operating procedures (SOPs) have been developed and transferability and within-laboratory reproducibility have

been assessed. For another three methods, the mechanistic relevance has been evaluated on the basis of 30 reference chemicals and available *in vivo* data.

In 2021, ESAC completed the peer review of the Genomic Allergen Rapid Detection (GARD) methods for the assessment of the skin sensitisation potential of chemicals. This is the first time a machine-learning algorithm has been independently reviewed for application in the field of regulatory toxicology. The ESAC also initiated the peer review of SENS-IS, another gene expression-based test method, using reconstructed human epidermis, for the assessment of skin sensitisation. Projects to develop OECD test guidelines based on these methods are already on the OECD work plan.

A major breakthrough in 2021 has been the adoption of an OECD guideline that includes three defined approaches (DA) for skin sensitisation that use data derived from combinations of validated chemistry-based and cell-based *in vitro* tests, and in some cases from computer models. These DAs have comparable or even better performance than the Local Lymph Node Assay (LLNA) using mice.

EURL ECVAM continues to support the work under the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) to include criteria for classification based on non-animal methods/approaches. After the successful revision of Chapter 3.3 on serious eye damage and eye irritation, EURL ECVAM is now leading the revision of GHS Chapter 3.4 on skin and respiratory sensitisation in collaboration with the Netherlands and the UK who co-chair the GHS informal working group on the use of non-animal test methods. In addition, EURL ECVAM is leading the work aiming at clarifying the classification criteria for germ cell mutagenicity in category 1B since the current criteria for category 1B have led to diverging opinions between experts when implementing the current GHS.

The Chemicals Strategy for Sustainability calls for extending the information requirements on chemicals under the Registration, Evaluation, Authorisation and Restrictions of Chemicals (REACH) regulation through a better assessment of so-called critical hazards, the introduction of a chemical safety assessment at all tonnage levels and a more extensive use of NAMs. EURL ECVAM is developing several options, which, if agreed, will be subject to an impact assessment in 2022, informing a legal proposal to amend the relevant parts of the REACH regulation. Another action under the Chemicals Strategy, supported by EURL ECVAM, is the improvement of use of academic data in regulatory safety assessments of chemicals under the “one substance one assessment” action.

According to the latest statistics from 2018 on the use of animals for scientific purposes in the EU and Norway, research remains the main area where animals are used for scientific experiments. Almost five million animals were used in basic research and three million in applied and translational research.

In 2021, EURL ECVAM published new reviews of advanced non-animal models in additional biomedical fields, including neurodegenerative diseases and immuno-oncology. EURL ECVAM also explored ways for improving collaboration within biomedical domains and evaluated the output and impact of biomedical EU-funded research.

Progressing its holistic approach for a transformative change, EURL ECVAM also pursued its 3Rs education initiatives by further developing 3Rs education and training resources for primary and secondary school teachers as well as for higher education, and by organising the third edition of its highly successful JRC summer school on non-animal approaches in science, held virtually in 2021.

Abstract

The 2021 EURL ECVAM status report describes research, development and validation activities, as well as initiatives that promote the uptake and use of non-animal methods and approaches in science and regulation.

The principle of the Three Rs, i.e. Replacement, Reduction and Refinement of animal use in basic, applied and translational research, as well as for regulatory purposes is firmly anchored in EU legislation, with full replacement of animal testing being the ultimate goal.

New approach methodologies including a variety of innovative technologies, such as *in vitro* methods using 3D tissues and cells, organ-on-chip, computational models (including artificial intelligence) and 'omics (genomics, proteomics, metabolomics), are developed, evaluated and integrated in assessment frameworks with a view to improve the efficiency and effectiveness of hazard and risk assessment of chemicals and products in a variety of regulatory contexts. Important activities to promote the development and use of non-animal approaches are also pursued in the areas of basic and applied research, where most of the animals are used, as well as for education purposes.

1. Introduction

EURL ECVAM publishes every year a report on the status of non-animal methods in science and regulation. This status report informs about EURL ECVAM activities to support the uptake and use of non-animal methods in the areas of basic, applied and translational research as well as regulatory testing. In addition, it informs about EURL ECVAM's education initiatives on the Three Rs (Replacement, Reduction and Refinement of animal testing).

The Three Rs principle is embedded in all relevant EU legislation, the ultimate goal being full replacement of animal procedures. Already today, if a non-animal method or testing strategy, recognised under Union law is available for obtaining the result sought in a study, it must be used instead of the animal procedure (Article 13 of Directive 2010/63/EU).

EURL ECVAM's mandate is broad and described in Directive 2010/63/EU on the protection of animals used for scientific purposes (Article 48 and Annex II). The duties include guiding and supporting research on alternative methods, coordinating validation on non-animal methods within the EU, disseminating information on the 3Rs[2], promoting stakeholder dialogue and promoting international acceptance.

EURL ECVAM is an integral part of the European Commission's Joint Research Centre.

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

2. Development

Through a range of EU-funded projects and research partnerships, considerable scientific and technical progress is being made on the development of non-animal methods.

EURL ECVAM contributes to such activities in a variety of ways, such as by offering expertise and advice, sharing best practices on method characterisation and standardisation. The overall aim is to identify promising methods and facilitate their progression to practical application and regulatory acceptance.

2.1 Systemic toxicity

2.1.1 EU-ToxRisk

As a contribution to the Horizon 2020 funded project EU-ToxRisk, EURL ECVAM has recently focused on a number of case studies aimed at assessing the regulatory applicability of high throughput new approach methodologies (NAM). The first case study consisted of the proposal of strategies to properly rank substances based on perturbation of *in vitro* human biological systems. The second case study explored an *ab initio* assessment of compounds with little or no prior safety information. The so-called *ab initio* approach aspires to run an entire chemical risk assessment workflow, based solely on data generated *in silico* and *in vitro*, without animal testing. The lessons learned in these case studies will be fed into the drafting of an advisory document.

With EU-ToxRisk nearing its end, EURL ECVAM was challenged with identifying the most innovative outcomes. This exercise has been carried out together with the project collaborators (coordinators and method developers). It was found that the project has developed methods for several critical regulatory endpoints. The innovation aspects of the developed methods range from the possibility to replace an animal test to the increased throughput and better human extrapolation than previous methods. Some of the methods have been implemented within integrated approaches to testing and assessment (IATAs) which have been reviewed by the OECD Working Party on Hazard Assessment (WPHA). The project has also established a strong academic-regulatory network. The main outcomes of the EU-ToxRisk are summarised in Box 2.1.

2.1.2 APCRA

Accelerating the Pace of Chemical Risk Assessment (APCRA) is a government-to-government initiative whose aim is to promote collaboration and dialogue on the scientific and regulatory needs for the application and acceptance of NAMs in regulatory decision making. It was initiated in 2016 and involves participants from the US, Canada, South Korea, Japan, Singapore, Australia, the OECD and the EU.

Two APCRA workshops were organised in 2021 to discuss the ongoing case studies in relation to data gaps and research needs for assessment of human health toxicity and ecotoxicity.

EURL ECVAM is involved in a case study aimed at investigating the qualitative and quantitative concordance of NAMs with traditional animal toxicity tests. The case study is a follow-up to an APCRA retrospective case study (Paul Friedman *et al.*, 2020) that used existing data. This prospective case study consists of two phases. The first phase of the case study resulted in the derivation of chemical points of departure (PoD) based on *in vitro* bioactivity data. The PoDs were adjusted with exposure predictions to develop a bioactivity:exposure ratio (BER) to provide a risk-based indicator. Moreover, the PoDs were compared with PoDs values derived by traditional approaches. In the second phase additional multi-omics data will be generated for a selected list of chemicals as a confirmative step for phase 1. The ultimate object of the case study is to explore the use of PoD derived by NAMs for the purposes of prioritisation and screening level risk assessment.

Box 2.1

Main outcomes of EU-ToxRisk

EU-ToxRisk is a EUR 30 million Horizon 2020-funded project that started in 2016 with a 6-year duration. The overall goal was to steer a paradigm shift in toxicology towards an animal-free, mechanism-based integrated approach to human chemical safety assessment. More specifically, the project aimed to establish: i) pragmatic, solid read-across procedures incorporating mechanistic and toxicokinetic knowledge; and ii) ab initio hazard and risk assessment strategies of chemicals with little background information.

The project addressed repeated dose systemic toxicity (liver, kidney, lung and nervous system) as well as developmental/reproductive toxicity. Different human tiered test systems including high throughput imaging-based test systems and 3D microtissues were integrated to balance speed, cost and biological complexity. All test methods and assays have been documented as detailed standard operation procedures to ensure full transparency and support the assessment of scientific validity. Adverse outcome pathways (AOPs) were central to the project, and advanced technologies, including high throughput transcriptomics, RNA interference, and high throughput microscopy, were applied to provide quantitative and mechanistic underpinning of AOPs and key events (KE). The project has combined *in silico* tools and *in vitro* assays by computational modelling approaches to provide quantitative data on the activation of KE of AOPs. This information, together with detailed toxicokinetic data, and *in vitro-in vivo* extrapolation algorithms should form the basis for improved hazard and risk assessments.

A particular focus of the project has been the application of *in silico* and *in vitro* NAMs for read across-based safety assessment. This work was structured along a broad spectrum of case studies. The results of the read across case studies have been discussed with stakeholders and regulators in workshops to define overall application of NAMs for read across. Moreover, the OECD WPHA has reviewed these case studies in depth and endorsed them for publication as IATA case studies. The learnings of the read across case studies have resulted in an advisory document which describes a strategy for applying NAMs in read across.Additional EU-ToxRisk case studies illustrate how NAMs can demonstrate the absence of an adverse effect. One of these case studies evaluated whether NAMs can differentiate between chemicals with high versus low liability for an adverse effect.

All data from EU-ToxRisk will become available in the public domain through Biostudies. Moreover, the test systems will be sustained in a commercialisation platform SaferWorldbyDesign to support the transition of scientifically valid test methods to applications in chemical safety assessment.

Coordinator: Bob van de Water, Leiden University.

▸▸ *EU-ToxRisk: http://www.eu-toxrisk.eu*

2.1.3 Mechanistic analysis of repeated dose toxicity studies

In a two-year EURL ECVAM-funded study, the Free University of Amsterdam (VU Amsterdam), together with Edelweiss Connect Gmbh, are collecting and analysing toxicological information provided by repeated dose systemic studies. The aim of the study, which kicked off in October 2020, is to "reverse engineer" repeated dose toxicity studies by describing how they capture the key characteristics of repeated dose systemic toxicants (Figure 2.1). A key characteristic is considered as any molecular or cellular event triggered by a chemical that is implicated in the development of an adverse event after repeat exposure.

During the first year, the work focused on gathering information and identifying potential key characteristics of chemicals that induce repeated dose systemic toxicity in target organs such as the liver, kidney, heart and lung. Relevant information on observed effects and target organs was collected through an online public survey and a series of interviews with experts from different product-type sectors and regulatory agencies. This led to insights into how

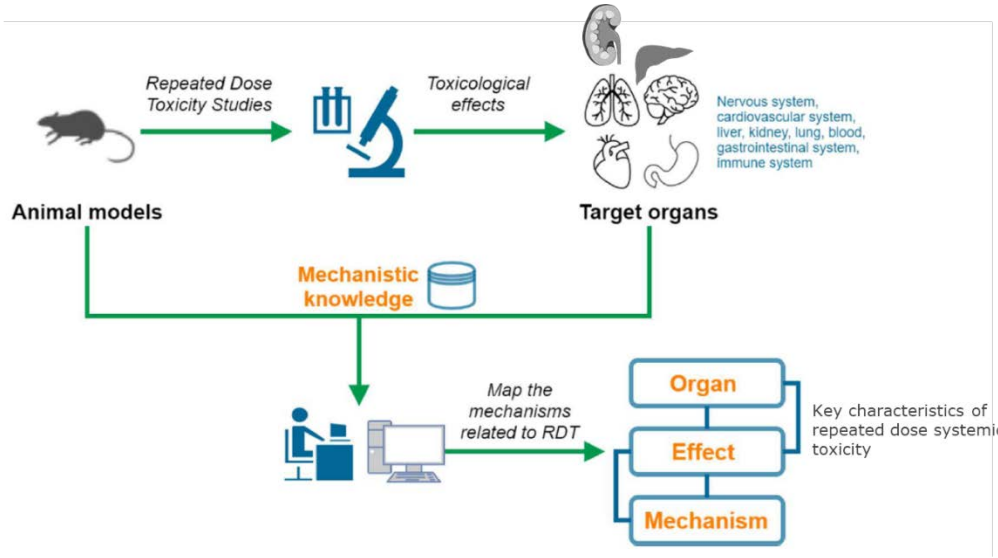

Figure 2.1: Mechanistic analysis of repeated dose toxicity studies: study plan (©Paul Jennings, VU Amsterdam).

industry incorporates alternative approaches when assessing chemicals. In the second year, the collection and curation of data from *in vivo* and *in vitro* studies will continue and will be used to refine and organise the mechanistic evidence in terms of key characteristics.

2.1.4 Making better use of toxicity data by extrapolating across endpoints

With a view to avoiding redundant *in vivo* studies, minimising reliance on apical endpoint tests and devising efficient testing strategies, EURL ECVAM has been exploring opportunities for evaluating hazards by combining information across different systemic toxicity endpoints (Da Silva *et al.*, 2020; Madia *et al.*, 2020; Prieto *et al.*, 2019).

In this context, EURL ECVAM recently developed a methodology and applied it to the carcinogenicity testing scenario, with the specific aim to organise and extrapolate toxicity information based on mechanistic knowledge across multiple sources and endpoints (Madia *et al.*, 2021). As illustrated in Box 2.2, it was possible to identify relevant types of information across different test methods (*in silico*, *in vitro*, *in vivo*) and endpoints, as well as to identify where new testing could be integrated to fill knowledge gaps (Figure 2.2).

In line with the above, EURL ECVAM has contributed to an international effort to review the current status of *in silico* tools for the assessment of each key characteristic of carcinogens and identify the data gaps that need to be addressed before a comprehensive *in silico* carcinogenicity protocol can be developed for regulatory use (Tice *et al.*, 2021).

Box 2.2

Carcinogenicity assessment by combining information across toxicity endpoints

Information provided by test methods across different toxicity endpoints was mapped to the key characteristics of carcinogens (Smith *et al.*, 2016) to build a matrix of toxicological data. Test methods were deconstructed to allow the information they provide to be organised in a systematic way, enabling the description of the toxicity mechanisms leading to the adverse outcome. Further information might be derived by other key characteristics under development (collaborative project lead by University of California, Berkeley, *https://keycharacteristics.org*).

Figure 2.2: Differential contribution of toxicity endpoint studies information to the ten key characteristics of carcinogens. Differential contributions, reported as percentages, of each toxicity endpoint to the properties (key characteristics) of carcinogens, in terms of provided information. Each regulatory toxicity endpoint can be assessed through different types of assays. The information is normalised (percent ratio) over the number of studies available for each single endpoint (each column adds up to 100%). Available toxicity studies for carcinogenicity endpoint (red line) were also organised on the basis of key characteristics. Standard toxicological information can also be enriched with information derived from more recent test protocols and/or investigative studies such as those (green dotted line) describing endocrine disruptor (ED) properties, epigenetic alterations (EpigeneticTox) or toxicity effects detected by high-throughput screening (ToxCast data). From Madia *et al.* (2021), CC BY 4.0.

2.1.5 in3 project

The in3 project, which ran from January 2017 to June 2021, was funded by the EU's Marie Skłodowska-Curie Action - Innovative Training Network (MSCA-ITN) with the aim of driving the development and use of *in vitro* and *in silico* tools for human chemical and nanomaterial (NM) safety assessment.

The project developed a broad range of NAMs including human induced pluripotent stem cells (hiPSC) differentiated into brain, lung, liver, kidney and vascular / blood brain barrier cells, as well as computational models (cheminformatics, AI, QSAR, biokinetic models and quantitative AOPs). The project employed 15 PhD students who carried out these activities in a coordinated and collaborative fashion. A key feature of the project was the emphasis placed on the training and mobility of the researchers, who spent short periods in most of the

beneficiary and partner organisations across Europe, including EURL ECVAM.

On 14 to 15 December 2020, the 6th in3 network meeting was organised virtually, with several EURL ECVAM scientists participating. The final presentations of the researchers were recorded and are available on the in3 project website.

▸▸ In3 project: *https://estiv.org/in3/index.php*

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It was a great privilege to coordinate the in3 ITN project. The main aim of the project was to provide the 15 early stage researchers (ESRs) an interdisciplinary training in the use of new approach methodologies for chemical safety assessment.

We believe we achieved this with our multifaceted in3 training and research program, which included an individual research project, intense research collaboration with the other in3 ESRs and institutes, hands on technical workshops, training workshops, a multi-disciplinary lecture series and secondments to beneficiaries and partner organisations.

The support of all our partner organisations, including the extremely active participation of the JRC, was really helpful and appreciated. While there was excellent research output, the main legacy is the ESRs themselves, whom I have no doubt will make a positive impact in their future careers.

Paul Jennings
in3 Project Coordinator

2.1.6 ASPIS

With a view to developing NAMs for chemical safety assessment, three research projects funded under Horizon 2020 started their activities in 2021, led respectively by the University of Birmingham (PrecisionTox), the Vrije Universiteit Brussels (ONTOX) and Leiden University (RISK-HUNT3R). The three projects have joined forces in the so-called ASPIS cluster ("aspis" means "shield" in ancient Greek), which gathers 70 scientific organisations towards the replacement of animal testing. While the three 5-year projects are complementary to each other, they have common elements that form the basis of collaboration at cluster level. In Box 2.3, the three projects are described by their leaders.

The JRC (EURL ECVAM) has put in place formal collaboration agreements with each of the three projects individually, as well as contributing to cross-cutting activities at cluster level. In particular, thematic working groups have been set up to tackle issues relevant across the cluster, including: 1) risk assessment and chemicals selection; 2) exposure assessment and kinetics, qAOPs of ASPIS target organs; 3) computational approaches to qualify and quantify molecular

Box 2.3

ASPIS – research to accelerate the transition towards non-animal testing

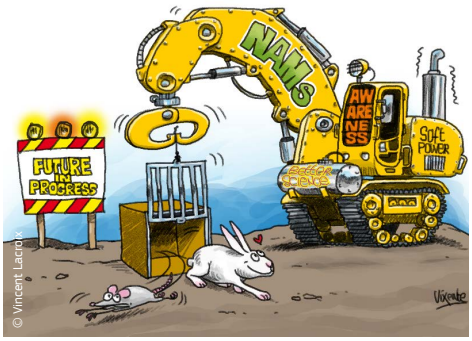
PrecisionTox

Despite having branched off into diverse forms through evolution, animals continue to share many genes that are regulated into functionally conserved pathways. Importantly, genes that govern disease and stress response are among the most likely to be shared among different animals. As a result, instead of using traditional mammal models like rodents, greater accuracy can be achieved by using a diverse range of biomedical model species — fruit flies, water fleas, round worms, and embryos of frogs and zebrafish — along with human cell lines to observe what happens when organisms are exposed to chemicals. Moreover, by using advanced approaches to analysing activity at the molecular level, it is possible to identify the fundamental biological mechanisms by which these organisms and humans respond to toxic chemical exposure.

The overarching goal of PrecisionTox is to propose a new assessment framework, which is based on observable mechanistic processes leading to toxicity, and yet which also factors evolutionary and quantitative genetic models of species-level and individual variation into the prediction of chemical toxicity.

Coordinator: John Colbourne, University of Birmingham.

▸▸ Website: *http://precisiontox.org*



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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.

Specifically, ONTOX will deliver a set of six innovative NAMs to predict systemic repeated dose toxicity effects that will enable human risk assessment, when combined with custom-fit exposure assessment. For proof-of-concept purposes, focus will be put on adversities in the liver (steatosis and cholestasis), kidneys (tubular necrosis and crystallopathy) and developing central nervous system (neural tube closure and cognitive function defects) induced by diverse types of chemicals, such as those from the pharmaceutical, cosmetics, food and biocide sectors.

The six ONTOX NAMs will each consist of a computational system based on cutting-edge artificial intelligence (AI) and will be mainly fed by available relevant biological/mechanistic, toxicological/epidemiological, kinetic and physicochemical data. Data will be collected and integrated in adverse outcome pathway networks and ontology frameworks. Data gaps, as identified by AI, will be filled by targeted *in vitro* and *in silico* testing. The six NAMs will be thoroughly evaluated and applied in collaboration with industrial and regulatory stakeholders to maximise end-user acceptance and regulatory confidence, and thus to expedite implementation in risk assessment practice as well as commercialisation.

Coordinator: Matthieu Vinken, Vrije Universiteit Brussels.

▸▸ Website: *https://ontox-project.eu/*

RISK-HUNT3R

The vision of the RISK-HUNT3R project is to enable a reliable and cost-effective chemical risk assessment, based entirely on non-animal approaches. RISK-HUNT3R has united the expertise of all relevant disciplines and stakeholders to bring non-animal safety assessment to regulatory acceptance. The critical challenges that RISKHUNT3R will address are the (*ab initio*) safety evaluation of chemicals without prior knowledge of their properties, and taking the step from hazard identification to a full risk assessment. The project will develop, validate and implement integrated approaches to next generation risk assessment (NGRA) using innovative, mechanism-based, human-relevant *in silico* and *in vitro* new approach methodologies.

The project is organised into five different modules that focus on: 1) from external to internal exposure; 2) internal metabolism, distribution and excretion; 3) hazard characterisation using high throughput approaches; 4) adverse response characterisation; 5) full risk assessment and IATA development. Through an iterative approach, the project will optimise the strategy to assess exposure and toxicokinetics,

as well as toxicodynamics. Adverse outcome pathway (AOP) network activation and *in vitro* to *in vivo* extrapolation will form the basis of human adversity prediction. The NGRA testing paradigm will be exemplified for acute and chronic health effects relevant to early and later human life stages with the following focus areas: target organ toxicity, developmental neurotoxicity, non-genotoxic carcinogenicity. The resulting NGRA strategy will be evaluated and refined by means of case studies relevant to regulators.

Coordinator: Bob van de Water, Leiden University.

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

initiating events (MIEs) and KEs; 4) 'omics and applications for risk assessment, and 5) common dissemination activities and regulatory impact.

To encourage the regulatory relevance of the research, EURL ECVAM took the initiative to set up the ASPIS Regulatory Forum. The first meetings of the forum were dedicated to the identification of methods that are potentially applicable under extended REACH information requirements, as foreseen in the European Commission's Chemicals Strategy for Sustainability. As described in Section 4.2, the JRC is leading the development of options for extending the REACH information requirements, in close collaboration with ECHA, DG ENV and DG GROW. The forum discussions also served to identify method gaps and methods in need of further evaluation and standardisation - information that will be useful for the further planning and prioritisation of cluster activities and future forum events.

2.2 EURION

The European Cluster to Improve Identification of Endocrine Disruptors, EURION, is a cluster of eight European research projects, aiming to develop new test methods for identification of endocrine disruptors. The cluster was established in order to optimise synergies and avoid overlaps between the projects selected for funding from the Horizon 2020 call SC1BHC-27-2018 'New testing and screening methods to identify endocrine disrupting chemicals'.

Each project focuses on different aspects of new testing and screening methods for ED identification including thyroid hormone disruption, metabolic disruption, female reproduction and developmental neurotoxicity. Transversal working groups share best practice on common issues such as validation and the process of chemical selection, development of AOPs and IATA. The concept behind the cluster is to develop reliable and relevant methods within the context of IATA underpinned by AOPs. Efforts to validate the methods are an essential part of each project.

In January 2021, a thematic workshop was organised in conjunction with the annual meeting of the Cluster. It focused on the development of AOPs and AOP networks and was successful in providing opportunities for initiating collaborations on AOPs across projects. A number of AOPs and AOP networks are emerging from these collaborations and being entered into the OECD's AOP Wiki. In May 2021, the EURION coordinators were also given the opportunity to present their research and candidate methods for test guideline development to the OECD's Endocrine Disrupter Testing and Assessment Advisory Group.

EURL ECVAM continues to support the Cluster by advising on aspects related to validating the methods and translating the methods towards regulatory use.

The projects are now mid-way through their 5-year term and with a possible six-month extension due to the COVID-19 pandemic are expected to deliver their final results in the first part of 2024. The most recent annual meeting of the EURION Cluster was held online from 20 to 21 January 2022.

▸▸ European Cluster to Improve Identification of Endocrine Disruptors, EURION: *https://eurion-cluster.eu*

2.3 PARC

The European Partnership for the Assessment of Risks from Chemicals (PARC) will support the development and implementation of a research and innovation programme to address current and future needs in relation to chemical risk assessment. PARC is a Horizon Europe public-public partnership, co-funded by the European Commission and the Member States with a budget of EUR 400 million and will run for 7 years. More than 200 partners from 28 countries have joined during the preparation of the proposal. The project is expected to start in spring 2022.

PARC will develop innovative methodologies and tools and generate data for exposure, hazard and risk assessment. This will include methods to monitor chemicals in human and environmental matrices as well as tools to increase the efficiency of toxicity testing. PARC will focus on methods such as high throughput *in vitro* test systems, 'omics, high content analysis and computational models. Another aim of PARC is to enable the chemical risk assessment community by supporting networking and providing training programmes to enhance the skills of scientists and risk assessors and managers to apply the new tools and methods.

The JRC (EURL ECVAM) and the PARC consortium intend to put a formal collaboration in place to facilitate very close cooperation and a practical working relationship. Several EU agencies and policy DGs of the European Commission will also be heavily involved in the partnership.

▸▸ Official call - European partnership for the assessment of risks from chemicals (PARC): *https://europa.eu/!qVRJmK*

▸▸ Concept paper (June 2020): *https://ec.europa.eu/info/files/european-partnership-chemicals-risk-assessment_en*

2.4 Quality control of vaccines – VAC2VAC project

The overall objective of the VAC2VAC ("Vaccine batch to vaccine batch comparison by consistency testing") project is to demonstrate proof of concept of the consistency approach for batch release testing of established vaccines. This means that physicochemical, immunochemical, cell-based and/or multi-parametric tests shall be used, instead of animal tests, to ensure that each vaccine batch produced is consistent with a batch already proven to be safe and efficacious.

Recent achievements were presented at the annual meeting that took place in September 2021. Three ELISA methods for potency testing of tick borne encephalitis vaccines (TBEV) have been selected to enter the validation phase. Moreover, the discussion on how to move from the current *in vivo* based control strategy to a consistency approach was continued with VAC2VAC internal and external experts.

Implementation of the consistency approach will lead to replacement, reduction or refinement of animal use and could result in a revision of the monographs for some vaccines. In addition, the consistency approach will shorten the overall release time so that vaccine batches will be available for vaccination much quicker. Global awareness about VAC2VAC is quickly growing thanks to a coordinated approach with international organisations, such as WHO, ICH/VICH, EDQM.

2.5 Applications of modelling

2.5.1 Guidance on physiologically based kinetic models

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

In a modern toxicological framework, PBK models are used alongside *in vitro* methods that characterise the hazardous properties of chemicals (see Box 4.4). By allowing extrapolation from *in vitro* data, this next generation of PBK models provides a means of conducting chemical risk assessments more efficiently and reliably, without testing on animals to measure tissue levels of chemicals. Even though PBK modelling technology is now mature, and has been used in other sectors (notably pharmaceuticals) for decades, the uptake of PBK models in the regulatory assessment of chemicals has been slow. Therefore, to accelerate the acceptance and use of PBK models, the JRC (EURL ECVAM) and the US Environmental Protection Agency (EPA) led an international working group at the OECD to draft a guidance document on the characterisation, validation and reporting of these models (OECD, 2021b).

The OECD guidance document builds on existing international guidance for PBK models, while emphasising aspects relevant for a next generation of models. Key features of the guidance include how to establish the validity of PBK models built using data from non-animal methods (*in vitro* and *in silico*), including in situations where *in vivo* data are lacking (the "read-across approach" to model validation). These features are described in a recent commentary (Paini *et al.*, 2021b). A further publication illustrates the read-across approach to model validation in cases where *in vivo* data are lacking (Paini *et al.*, 2021c). To promote the guidance document, a dedicated webinar was hosted by the OECD on 10 May 2021 and is publicly accessible.

▸▸ Webinar on Physiologically Based Kinetic (PBK) Models: *https://www.oecd.org/chemicalsafety/testing/webinars-on-testing-and-assessment-methodologies.htm*

2.5.2 Top dose selection for repeated dose toxicity studies

The purpose of top dose selection in animal studies is to detect effects for hazard identification. In repeated dose studies, top dose selection has focused on identifying apical endpoints, and determining the maximum tolerated dose (MTD) or using the limit dose. An alternative approach, the kinetically derived maximum dose (KMD), has been proposed as a means of integrating toxicokinetic (TK) data into the dose selection process. The KMD refers to the dose above which the systemic exposures are no longer directly proportional to the external dose. This non-linear external-internal dose relationship arises from saturation or limitation of TK processes, such as absorption or metabolism. Since there have been differing opinions related to the use and application of the KMD concept, a multi-stakeholder working group was formed to address commonly raised scientific and technical issues. The working group has recommended a weight of evidence approach to inform the design and dose selection for repeated dose animal studies (Tan *et al.*, 2021).

2.5.3 EPAA funded projects on toxicokinetics

EURL ECVAM is a "champion" for two projects funded by the European Partnership for Alternative Approaches to Animal Testing (EPAA).

One project, carried out by Liverpool John Moores University, is developing Tools to support application of PBK modelling. The aim is to review the current status of PBK models (for rodents and humans), assess the chemical space coverage of the models, and propose methods to identify 'similar' chemicals (Thompson *et al.*, 2021). The results will help to find suitable analogues for PBK model development/validation as well as gaps in available models.

Another project, carried out by the UK Health and Safety Laboratory, is focused on quantitative *in vitro* to *in vivo* extrapolation (QIVIVE). The aim is to assess the ability of a computational algorithm to convert *in vitro* concentration-response data to *in vivo* dose-response data. Case studies are being developed for three chemicals - perfluorooctanoic acid (Loizou *et al.*, 2021), bisphenol A (Loizou *et al.*, 2022) and chlorpyriphos (manuscript in preparation).

2.5.4 Modelling AOPs and AOP networks

Quantitative AOPs (qAOPs) are toxicodynamic models based on adverse outcome pathways. As a follow-up to the 2019 Lorentz workshop entitled 'e-Resources to Revolutionise Toxicology: Linking Data to Decisions', co-organised by EURL ECVAM together with the Liverpool John Moores University and RIVM, a framework for qAOP development was published (Paini *et al.*, 2021a). The proposed framework, illustrated by three case studies, provides a harmonised approach for both regulators and scientists working in this area. One of the case studies, on developmental neurotoxicity, was further elaborated in a PhD thesis (Spînu, 2021) supported by the in3 project (see Section 2.1.5), and has been published separately (Spinu *et al.*, 2021).

One of the challenges in developing qAOPs is that *in vitro* experiments are typically conducted over short durations with measurements of the toxicological response at a single time point. This limits the usefulness of such data since potential chronic effects that cumulate over time are not usually considered. To address this, EURL ECVAM scientists developed and demonstrated a

methodology that provides a means of conducting a short-term *in vitro* assay and extrapolating the results to characterise chronic toxicity (Macko *et al.*, 2021).

2.5.5 From in silico medicine to in silico toxicology

The universal immune system simulator (UISS) is an *in silico* modelling platform developed by the University of Catania and designed to simulate the human immune system. The UISS has been developed to simulate general immune system behaviour connected to multiple immune system responses, triggered by drugs, viruses, bacteria, tumors, and auto-immune diseases. In a collaboration between EURL ECVAM, the University of Catania, and the University of Milan, the UISS is being explored for its applicability in a different use context - the immunotoxicity risk assessment of chemicals. Initial work is focussing on the ability of the UISS to reproduce the immunotoxic effects of PFOA/PFOS, BPA, and selected skin sentisisers.

2.6 Contributing to methodology development for mixtures

2.6.1 Assessing DNT effects of mixtures

Infants and children are undoubtedly co-exposed to multiple chemicals, which may have deleterious effects on the developing nervous system. In a study published in 2021 (Pistollato *et al.*, 2021b), EURL ECVAM scientists assessed the effects of different chemical mixtures on human-derived neuronal/glial cells. The mixtures contained different combinations of bisphenol A, chlorpyrifos, lead(II) chloride, methylmercury chloride, PCB138, valproic acid, 2,2′4,4′-tetrabromodiphenyl ether (BDE47), ethanol, vinclozolin and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). These chemicals were studied because they have been found in human biological samples, such as breast milk or children's and maternal blood.

After clustering the chemicals into similar or dissimilar modes of neurotoxic action (MoA), it was found that mixtures comprising chemicals of similar MoA caused more dramatic neurotoxic effects, as shown by decreases in synapse formation and electrical activity. Additionally, the use of mathematical modelling allowed an understanding of whether the mixture effects were additive or synergistic (more than additive). Overall, the findings showed that the use of human relevant test systems combined with mathematical modelling represents a suitable testing strategy to assess *in vitro* the possible developmental neurotoxic (DNT) effects induced by chemical mixtures.

2.6.2 Exposure to mixtures of halogenated persistent organic pollutants (POPs) alters neurodevelopmental processes

Halogenated persistent organic pollutants (POPs) are toxic chemicals that are persistent and tend to accumulate in biological organisms, posing risks to health and wildlife. These chemicals may impact brain development, increasing the risk of neurodevelopmental disorders. In collaboration with the Norwegian Institute of Public Health, EURL ECVAM conducted a study aimed at better understanding the effects of POPs on brain development (Davidsen *et al.*, 2021). By using human-derived neuronal/glial cells and a mathematical model, the results of the study showed that repeated exposure to POPs may affect some biological processes critical for human brain development, such

as synapse formation and neuronal differentiation. This suggests that POPs could contribute to learning and memory impairment in children. This is important, since it is known that the prevalence of learning disabilities, autism and attention-deficit hyperactivity disorder, have been increasing over recent years.

2.6.3 Towards a systematic use of effect biomarkers in population and occupational biomonitoring

EURL ECVAM participates in an interdisciplinary network of experts from the European chapter of the International Society for Exposure Science (ISES Europe) and the OECD Occupational Biomonitoring activity (under the Working Parties for Hazard and Exposure Assessment). The aim is to provide an overview of available effect biomarkers for monitoring chemical exposures in the general and occupational populations and to highlight their potential use in monitoring human exposure to chemical mixtures. The outcome of this work, a tiered risk assessment approach for occupational biomonitoring, has been published by Jeddi *et al.* (2021). The EURL ECVAM contribution focused on potential biomarkers for developmental neurotoxicity, the role of AOPs and physiologically based kinetic and dynamic (PBK/D) modelling in strengthening the mechanistic understanding of effect biomarkers.

As a follow up, a new project proposal titled: Using adverse outcome pathways (AOP) to address combined exposures to chemicals with relevant effect-biomarkers has been adopted by the OECD WPHA and OECD Working Party on Exposure Assessment (WPEA) and will start in mid-2022.

EURL ECVAM is also contributing to the drafting of guidance on occupational biomonitoring under the OECD WPHA/WPEA. This project aims at developing guidance for deriving internal dose level associated with no effect based on scientific and regulatory studies. It is expected that the guidance will increase the acceptance of health based human biomarker values and their use in biomonitoring programmes to reduce exposures to workers, ultimately resulting in a reduction in occupational diseases.

2.7 Development of safety assessment workflows

2.7.1 Derivation of PNEC values for environmental risk assessment

In collaboration with the Health and Environmental Sciences Institute (HESI), EURL ECVAM contributed to the development of an ecotoxicity database EnviroToxdatabase (Kienzler *et al.*, 2019) and the development of standardised workflows for the derivation of Predicted No Effect Concentration (PNEC) values for aquatic systems. In environmental risk assessment, the PNEC is compared with exposure levels to determine the level of concern (risk characterisation ratio). However, different regulatory jurisdictions apply 'Application Factors' (AFs) to the most sensitive measured endpoint to derive the PNEC for a chemical. The latest work stemming from this collaboration (Belanger *et al.*, 2021) explores how chemical structures and mode of action information can be used to refine the derivation PNEC values.

▸▸ EnviroTox database: *http://www.envirotoxdatabase.org*

2.7.2 Next generation risk assessment of cosmetic ingredients

Building on the proposed framework (Berggren *et al.*, 2017) for a tiered (also called *ab initio*) approach to next generation risk assessment (NGRA), EURL ECVAM collaborated with Cosmetics Europe to illustrate how this approach can be practically applied, using caffeine as a case study. The NGRA methodology (Bury *et al.*, 2021) illustrates how PBK modelling can be incorporated along with *in vitro* toxicity data to derive an internal margin of internal exposure. This work also illustrates how the uncertainties underlying the NGRA methodology can be characterised in a qualitative manner.

3. Validation

Validation ensures a rigorous science-based evaluation of test methods and approaches, independent of special interests, establishing their overall performance and fitness for a given purpose, i.e. their scientific validity. While science progresses, there is a need to keep on innovating in the way validation is approached. There is a need to be smart on how to split the efforts between validating individual components on one side, and validating the entire integrated approach on the other.

It is also critical to ensure that reliable and relevant datasets are available to benchmark against, and at the same time to find the best way of building confidence in new approach methodologies, e.g. through mechanistic reasoning. Our efforts should continue to ensure that validation studies are assessing methods for a relevant purpose, in particular to deliver the actual information needed for regulatory decisions.

This chapter describes some selected activities directly related to the EURL ECVAM validation process. However, various aspects of validation are evaluated in several of the projects presented throughout the report. In particular, they range from those aiming at developing and optimising new approach methodologies (Section 2) to those contributing to international standards (Section 4).

3.1 Test method submissions

In 2021, EURL ECVAM received two full test submissions on methods for genotoxicity testing using reconstructed skin. A comprehensive list of test method submissions and their status can be found in EURL ECVAM's tracking system for alternative methods towards regulatory acceptance (TSAR; *https://tsar.jrc.ec.europa.eu,* see also Section 3.5).

3.1.1 Genotoxicity testing

In order to improve the predictivity of the genotoxicity *in vitro* test battery, Cosmetics Europe has led the development and validation of *in vitro* human skin-based genotoxicity assays for topically exposed substances, including cosmetics ingredients. An international validation study was carried out specifically for the reconstructed skin (RS) comet assay and micronucleus test (RSMN). Both test methods were initially submitted to EURL ECVAM for consideration to undergo peer review by ESAC, in May and June 2020, respectively. A proposal for the development of new OECD test guidelines for both assays has been already included in the OECD work plan.

The RS Comet Assay is an adaptation of the known alkaline comet assay to address the potential of test items to cause genotoxicity in the form of DNA strand breaks, which can result in clastogenic effects, and DNA lesions leading to gene mutations (Pfuhler *et al.*, 2021b). The method uses specifically the Phenion® Full-Thickness Skin Model, which is composed of primary and p53 competent keratinocytes and fibroblasts of human origin. The RSMN assay applies the known micronucleus test in a three-dimensional reconstructed human skin model and addresses the potential of test items to cause genotoxicity in the form of chromosomal damage (clastogenicity and aneugenicity; (Pfuhler *et al.*, 2021a). The method uses the EpiDerm™ Skin Model, composed of normal human epidermal keratinocytes derived from neo-natal foreskin tissue. Of note, regulatory guidance documents within the pharmaceuticals and cosmetics sectors (i.e., SCCS, ICH) have expressed favourable opinions with regard to the appropriateness of these test methods (ICH, 2011; SCCS, 2021). Thus the results of these methods are sometimes already considered within safety evaluations.

In summer 2020, EURL ECVAM invited the submitter to put forward a full submission for the two test methods, taking into consideration the information provided, the implications of the tests to predict the genotoxic potential of primarily dermally exposed substances and their potential regulatory relevance.

Between March and September 2021, EURL ECVAM received the full test method submissions, for which the assessment is currently on-going.

3.2 Validation studies

EURL ECVAM continued the validation of methods relevant for the thyroid signalling pathway.

3.2.1 Thyroid validation study

The multi-laboratory study aiming at validating a number of *in vitro* methods (OECD, 2014) for different modes of action relevant for the thyroid system

was continued by fifteen laboratories from the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL).

Part 1, aiming to characterise the methods through the development of standard operating procedures (SOPs) and the assessment of transferability and within-laboratory reproducibility was finalised for seven methods (see Figure 3.1). Data sets are available for the reference and positive/negative control chemicals and in some cases for additional chemicals. The assessment of one of the two methods measuring thyroid peroxidase (TPO) inhibition was stopped before the start of Part 1, due to lack of specificity of the method to distinguish TPO from other peroxidases. Part 2, which aims to assess the overall relevance of selected test methods using a set of 30 reference chemicals (selected on the basis of known modes of action and available animal and human study data) was finalised for the human liver microsome-based diodinase 1 (DIO 1) inhibition method (see Box 3.1).

Figure 3.1: Validation status of methods included in the thyroid validation study (ref. date 01/12/2021).

To increase the capacity of data production for 30 chemicals it was recently decided to implement three methods on the robotic platforms in the EURL ECVAM *in vitro* methods facility to perform the part 2 study.

The following methods have entered in the experimental phase for part 2:

- method 2a, thyroperoxidase (TPO) inhibition method based on oxidation of Amplex UltraRed,
- method 3a, thyroxine-binding prealbumin (TTR) binding method using ANSA fluorescence displacement (performed on the robotic platform),
- method 3b, thyroxine-binding prealbumin (TTR) binding method using T4-FITC fluorescence displacement.

For most of the methods the assessment will continue until the planned end of the study in October 2022, by which time the finalised SOPs, data analysis forms, data recording forms, study plans, study reports and assessment reports should be submitted to EURL ECVAM. The intention is to make all the validation

Box 3.1

Principle of the DIO1-SK assay; colorimetric method for assessing deiodinases activity based on Sandell-Kolthoff reaction with human microsomes

Diodinases are important regulators of systemic and local thyroid hormone balance by activation of T4 to T3 and degradation of thyroid hormones via deiodination. DIO1, one of the three isoforms, serves as one main source for circulating T3 via deiodination of T4 in liver, kidney and thyroid and has a role in rescuing thyroid hormone bound iodide from biliary excretion.

Jointly with the original developers (Charité Berlin and BfR, Germany), a non-radioactive approach to determine substance induced DIO1 inhibition based on iodide release activity (Renko *et al.*, 2012) of human liver microsomes was established. Standardisation efforts regarding batch specific enzyme activity further improved the performance of the human liver microsome-based DIO inhibition *in vitro* method.

In the method, human liver microsomes are used as the test system. In the presence of dithiothreitol (DTT), DIO-1 will transform the rT3 into T2 and free iodide. After incubation of the microsomes with the test chemical, the released iodide is separated from the incubation mixture on a Dowex resin filter. The free iodide concentration can be measured with the colorimetric "Sandell-Kolthoff-reaction" reaction, based on the reduction of yellow cerium (IV) to colourless cerium (III). The more iodide that is released, the more the yellow colour is reduced, which can be quantified with optical density (OD) measurements. The inhibition of another enzyme, unrelated to this mechanism of action, provides information on the specificity of the method and interference with enzymatic activity. For potent deiodinase inhibitors, an IC50 value can be measured.

information on the methods available as soon as possible and to engage with the OECD Advisory Group for Endocrine Disruptors Testing and Assessment to explore how the methods could be best used and further optimised within a battery to support thyroid disruption related regulatory assessments. The status of the different methods is reported in TSAR (see Section 3.5).

▸▸ Thyroid methods available on TSAR: *https://europa.eu/!Cp939t*

3.3 Peer reviews conducted by the EURL ECVAM Scientific Advisory Committee (ESAC)

In 2021, ESAC completed the peer review of the Genomic Allergen Rapid Detection (GARD) methods comprised of GARDskin for the assessment of the skin sensitisation potential of chemicals and of GARDpotency for the assessment of skin sensitisation potency (see Section 3.3.1). As far as EURL ECVAM and the ESAC are aware, this is the first time a machine-learning algorithm has been independently reviewed for application in the field of regulatory toxicology. The ESAC also initiated the peer review of the SENS-IS method for the assessment of skin sensitisation (see Section 3.3.2).

The three-year mandate of the ESAC ended on 15 April 2021 but the Committee was authorised by the JRC to complete their reviews of both methods beyond their official mandate. A new open call for applications will soon be launched to renew the ESAC membership. The next Committee will be given a five-year mandate.

3.3.1 GARD

The ESAC Opinion on the peer-review of the GARDskin and GARDpotency *in vitro* test methods for skin sensitisation was endorsed by written procedure on 8 July 2021 (Corsini *et al.*, 2021). The ESAC concluded that the evidence provided

on GARDskin is sufficient and adequate to support its scientific validity. Thus, the ESAC considered that GARDskin is ready to progress to further consideration by the OECD for test guideline development. GARDskin can contribute to skin sensitisation hazard identification in a weight-of-evidence approach. Depending on the regulatory context, positive results obtained with GARDskin may be used on their own to identify skin sensitisers. However, a negative result obtained with this assay may not be sufficient to identify non-sensitisers and should be considered together with additional evidence. Thus, the ESAC did not consider the information currently available on GARDpotency to be sufficient, at present, to recommend its use for regulatory purposes. The use of the GARDpotency assay for discriminating Cat. 1A and Cat. 1B sensitisers is currently prevented by identified issues in reproducibility and predictive capacity due to the design of the validation study. In addition, the ESAC concluded that the evidence supporting the validity of GARDskin and GARDpotency support vector machines (SVMs) is sufficient and adequate.

3.3.2 SENS-IS

The SENS-IS assay is a patent-protected gene expression-based test method using Reconstructed human Epidermis (RhE) proposed to discriminate between skin sensitisers and non-sensitisers. In addition, the test method allows the classification of sensitisers into potency categories on the basis of the concentration of chemical needed to induce a positive response.

In February 2021, the ESAC was mandated to peer review the SENS-IS test method for the assessment of the skin sensitisation potential and potency of chemicals. The ESAC working group that peer reviewed the GARD was also tasked by EURL ECVAM to review the SENS-IS. It met on several occasions during 2021 and had interactions with the test submitter in order to explain certain technical and scientific aspects of the method and the validation dataset. As part of the additional information submitted to the ESAC working group by the method developer, there were new data on the reproducibility of the method generated in laboratories other than those that participated in the original validation study. The working group is considering this additional data and has requested some clarification from the method developer. Further updates on the progress of this ESAC peer review will be communicated through TSAR (see Section 3.5 and Box 3.3) as they become available.

3.4 Meeting of the network for Preliminary Assessment of Regulatory Relevance (PARERE)

The 10th PARERE meeting was held online on 25 November 2020. The purpose of the meeting was to provide updates on 3Rs activities undertaken in EU Member States and by Commission services and EU agencies; to seek input on a possible validation framework for non-animal methods in the area of respiratory sensitisation; and to have an exploratory discussion on organ-on-chip technologies and their potential for translation into the regulatory arena.

The session on respiratory sensitisation had been inspired by the informative feedback received during the PARERE consultation on the ALISENS test presubmission (see 3.1.1 of Zuang *et al.* (2021)). The session continued the discussion

on what elements would be necessary and important for the validation of methods for respiratory sensitisation.

The session on organ-on-chip (OoC) introduced this emerging technology and explored its potential usefulness for testing some complex toxicological or related effects such as non-genotoxic carcinogenicity, developmental neurotoxicity and metabolism. The next steps needed to deploy OoC in the regulatory arena was then investigated (see also Section 3.6). The next PARERE meeting will be held on 17 February 2022.

▸▸ Summary record of PARERE meeting 2020: *https://europa.eu/!yMG6NY*

Box 3.2

The Preliminary Assessment of Regulatory Relevance (PARERE) network

Directive 2010/63/EU requires that Member States nominate a single point of contact to provide advice on the regulatory relevance and suitability of alternative approaches proposed for validation. The PARERE network is trans-sectoral and includes regulators of the EU Member States, representatives from EU agencies such as the European Medicines Agency (EMA), the European Chemicals Agency (ECHA) and the European Food Safety Authority (EFSA), and relevant policy services of the EC. Regulators operating within all sectors of relevance to alternative methods are involved as early as possible in providing a preliminary view on the potential regulatory relevance of methods and approaches submitted to EURL ECVAM for validation or peer review or evaluation.

PARERE members are consulted on several occasions over the year, either on the regulatory relevance of individual methods or approaches that are submitted to EURL ECVAM or on other topics such as e.g. EURL ECVAM Recommendations, standardisation and validation frameworks for novel technologies developed within research projects funded by the EU framework programme for research and innovation. More information on the PARERE network can be found here: *https://europa.eu/!VdP8nB*.

3.5 TSAR

TSAR is a web-based tracking system, which provides the latest information on the status of alternative methods proposed for validation with a view to their use for a regulatory purpose. It contains methods under evaluation by EURL ECVAM and by members of the International Cooperation on Alternative Testing Methods. In 2020 for example, JaCVAM (Japan) submitted 9 methods in the areas of topical toxicity and endocrine disruptors.

TSAR currently contains information on 137 alternative methods that underwent or are currently undergoing a validation process for different topics such as endocrine disruptors, skin irritation, skin sensitisation, genotoxicity or reproductive toxicity.

TSAR has recently been technically revamped to enhance the user experience (see Box 3.3).

▸▸ The nine methods submitted by JaCVAM (Japan) are avaiable on TSAR: *https://europa.eu/!JNHwx7*

Box 3.3

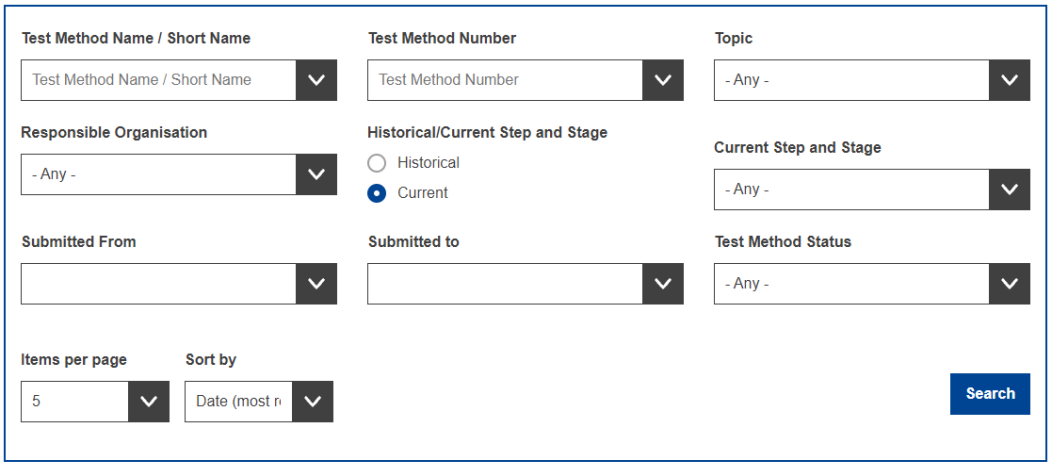
New TSAR layout

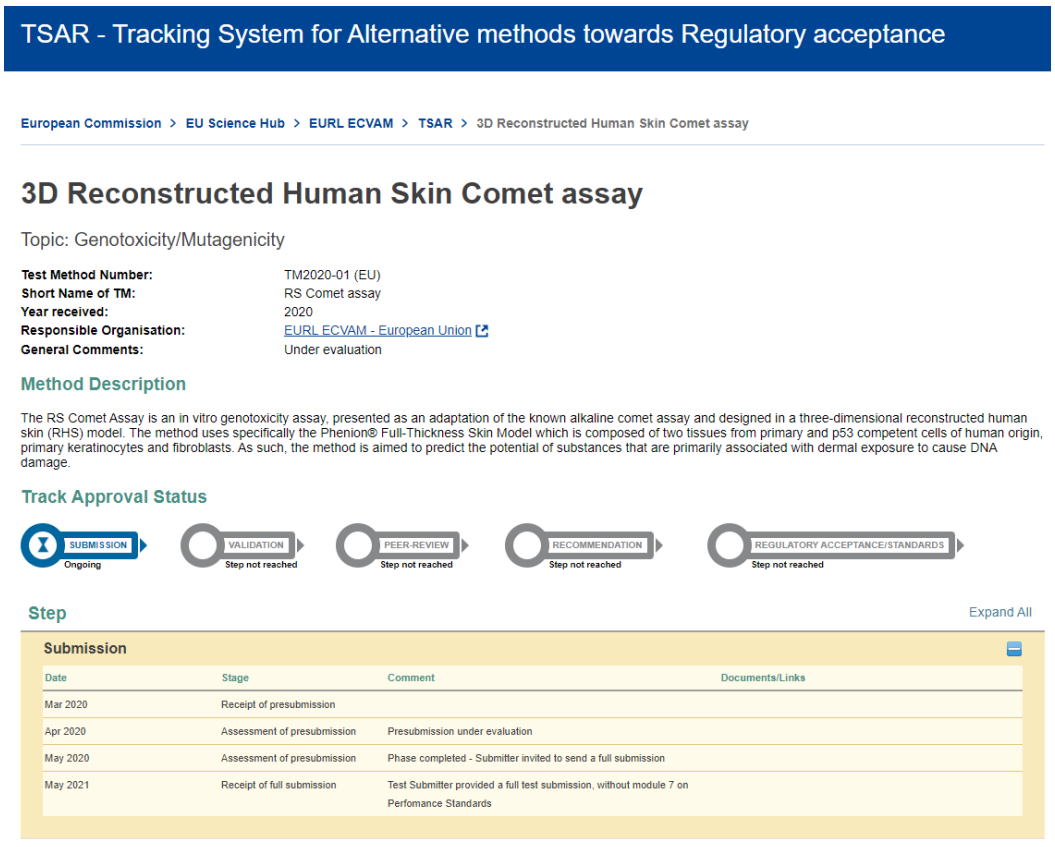
With a new design, TSAR allows for retrieving methods according to different filtering options such as the submitting organisation, the status of the validation or the topic the methods relates to. It is now also possible to know if the validation process of a method is actively on-going (open or closed).

TSAR methods pages are divided into two distinct separate sections:

- **Method description** that provides information related to the method, such as test method (TM) number, title, year submitted for validation, description, and an overview of the tracking status.

- **Method tracking** that provides details on the individual tracking steps and stages. Five pre-defined steps are built into TSAR: submission, validation, peer-review, recommendations, and regulatory acceptance/standard. One or more status steps may be available for each of the pre-defined steps (depending on relevance and position in the validation process). When available, files or links are added to the stages providing additional information.





3.6 Supporting the scientific validity of organ on chip devices

Organ on chip (OoC) devices represent the cutting edge of biotechnologies, combining advanced cell and tissue culture with micro-engineering. They have the potential to revolutionise many fields including biomedical research, drug development and chemical risk assessment. In order to establish OoCs as a credible alternative to animal testing, standardisation plays a key role in characterisation of devices, including benchmarking against appropriate references and supporting communication among stakeholders. An advisory board of the European Organ-on-Chip Society (EUROoCS), chaired by EURL ECVAM, aims at supporting and facilitating the regulatory acceptance of OoC.

Recently, EURL ECVAM analysed the state of the art of standards in OoC (Piergiovanni *et al.*, 2021c) that describes the technical and biological aspects of OoC, focusing on standardisation needs and opportunities. About 90 standards were identified as relevant in related fields, including microtechnologies, medical devices and *in vitro* cell culture.

> *The PSIS workshop on organ-on-chip showed a strong interest from the scientific community to take part in shaping future standardization in the field of OoC. As a result, CEN and CENELEC have decided to create a focus group on OoC that will ensure interaction between all relevant European stakeholders and will define a roadmap for future standardization.*
>
> *Ashok Ganesh*
> *CEN-CENELEC Innovation Director*

To further discuss the role of standards in the OoC domain, the JRC organised the 2021 PSIS (Putting Science into Standards) workshop, together with CEN/CENELEC. The workshop was very well received by both standards experts and scientists and the main outcomes are outlined in a JRC workshop report (Piergiovanni *et al.*, 2021b) and a scientific paper (Piergiovanni *et al.*, 2021a).

> *The successful exploratory PSIS workshop on 'Standardization of organ-on-chip' has been an important step towards community-driven acceleration of the maturation and acceptance of organ-on-chip technology.*
>
> *Next the EUROoCS community of developers, end users and regulators will join forces to define the strategy for bringing standards in the organ-on-chip field, as necessary tools for large-scale application.*
>
> *Janny van den Eijnden-van Raaij*
> *Executive Board Member EUROoCS*

To build a case study on OoC relevance for regulatory purposes, the EURL ECVAM laboratory is now equipped with a commercial device that can integrate multiple model organs on the same chip and connect them with a physiologically relevant fluid flow. EURL ECVAM recently initiated an experimental campaign with such device, combining liver and brain 3D models to study the neurotoxicity of pesticides for DNT applications. This study will also contribute to gaining experience in the different steps necessary to assess the reliability and relevance of an OoC for its intended purpose.

- European Committee for Standardization (CEN): *https://www.cen.eu/about/Pages/default.aspx*
- European Committee for Electrotechnical Standardization (CENELEC): *https://www.cencenelec.eu*

3.7 EURL ECVAM validation resources

Over the past years, EURL ECVAM has collected a number of resources that can be used to facilitate the validation of test methods by applying good practices in the development of new methods. Among these, a library of reference chemicals has been made available.

3.7.1 EURL ECVAM library of reference chemicals

Chemical selection is typically one of the most important and challenging aspect of research projects and validation studies supporting the development and application of alternative methods. Essentially, chemical selection is the basis of an appropriate characterisation, standardisation and qualification exercise. It also enables comparability of methods and models. A chemical selection process requires many resources in terms of time, money and expertise. To facilitate this process by others and to avoid duplication of work, EURL ECVAM has published a new resource titled 'EURL ECVAM library of reference chemicals'. This resource contains chemical lists used in various research and validation projects including JRC, EU-funded, and international projects. Proficiency chemicals from OECD test guidelines and chemicals that have been classified within various regulatory contexts (e.g. pesticides, carcinogenic and endocrine disrupters) are also included. The chemicals in the lists have been extensively characterised and for the most part have been selected by domain experts working within chemical selection groups. The chemical lists are grouped into toxicological-effect categories such as endocrine disruption, skin corrosion, acute toxicity, developmental toxicity and carcinogenicity. EURL ECVAM library of reference chemicals is fully interactive so that the chemical lists can be filtered and searched using keywords. It is also possible to download the whole library as an Excel file. The resource is a non-exhaustive living database and it is updated whenever new reference chemical lists are identified. The EURL ECVAM library of reference chemicals aims to facilitate the work of the scientific community (e.g. academia, research consortia, method developers and contract research organisations) in the area of method development and validation. It enhances the re-use of existing highly curated information and makes better use of valuable resources in the chemical selection process.

▸▸ EURL ECVAM library of reference chemicals: *https://europa.eu/!GW4wjv*

3.8 Training

3.8.1 Training the research community

During the last two years, EURL ECVAM performed a series of training events on GIVIMP (OECD, 2018c) and validation. This training was given on demand and within the existing collaboration between JRC and four EU-funded projects, EURION, PATROLS, REMODEL, and SCREENED. The goal was either to increase the researchers' knowledge on validation or to help them complete the validation related tasks within their projects.

With these training activities, EURL ECVAM helps the research community to better understand and apply the principles of validation in their particular

context. Appropriate material, language and examples familiar to the audience were used.

Each training event had around 40 participants including young and senior researchers. Before each training, the participants were asked to reply to a 6-question survey to help EURL ECVAM understand the test systems used and the general knowledge of the group related to reference and control chemicals, possible limitations of the method, what is needed to transfer a method to another laboratory and how to perform in-house validation. Whenever possible, trainings also included interactive sessions allowing an open discussion. The positive feedback received confirmed that the trainings were useful and well appreciated by the project consortia.

- EURION: *https://eurion-cluster.eu*
- PATROLS: *https://www.patrols-h2020.eu*
- REMODEL: *http://remodelproject.eu*
- SCREENED: *https://www.screened-project.eu*

3.8.2 e-learning modules

In July 2021, Directorate-General for Environment launched six open access e-learning modules mainly based on EU functions under Directive 2010/63/EU on the protection of animals used for scientific purposes. EURL ECVAM supported the development of two of the modules, one of which provides guidance to *in vitro* test method developers and others interested in ensuring the quality of new methods or approaches and in improving the efficiency with which these are developed, tested and optimised (see Section 6.4 in Zuang *et al.* (2021)).

The module covers 1) context and needs for reliable and relevant *in vitro* methods; 2) method development and implementation based on Good *In vitro* Method Practices (GIVIMP), 3) demonstrating the scientific validity of a new method or approach and 4) knowledge assessment.

Students will learn important aspects when designing, optimising and ensuring that the newly developed *in vitro* methods are reliable, relevant and fit-for-purpose. Furthermore, they will get an overview of the different steps and target groups involved in the process of test method development, optimisation and, if applicable, in the validation of new methods and the pathways to regulatory acceptance.

All the e-learning modules can be found on the Education and Training Platform for Laboratory Animal Science (ETPLAS).

- Directorate-General for Environment: *https://ec.europa.eu/environment/chemicals/lab_animals/index_en.htm*
- ETPLAS e-learning modules: *https://etplas.eu/learn*

4. Regulatory application

Effective regulation of chemicals is crucial for health, the environment and commerce. Leading global economies strive to balance the benefits of using chemicals in practically every industrial, agricultural and consumer product, against the potential risks they pose to people and our ecosystem. Striking this balance is also an overarching goal in the European Commission's Chemicals Strategy for Sustainability (EC, 2020) - a central element of the European Green Deal (EC, 2019c) and its 'zero pollution' ambition.

Standardised methods that can reliably measure parameters relevant to a chemical's hazardous properties along with smart knowledge management solutions are essential in order to deliver on this ambition. Efforts to increase and promote the development and uptake of new approach methodologies and comprehensive knowledge bases on chemicals to support chemicals policies at EU and global level are described in this chapter.

4.1 Test methods and integrated approaches to testing and assessment

4.1.1 Defined Approaches for skin sensitisation

In April 2021, the WNT endorsed Guideline n. 497 on Defined Approaches for Skin Sensitisation (OECD, 2021a) (see also Box 4.1). The guideline describes three defined approaches (DAs) that have been shown to provide information that is equivalent to that provided by the Local Lymph Node Assay (LLNA). The DAs use method combinations intended to overcome some of the limitations of the individual, stand-alone methods in order to provide increased confidence in the overall result obtained. The three DAs currently included in the guideline are: 1) the "2 out of 3" (2o3) defined approach to skin sensitisation hazard identification based on *in chemico* (KE1) (OECD, 2021c) and *in vitro* (KE2/KE3) data (OECD, 2018a, b); 2) the integrated testing strategy (ITSv1) for UN GHS potency categorisation based on *in chemico* (KE1) (OECD, 2021c) and *in vitro* (KE3) data (OECD, 2018a), and *in silico* (Derek Nexus), and 3) a modification of the integrated testing strategy (ITSv2) for UN GHS potency categorisation based on *in chemico* (KE1) and *in vitro* (KE3) data, and *in silico* (OECD QSAR Toolbox) predictions. Results generated with these DAs are covered by Mutual Acceptance of Data (see Box 4.1).

A comprehensive dataset of 196 chemicals with DA predictions, data on individual information sources, highly curated LLNA and Human Patch Predictive Test (HPPT) data, and physicochemical properties, was compiled and was used for the evaluation of the DAs. The dataset is chemically diverse in relation to the physicochemical properties covered: it contains small and large molecules, hydrophobic and hydrophilic substances, solids and liquids, volatile and non-volatile substances.

The known limitations and applicability domains of the individual information sources in the DAs were used to design workflows for assigning confidence to each of the predictions produced by the DAs described in the guideline. In order to have a high confidence prediction, the underlying data must meet criteria in the respective test guidelines. DA predictions with high confidence for hazard identification and/or potency are considered conclusive. DA predictions with low confidence are considered inconclusive. These 'inconclusive' predictions may nevertheless be considered in a weight of evidence approach and/or within the context of an IATA together with other information sources.

ECHA has published additional guidance (ECHA, 2021) on how the DAs can be used to fulfil REACH requirements.

4.1.2 Developmental neurotoxicity in vitro assays

Regulatory *in vivo* studies for generating data relevant to developmental neurotoxicity evaluation following the OECD developmental neurotoxicity (DNT) TG 426 are not efficient, since they are costly and use many animals and are therefore not often performed. EFSA, in collaboration with the OECD and DNT academic scientists, has proposed an *in vitro* battery of DNT assays to be included in an AOP-driven IATA, for permitting more efficient and predictive regulatory DNT testing.

Box 4.1

Spotlight on the OECD Test Guidelines Programme and 2021 highlights

The OECD Test Guidelines (TG) for the testing of chemicals are a collection of internationally harmonised and agreed testing methods used by governments, industry[3], research or contract laboratories and academia to assess the safety of chemicals and products. They are primarily used in regulatory safety testing and subsequent chemical notification and registration. The set of test guidelines is updated on a regular basis to keep pace with scientific developments and Member Countries' regulatory needs. OECD-wide networks of national coordinators provide input from scientists in government, academia and industry.

The OECD Test Guidelines Programme, with the Mutual Acceptance of Data (MAD) agreement is the main instrument to ensure a globally harmonised regulatory safety testing of chemicals. This supports an open global market, avoids creating non-tariff barriers to trade for the chemicals industry and supports protection of the safety of workers, consumers and the environment. OECD also considers animal welfare and is committed to the implementation of the 3Rs principles in the development of TG. The MAD system saves governments and industry around 309 million euro each year (OECD, 2019) and thousands of animals by avoiding duplicate testing for different jurisdictions. The programme is overseen by the Working Party of National Coordinators of the Test Guidelines Programme (WNT).

Anne Gourmelon, Principal Administrator of the OECD Test Guidelines Programme provides the highlights of the 33rd meeting of the WNT:

> *The meeting of the WNT was held over 4 days in April 2021 via a web-platform. Eighty members of the WNT representing twenty member countries, the European Commission (incl. ECHA and EFSA), observer countries: the People's Republic of China and the Russian Federation, BIAC and animal welfare NGO representatives.*
>
> *Among the highlights from this year: a new Guideline on Defined Approaches on Skin Sensitisation (DASS) was approved, which contains three defined approaches for hazard identification (1) and potency categorization (2). A defined approach (DA) consists of a selection of information sources (e.g. in silico predictions, in chemico, in vitro data) used in a specific combination, and the resulting data are interpreted using a fixed data interpretation procedure (DIP).*
>
> *The project to develop this guideline was led jointly by the United States (EPA and NICEATM), the European Commission (EURL ECVAM) and Health Canada. It all started in 2017 after a workshop of ICATM partners and regulators. It became obvious that there was much to gain in having agreed defined approaches at the OECD level to maintain the benefits of Mutual Acceptance of Data across countries. The project leads and volunteers from the dedicated Expert Group carried out a deep curation of LLNA and human data, and much work on a supporting document published with the Guideline. This is the first Guideline that contains a combination of methods and in silico tools. This type of guideline is one of the innovative new approach methodologies that can be developed at*

3 At OECD, industry is represented by BIAC, an international business network with a global membership representing over 7 million companies of all sizes.

OECD level for complex endpoints that cannot be recapitulated by single data sources and test systems.

Other new test guidelines were adopted by OECD countries this year:

- *new Test Guideline using fish gill cell line for predicting acute fish toxicity was approved, a potential alternative to the fish acute toxicity test together with other sources of information;*
- *new Test Guideline using zebrafish embryo for detecting chemicals that disrupt estrogenic-receptor mediated activity;*
- *new Test Guideline on phototoxicity testing using a reconstructed human epidermis model.*

Below is a sample of documents approved by the WNT and published in the Series on Testing and Assessment addressing a very diverse set of knowledge and methodologies:

- *A new Guidance Document on honeybee homing flight test following single exposure to sub-lethal doses of chemicals; this document describes a new methodology to evaluate a sub-lethal endpoint; the capacity of the bees to return to their hive after chemical exposure.*
- *A new Guidance Document on leaching of nanomaterials from soil columns, adapted from Test Guideline 312.*
- *A draft Detailed Review Paper on the retinoid pathway disruption impacting several organ systems; this document opens the door to a series of potential new methods for this complex pathway.*

Anne Gourmelon
Principal Administrator of the OECD Test Guidelines Programme

This *in vitro* battery (IVB) of test methods has been proposed based on the recently published EFSA report where the results of testing 119 chemicals is described using neuronal models derived mainly from human stem cells and anchored to key neurodevelopmental processes permitting the evaluation of the impact of a chemical on various stages of brain development (Masjost-husmann *et al.*, 2020).

At the same time a collaborative effort between EFSA, OECD, US EPA and Danish EPA, with engagement of academic and industry researchers, has provided a roadmap to develop an OECD guidance document (GD) for *in vitro* DNT testing which should be finalised at the end of 2021. The GD will focus on use and interpretation of data obtained from the DNT IVB.

To facilitate the preparation of the GD, the EFSA DNT Working Group has developed an AOP-informed IATA as a tool to conduct DNT hazard characterisation

for two pesticides, deltamethrin and flufenacet (EFSA Panel on Plant Protection Products and their Residues *et al.*, 2021). Overall, these case studies showed that the integration of mechanistic data obtained from the DNT-IVB reduces the uncertainty for DNT hazard identification and characterisation. Therefore, the EFSA Panel on Plant Protection Products concluded that the two IATA case studies should be included in the OECD GD on use and interpretation of DNT *in vitro* assays.

4.1.3 IATA for non-genotoxic carcinogens

The assessment of non-genotoxic carcinogens has been identified as a regulatory gap (Jacobs *et al.*, 2016). This prompted the OECD to set up an expert group to develop an integrated approach to testing and assessment (IATA) for non-genotoxic carcinogens.

The expert group has recently agreed on the overarching structure of the IATA, which aims to form a transparent basis to distil, evaluate and organise appropriate assays (Jacobs *et al.*, 2020). It is made up of different modules addressing the main key events in the development of cancer through non-genotoxic pathways.

The evaluation of the assays that address the respective key events is continuing and aims at assessing their readiness for use in the regulatory context, based on defined criteria. The assays are sub-divided into blocks according to cancer hallmarks that address early to mid and later key events. For the assay blocks already evaluated, manuscripts are in preparation.

4.1.4 Metal/metalloid release using a simple simulated gastric fluid

The EC (through JRC / EURL ECVAM) is leading a project for the development of an OECD Test Guideline (TG) for the determination of relative metal/metalloid release using a simple simulated gastric fluid (0.032 M HCl). An expert group composed of regulators, industry and academia is supporting the project (see Zuang *et al.*, 2021). The first WNT commenting round on the draft TG was launched in February 2021. Two expert group meetings were organised in May to discuss several technical aspects related e.g., to exposure time, pH, complexation, the need to set up a repository for reference and proficiency materials, surface area and selection of representative samples and particle size. An updated draft TG was provided to WNT members for commenting at the end of 2021.

The exchange of views on legislative and policy issues related to the use of the relative metal release data continued within the CARACAL sub-group. After the first meeting in 2020, DG ENV identified issues that needed attention in relation to the use of the data for classification and labelling that the group discussed at the second meeting in May 2021. Some Member States highlighted as problematic the lack of *in vivo* data on alloys and of data on the influence of particle size on the relative metal results. The latter was also discussed with the OECD expert group. EURL ECVAM asked for a clear position of Member States regarding the need to generate additional data and *in vivo* testing, since that would have clear consequences on the development of the TG at OECD level.

4.1.5 OECD detailed review paper on the miniaturised Ames test

An OECD Expert Working Group (EWG) on the development of the miniaturised Ames test was established in November 2016 with the aim to conduct a retrospective analysis of data obtained from the different miniaturised versions of the Ames test and a comparative evaluation with Ames results from the classic test method. The inclusion of these versions of the Ames test in an updated version of the TG would allow more regulatory acceptance and integration of genotoxicity testing.

Following an exploratory survey (May 2017) to gain better insights into the use and the amount of data available for the different miniaturised Ames tests and the collection and curation of data (May 2017 and March 2018), the expert group agreed on the drafting of a detailed review paper (DRP).

Chapters of the DRP cover data on performance, dynamic range of each miniaturised version and characterisation of reference data, i.e., chemical space analysis; advantages of various assays; acceptability criteria (linked to the recent work of the International Workshop on Genotoxicity Testing, (Martus *et al.*, 2020; Schoeny *et al.*, 2020); quantitative vs qualitative analysis; and applicability domain.

The document including recommendations from the EWG on the way forward on the applicability of miniaturised Ames assays was circulated for a first WNT commenting round on 3 September 2021. The EWG convened on 29 to 30 November to discuss the outcome of the WNT commenting round, address the comments and prepare a revised version of the draft DRP.

4.2 Updating REACH information requirements: introducing NAMs in REACH information requirements

To support one of the actions under the Chemicals Strategy for Sustainability (CSS) (EC, 2020), EURL ECVAM has been tasked by DG ENV to carry out an exercise to develop options for extending the information requirements under REACH. Aims of the exercise include the better assessment of so-called critical hazards including endocrine disruption, the introduction of Chemical Safety Assessment at all tonnage levels (1 t.p.a upwards), and a more extensive use of NAMs. As a preparatory step, EURL ECVAM conducted a survey from June to October 2021 to evaluate the state of play in the practical implementation of NAMs for chemical safety assessment. The options eventually agreed by the Commission services will be subject to an impact assessment in 2022, which will inform a legal proposal to amend the relevant parts of the REACH regulation.

At the same time in response to a further action under the CSS the Commission has developed two different options for strengthening standard information requirements across REACH annexes VII-X in order to improve the possibilities to identify endocrine disrupting properties of registered substances. Both options include *in vitro* mechanistic assays providing information on endocrine activity at the lowest tonnage level (1 t.p.a.)

upwards with increasing requirements for ED-relevant information from *in vivo* assays with either increasing tonnage or as a follow up to positive results. An impact assessment of the regulatory options is currently ongoing, including a consultation to gather the views of key stakeholders (closed 15 October, 2021) which will again inform a proposal for an amendment to REACH by the end of 2022. The information generated according to these requirements will be the basis for the application of the criteria for hazard classes for endocrine disrupting chemicals to be introduced into the CLP Regulation (see Section 4.4.1).

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

A new EPAA project aiming to provide a cross Industry/EC environment for appraisal of the current use of new approach methodologies (NAMs) for decision-making was launched early September 2021. The project builds on discussions among EPAA members in May 2021, which foresees to define the needs to increase the confidence to use NAMs more routinely for chemicals registration in the EU, especially in the context of the EU Chemicals Strategy for Sustainability (see Box 4.2).

The intent is to explore whether:

a) there are circumstances where NAMs could be used for safety assessments in different chemical sectors to provide information for the classification and labelling of ingredients in the EU – regardless of the tonnage – across different safety endpoints;
b) NAMs could be used to provide alternate DNELs (derived no-effect levels of exposure) for decision-making in a different way from traditional toxicology testing whilst still providing robust information on safety;
c) a and b could contribute significantly and help improve the speed of assessment under the EU Chemicals Strategy for Sustainability, particularly in the context of “one substance – one assessment”.

Thus, the project focus is on actual experience of EPAA partners in the use of NAMs for decision-making and exchange of this experience between the industry sectors and Commission partners in EPAA.

The project kicked off with a ‘deep-dive’ online workshop on NAMs held on 23-24 November 2021. Different groups currently using NAMs for various regulatory purposes shared their experiences through relevant case studies that provided the basis for a useful exploration of the topic. Presentations and dynamic discussions helped to identify the opportunities and challenges that policy makers and NAM users face today and the main areas for follow-up actions. The input from the workshop will help shape the project activities for 2022 and beyond. A workshop report will be delivered in the form of a publication.

The project steering team aims to find ways to establish a regular contact with other relevant projects such as PARC and ASPIS to exchange views and coordinate the work in order to be complementary.

▸▸ Flash report: EPAA Deep-Dive Workshop on «Use of New Approach Methodologies (NAMs) in Regulatory Decisions for Chemical Safety»: *https://ec.europa.eu/docsroom/documents/48034*

Box 4.2

EPAA promotion of the regulatory acceptance of alternative methods

The European Partnership for Alternative Approaches to Animal Testing (EPAA), a partnership between seven industry sectors and different Commission services, aims to replace animal testing by innovative, non-animal testing methods, to reduce the number of animals used and to refine procedures where no alternatives exist or are not sufficient to ensure the safety of substances (the ‹Three Rs principle›). The partners bring together their knowledge and resources to accelerate the development, validation and acceptance of alternative approaches at European and global level.

At the core of the EPAA work are the scientific projects that cover different areas. EURL ECVAM co-chairs the project platform and also co-chairs a number of the individual projects within the platform itself. In 2021, the project platform has supported ten project teams, which synergistically combine the expertise and collaboration available across industry sectors, academia, NGOs and regulatory agencies.

A detailed progress update can be found in the EPAA annual report. In the past year, a new project on new approach methodologies (NAMs) in regulatory decisions for chemical safety in the EU was initiated with the organisation of a deep-dive NAMs workshop (Section 4.3). Two additional workshops have been held related to clostridial vaccines (March 2021) and monoclonal antibodies (April 2021). In the field of skin sensitisation, three users fora took place to discuss a number of case studies on “How defined approaches (DAs) can be used to create a weight of evidence for skin sensitisation potential and potency assessment in absence of *in vivo* data”.

This year’s EPAA annual conference focused on “How can EPAA help the successful implementation of the EU Chemicals Strategy for Sustainability”. It also aimed at presenting EPAA’s achievements, as well as the 2021 Refinement Prize.

- EPAA annual report: *https://europa.eu/!KJfy3g*
- EPAA annual conference: *https://europa.eu/!hdtGfb*

4.4 Classification and labelling

4.4.1 Inclusion of endocrine disruptors in the CLP Regulation

A further action in the Commission’s Chemicals Strategy for Sustainability states that “*the Commission will […] propose to establish legally binding hazard identification of endocrine disruptors, based on the definition of the WHO, building on criteria already developed for pesticides and biocides, and apply it across all legislation*”. It is proposed to do this through amending Regulation (EC) No 1272/2008 on hazard classification, labelling and packaging of chemicals (the CLP Regulation). In March 2021, the Commission presented an initial draft proposal to gather further inputs from the experts of the CARACAL sub-group on endocrine disruptors. The proposal outlines criteria for a new hazard class for human health and one for the environment including categories; Category 1: Known or presumed endocrine disruptors and Category 2: Suspected endocrine disruptors. In addition, new labelling elements will be included (Hazard and Precautionary statements) potentially combined with existing ones.

An impact assessment will be carried out to identify and assess the impact of the proposal on the protection of human health and the environment, at the same time as considering the economic costs, impact on the internal market and other social impacts[4].

4 *https://ec.europa.eu/info/law/better-regulation/have-your-say/initiatives/12975-Revision-of-EU-legislation-on-hazard-classification-labelling-and-packaging-of-chemicals_en*

4.4.2 GHS guidance on severe eye damage/irritation

An important activity under the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) towards replacement of animal testing is the work carried out by the Informal Working Group (IWG) on the use of Non-Animal Test Methods (NATM) for classification of health hazards, which is co-chaired by the Netherlands and the United Kingdom (see also Box 4.3). In the last biennium (2019-2020), the IWG NATM worked on the revision of Chapter 3.3 on serious eye damage and eye irritation, under the lead of EURL ECVAM and the IWG co-chairs, to include criteria for classification based on non-animal methods/approaches. The work was completed in the second quarter of 2021 and the final revised chapter was adopted by the subcommittee of experts on the GHS during its 40th session in July 2021. The resulting text will be included in the 10th revision of GHS to be published in 2023 but the approved revisions can already be consulted at: *https://unece.org/sites/default/files/2021-04/UN-SCEGHS-40-INF3_0.pdf* (informal document) and *https://unece.org/sites/default/files/2021-04/ST-SG-AC10-C4-2021-4e.pdf* (working document).

The revised chapter retains a tiered approach to the evaluation of available information but *in vitro*/*ex vivo* data have been given a higher weight than appears in version 9 of GHS, being placed in Tier 2 above *in vivo* skin corrosion and other animal data. New sub-sections have been added on how to classify for serious eye damage or eye irritation based on *in vitro*/*ex vivo* data and on non-test methods. Importantly, *in vitro*/*ex vivo* data can now be used on their own to identify chemicals not requiring classification. Another important revision is the introduction of the concept of defined approaches (DAs) into the chapter, which now feature in Tier 2 together with *in vitro*/*ex vivo* data. This is the first time the concept of DAs is introduced in GHS and it will have a major impact on follow up chapters covering more complex endpoints.

The principle of test method neutrality described in paragraph 1.3.2.4.3 of the GHS was maintained and several validated methods/approaches other than OECD test guidelines are also mentioned in the revised chapter as being potentially useful for classification purposes. The currently available *in vitro*/*ex vivo* OECD Test Guidelines for serious eye damage/eye irritation often result in an outcome that is inconclusive according to the test guidelines' criteria and cannot be used directly to identify Category 2, eye irritation. The use of non-guideline methods, on the other hand, may be particularly useful for this purpose.

Finally, the IWG was able to resolve an issue that was still pending from Chapter 3.2 namely, the current ambiguity on whether the appropriate classification is Category 1 or inconclusive where a substance/mixture has extreme pH and non-significant acid/alkaline reserve. For substances, an extreme pH with non-significant acid/alkaline reserve now leads to Category 1 classification in Tier 7 (classification based on an overall weight of evidence assessment) if no other information is available or if the overall weight of evidence remains inconclusive. For mixtures, it leads to Category 1 classification only if the bridging principles cannot be applied.

A series of consequential amendments were also introduced in Chapter 3.2 so that it is fully aligned with Chapter 3.3. The approved conforming changes to Chapter 3.2 can be consulted at: *https://unece.org/sites/default/files/2021-04/*

UN-SCEGHS-40-INF4_0.pdf (informal document) and *https://unece.org/sites/default/files/2021-04/ST-SG-AC10-C4-2021-5e.pdf* (working document).

Box 4.3

UN Subcommittee on the Globally Harmonized System of Classification and Labelling of Chemicals (GHS)

The Globally Harmonized System of Classification and Labelling of Chemicals (GHS) addresses classification of chemicals by types of hazard and proposes harmonised hazard communication, including labels and safety data sheets. It aims at ensuring the protection of human health and the environment during the handling, transport and use of chemicals. It therefore includes criteria for identifying physical hazards as well as hazards to human health and the environment. The first edition of the GHS, which intended to serve as the initial basis for the global implementation of the system, was adopted in December 2002.

Since then, the UN subcommittee on the GHS meets twice a year in the UN palace in Geneva, and based on their work the GHS is updated, revised and improved every two years. Several Informal Working Groups are set up to progress different activities of the subcommittee between the plenary sessions. The Informal Working Group on Non-animal Test Methods (IWG NATM) was established in 2016 on the initiative of the Netherlands and the United Kingdom. Since then they have been co-leading the work on the introduction of criteria based on non-animal approaches as an alternative to the traditional animal testing for health and environmental hazard classes.

The ninth revised edition of the GHS (GHS Rev.9), published in 2021, is the most recent revision: *https://unece.org/node/359591*.

4.4.3 GHS guidance on skin sensitisation

On behalf of the European Commission, EURL ECVAM is providing scientific support for the revision of GHS Chapter 3.4 on skin and respiratory sensitisation to include classification based on non-animal methods/approaches. EURL ECVAM produced a scoping document outlining the issues for discussion by the IWG NATM. In this document the proposal was made to also revise chapter 3.4 in relation to respiratory sensitisation although this has not been agreed by the GHS subcommittee.

The IWG NATM discussed this proposal and decided to focus for the time being only on the skin sensitisation section of the chapter, nevertheless the possibility of revising the respiratory sensitisation part of the chapter was kept on the agenda and will be re-discussed if time allows within the biennium but only once the skin section is finalised.

A proposal was made for a revised decision logic for skin sensitisation to give to the recently adopted DAs (OECD, 2021a) the same weight as *in vivo* human and animal data. Consideration will also be given in the decision logic on the possibility of using validated individual *in chemico* and *in vitro* methods for classification. The proposed revised decision logic is currently under discussion by the IWG NATM. Once agreed, a proposal on how the text of the chapter should be revised will be made.

4.4.4 Clarification of the GHS classification criteria for germ cell mutagenicity

EURL ECVAM proposed to create the IWG on germ cell mutagenicity to clarify the classification criteria for germ cell mutagenicity in category 1B supported by the UN GHS subcommittee at their meeting in December 2020. The subcommittee further adopted the Terms of Reference of the group in July 2021,

which on proposal of, and in agreement with, other delegates was extended to also revise the criteria for classification in category 2 and category 1A. Moreover, the chapter will include an update with respect to the current state of the art including newly available test guidelines.

The problem with the current criteria in category 1B is that they have led to diverging opinions between experts when implementing the current GHS. In particular, the requirement for "demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells" has caused difficulties in enabling classification in category 1B. In fact, data proving the substance's molecular interaction with germ cell DNA are so far only occasionally available. Therefore, this wording is prone to different interpretations.

To assist the scientific discussion, HESI's Genetic Toxicology Technical Committee (GTTC) agreed to re-visit any existing data and provide their opinion on what positive endpoints in somatic tissues and/or level of exposure in gonads would allow classification and labelling as a germ cell mutagen without germ cell testing. In addition, also the OECD genotoxicity expert group will be consulted on the criteria proposed by the Informal Working Group, prior to submitting them for agreement to the GHS subcommittee.

4.5 Data and knowledge management

Smart data and knowledge management solutions are crucial elements of mature science-policy frameworks. The field of regulatory toxicology is undergoing profound changes with respect to methods and approaches for generating evidence. The shift towards new assessment methodologies that link together mechanistic information to adverse outcomes brings new challenges as well as opportunities for data and knowledge management.

In the EU, the European Commission's Chemicals Strategy for Sustainability (EC, 2020) aims at improving the science-policy interface through the creation of a comprehensive knowledge base on chemicals. At international level, the OECD AOP framework has established itself as the core knowledge base for mechanistic information on chemical effects. The JRC is making substantial contributions to these important initiatives.

4.5.1 AOP knowledge base

The adverse outcome pathway knowledge base (AOP-KB) is the central hub for real life application of the AOP framework. AOP authors draft, finalise and publish their AOPs in the KB, reviewers apply their comments here, and knowledge consumers browse through the KB to find the AOPs or AOP elements they need. EURL ECVAM co-chairs the OECD subgroup dealing with the maintenance and further development of the AOP-KB.

In 2021, the AOP-KB subgroup at OECD introduced a clear separation between the data and the application aspects of the AOP-KB, see Figure 4.1.

- The AOP-KB as such is the collection of data and knowledge managed at central level by the OECD subgroup: This includes the underlying data model, as well as the physical SQL DB and the XML format used to import

and export knowledge. The AOP-KB now features almost around 340 AOPs, an increase of 20% compared to 2020.

- On the other hand, there are a series of tools that can interact with the central knowledge base: official tools (co-)managed by the OECD subgroup, and third party tools (both public and commercial) with which the subgroup is examining overlaps and synergies.

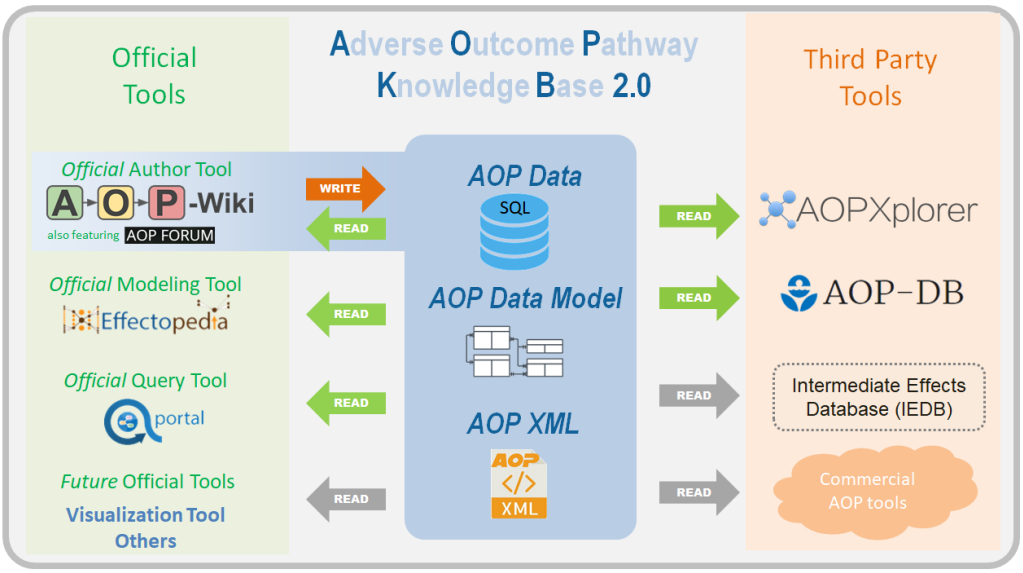


Figure 4.1: Modules and third party tools of the AOP knowledge base.

Among the official tools, the most prominent is the AOP-Wiki. Read access is open to anyone without registration, and there are 500 registered users who can comment on AOPs and an additional 200 who are able to enter AOP knowledge. In 2021, the subgroup started the design of a series of new features for the AOP-Wiki, which will be implemented (subject to resource availability) in the coming years.

The AOP forum, which was introduced in 2020, continues to facilitate the liaison between AOP authors, users and the OECD subgroup. Discussion threads deal with technical questions around the use of the AOP-Wiki, issues concerning the science underpinning the AOP Framework and general questions of collaboration. Everyone is welcome to join the discussion in the forum.

- AOP-Wiki: *https://aopwiki.org/*
- *AOP forum:* *https://aopwiki.org/forums/index.php*

4.5.2 AOP framework analysis study

The transition towards a comprehensive knowledge base on chemicals supporting chemicals policies at EU and global level comes at a time when the field of regulatory toxicology is undergoing profound changes with respect to methods and approaches for generating evidence. At the same time, in the public arena, chemical-related policies and regulations are coming under greater public scrutiny, and the COVID-19 pandemic has focused attention on how evidence is used for policy-making.

These issues were addressed in the AOP Framework Study commissioned by EURL ECVAM, the results of which are now available as a science for policy report (Carusi *et al.*, 2021). The study aimed to gain insights into stakeholders' perceptions of i) the main challenges facing chemicals regulation, ii) alternative approaches to conducting toxicological studies, and iii) the role and added-value of the AOP framework. The focus was on key stakeholders who are directly involved in decision-making in regulatory or industry contexts: regulatory toxicologists, risk assessors and risk managers.

The study revealed that the main challenges of current chemicals regulation are:

- the science directly informing policy and regulatory decision-making often lags behind current science;
- there is a lack of consensus on different methods and approaches in toxicological sciences, exacerbated by the difficulty of access to large quantities of dispersed and non-standard data;
- there is mistrust among stakeholders in different sectors;
- there is not a shared understanding of how data is constituted as evidence for regulatory decisions, or for current and future policy regarding chemicals;
- in view of the likely increasingly contentious nature of chemicals and other potential stressors, transparency of the decision-making process in regulation and policy, for all stakeholders, becomes an ever greater challenge.

Main recommendations to address these challenges are shown in Figure 4.2.

4.5.3 Transcriptomics and metabolomics reporting framework

In the last few years, EURL ECVAM has been involved, together with experts from government agencies, industry and academia, in devising a framework suitable for the reporting of transcriptomics and metabolomics data to be used in regulatory toxicology (see Box 4.4). This collective effort resulted in the publication of the OECD draft guidance documents: "Transcriptomics reporting framework" and "Metabolomics reporting framework", supplemented by specific reporting templates. These publications represent a crucial step towards facilitating the use of 'omics technologies in chemical risk assessment, since transparency in the reporting of 'omics data was one of the barriers to their wider regulatory application.

The guidance documents describe in detail the four types of reporting modules constituting the harmonised framework:

1. the study summary reporting module, providing a high-level overview of the regulatory toxicology and 'omics experiment;
2. the toxicology experiment reporting module, reports the key descriptors of the *in vivo* or *in vitro* toxicology study;
3. the data acquisition and processing reporting modules, reporting descriptions of the 'omics assays, data acquisition and associated data;
4. the data analysis reporting modules, describing the statistical analysis that has been undertaken in the 'omics study.

The harmonised transcriptomics and metabolomics reporting framework has been tested in different regulatory relevant scenarios to prove its applicability. More information can be found in Harrill *et al.* (2021).

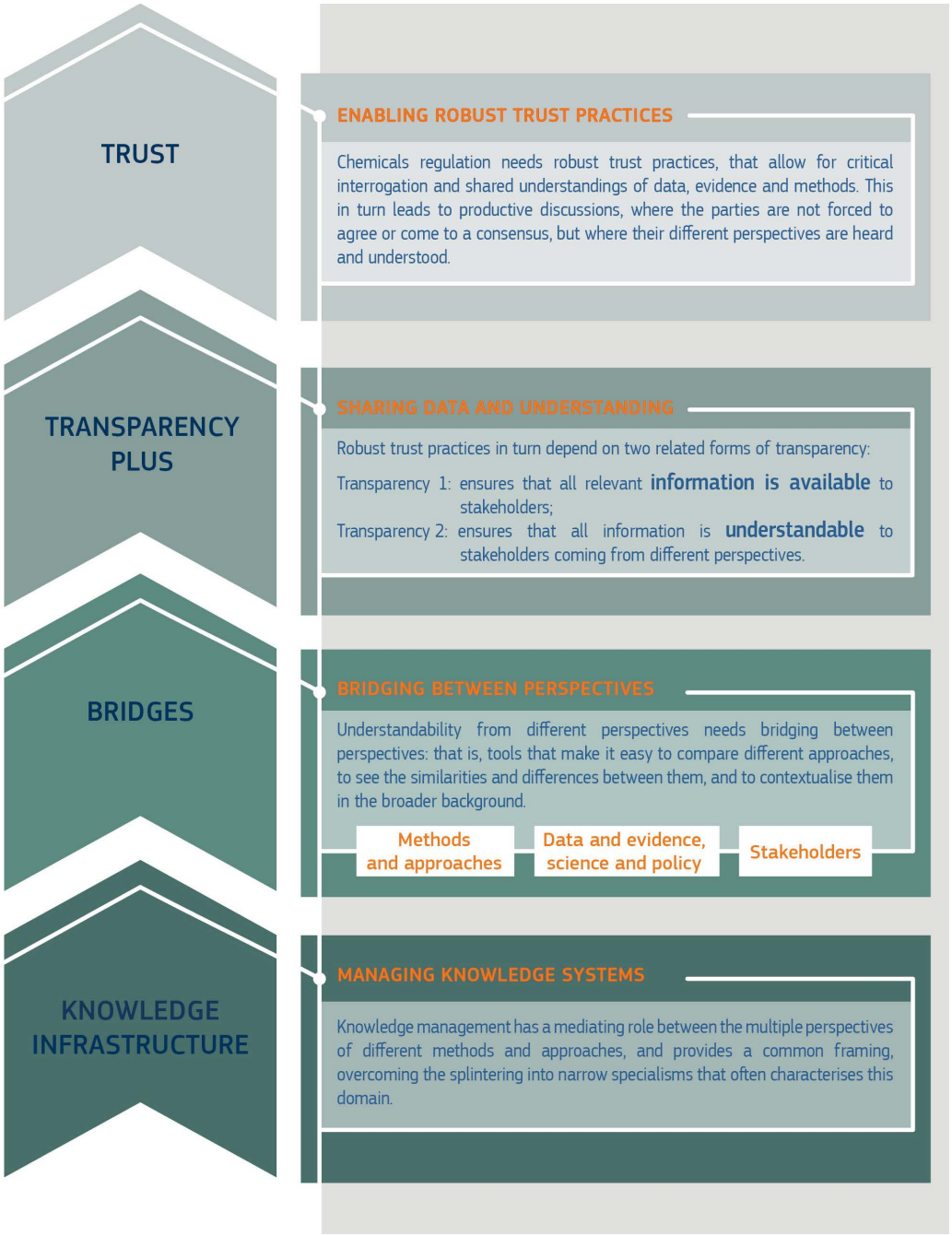


Figure 4.2: AOP framework study recommendations, © Carusi et al., 2021, CC BY 4.0.

- Transcriptomics reporting framework: *https://www.oecd.org/chemicalsafety/testing/transcriptomic-reporting-framework.pdf*
- Metabolomics reporting framework: *https://www.oecd.org/chemicalsafety/testing/metabolomics-reporting-framework.pdf*

Box 4.4

Highlights from the OECD Working Party on Hazard Assessment 2021

The 5th Meeting of the OECD Working Party on Hazard Assessment (WPHA) was held virtually on 24 to 25 June 2021. Topics included country updates, progress on OECD-related IT tools (IUCLID, eChemPortal, QSAR Toolbox), an update from the Accelerating the Pace of Chemical Risk Assessment (APCRA) project, the IATA Case Studies project (endorsement of Case Studies and Considerations Document from 6th review cycle), and the Occupational Biomonitoring project. Progress was also presented on the QSAR Assessment Framework project, led by Italy. This project, to which EURL ECVAM is contributing, is carrying out a targeted update of the 2007 QSAR Validation Guidance Document, with an emphasis on the identification and characterisation of uncertainties in QSAR predictions.

On 23 June 2021, the WPHA held a joint session with the EAGMST group, to discuss the metabolomics and toxicogenomics reporting frameworks, as well as opportunities for further 'omics related work.

Important contributions from the EURL ECVAM to the work of the WPHA included the finalisation of an "Overview of Concepts and Available Guidance related to Integrated Approaches to Testing and Assessment (IATA)" (OECD, 2020) and the Guidance Document on the Characterisation, Validation and Reporting of PBK Models for Regulatory Purposes (OECD, 2021b).

▸▸ OECD IATA webpage: *https://www.oecd.org/chemicalsafety/risk-assessment/iata-integrated-approaches-to-testing-and-assessment.htm*

4.5.4 EU Open data platform on chemicals including IPCHEM

Chemical safety assessments are performed under various pieces of legislation, by various actors and at different points in time. The EU Chemicals Strategy for Sustainability (EC, 2020) therefore announced the future 'one substance, one assessment' approach. It aims to improve the coordination and transparency of chemical safety assessments.

One element of the 'one substance, one assessment' approach is the sharing and reuse of data. The Commission will develop a common open data platform on chemicals to facilitate the sharing, access and re-use of information on chemicals coming from all sources. Data will be made available in appropriate formats and tools to ensure interoperability, i.e. IUCLID6 for hazard data and IPCHEM for chemical monitoring data.

IPCHEM, the Information Platform for Chemical Monitoring, provides a wealth of occurrence data on chemicals present in our environment, food, indoor air, and even in our bodies. It is a single entry point, where EU authorities, national and regional authorities, and researchers can find and share information.

IPCHEM allows the user to discover, access and retrieve information on chemical concentrations throughout Europe across different media. As the scientific and technical lead of the platform, the JRC has integrated data of more than 170 studies. Many more are in the pipeline.

One recent highlight is that data from human biomonitoring studies that were collected by partners of the HBM4EU project under the lead of VITO, are now publicly available in IPCHEM.

▸▸ IUCLID6: *https://iuclid6.echa.europa.eu/home*
▸▸ IPCHEM: *https://ipchem.jrc.ec.europa.eu/*
▸▸ HBM4EU: *https://www.hbm4eu.eu/*
▸▸ VITO: *https://vito.be/en*

4.5.5 Improving the use of academic data in regulatory assessment

The European Commission has committed to improve the implementation of legal provisions that require considering all available scientific information in regulatory assessments. Findings of recent policy evaluations (EC, 2019a, b) motivated the initiative, announced in October 2020 under the Chemicals Strategy for Sustainability (EC, 2020). The JRC is leading the work in collaboration with ECHA, EFSA and other scientific, regulatory and industry stakeholders. The goals of the initiative are:

- to improve the uptake and value of data derived from academic studies in regulatory safety assessments of chemicals;
- to improve the implementation of the requirements of chemical legislation to consider all available information, including academic data, in safety assessments.

The JRC will engage with interest groups that share responsibility and ownership of the challenge to make research as valuable as possible to regulatory decision-making (Figure 4.3). Broad stakeholder engagement is foreseen to leverage existing resources, expertise and related initiatives at EU agencies, the OECD, and EU funded projects. The results, expected by 2023 include:

- a guidance setting minimum quality and reporting requirements for consideration of academic studies in regulatory assessments. The guidance will help researchers to design, perform and report studies in a way that facilitates regulatory use;
- a search guide to aid the implementation of the requirement to consider all available information in regulatory assessments. The search guide will help assessors to find, access and evaluate academic data from peer-reviewed studies.

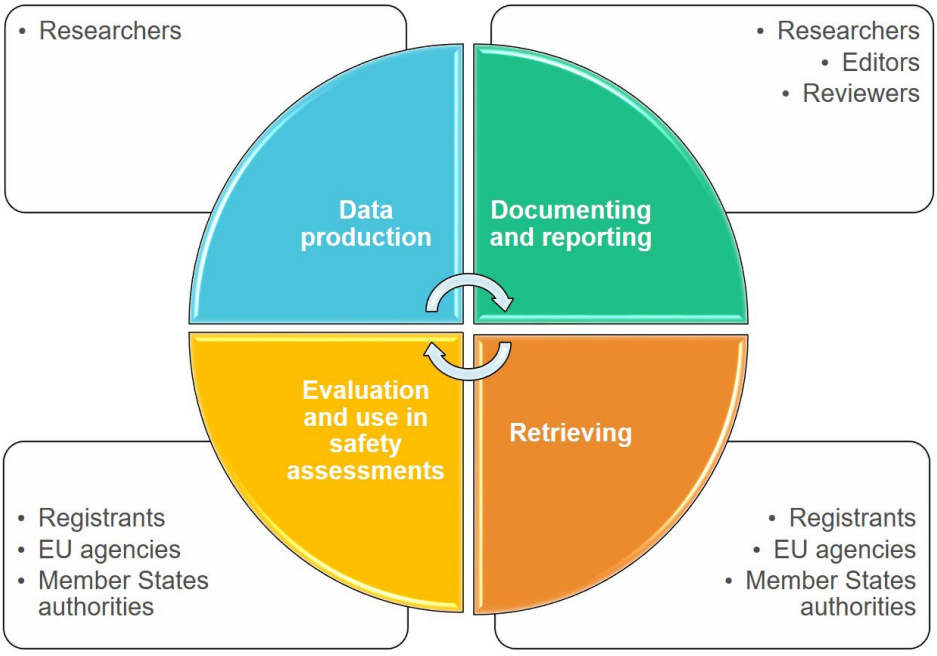


Figure 4.3: Stakeholders and steps involved in the generation and regulatory uptake of academic data.

5. Alternatives in research and education

Research is the main domain where animals are used for scientific experiments, with almost five million in basic research and three million in applied and translational research being used annually in the EU and Norway (see Box 5.1). It is therefore essential to understand how these animals are used and where alternative methods could be further developed or promoted.

Biomedical research is one important area that accounted for five million uses of animals which represents half of the animal uses in 2018. In 2021, EURL ECVAM continued the work initiated during the past few years looking at biosciences in particular, with the identification of existing non-animal models. It also worked on identifying ways of building bridges between different scientific communities, providing concrete innovative approaches such as modelling the pathogenesis of COVID-19 using adverse outcome pathways.

Because changing the current paradigm in research is a long-term process, EURL ECVAM also focused on further developing education and training resources. Reinforcing existing knowledge bases for primary and secondary school teachers, will pave the way for a future where research fully embeds the Three Rs.

5.1 Biomedical research

In 2021, EURL ECVAM pursued its work in the biosciences, analysing current approaches to health-related research, publishing new reviews of advanced non-animal models in different fields of disease research, assessing ways of building bridges within biomedical domains and evaluating the output and impact of biomedical EU-funded research. Moreover, EURL ECVAM established a basis for complementing the monitoring mechanisms set out by Directive 2010/63/EU in relation to animal use (see Box 5.1 for the statistics on the use of animals for scientific purposes in 2018), by developing a framework for the creation of indicators measuring the uptake of non-animal methods for scientific purposes.

5.1.1 Review of advanced non-animal models in biomedical research

Two additional technical reports have been published in the areas of neurodegenerative diseases and immuno-oncology, as well as new collections of models in those areas.

The study on neurodegenerative research has produced a freely available knowledge base describing 568 different models that can be used to investigate the underlying mechanisms leading to neurodegenerative diseases and to find new therapies. The types of models reviewed are based on human derived cells and tissues cultured in the laboratory (*in vitro*), computer modelling and simulation (*in silico*), or cells and tissues explanted from patients (*ex vivo*).

In the area of cancer research, in order to explore the trends of human-based *in vitro* and *in silico* models being used fruitfully in immuno-oncology, around 130,000 peer-reviewed papers published from January 2014 to March 2019 were initially retrieved and screened, from which 542 scientific peer-reviewed articles were selected for a deeper analysis of the non-animal models used.

- Neurodegenerative diseases: *https://europa.eu/!bjY7Bp*
- Immuno-oncology: *https://europa.eu/!myNbWr*

5.1.2 CIAO – modelling the pathogenesis of COVID-19 using AOPs

The adverse outcome pathways (AOP) framework provides a publicly accessible platform for organising and reviewing knowledge across multiple biological levels. While AOPs are widely acknowledged in chemical safety assessment and regulatory toxicology, they are foreseen to become of great value also for biomedical research. The COVID-19 pandemic is a unique opportunity to introduce the biomedical community to the AOP framework.

As a hands-on initiative in that direction, EURL ECVAM is facilitating an interdisciplinary crowd-sourced project, called CIAO, towards a better understanding of COVID-19 by applying the AOP paradigm. CIAO is based on the postulation that AOPs can provide an integrative means for organizing the abundant, fast evolving and dispersed knowledge on COVID-19 pathogenesis (Nymark *et al.*, 2021). A mechanistic understanding of the disease permits the combination of data from *in vitro* models for virus characterisation with data from animal and human studies depicting the inflammatory response and various adverse outcomes. This AOP-based description of how the virus enters and infects the body also helps to capture the various factors modulating the clinical outcomes,

Box 5.1

2018 statistics on the use of animals for scientific purposes in the EU and Norway

For the second time, after the publication of the 2015-2017 statistics on the use of animals for scientific purposes in the EU, EURL ECVAM supported Directorate-General for Environment in the analysis of the data reported by Member States in the context of Directive 2010/63/EU. For the first time the report features data reported by Norway which implemented the Directive in 2016. In 2018, we observed a slight decrease of the total number of animals used for the first time.

The provision of such statistics is essential to monitor the progress of Three Rs implementation in the EU. Thanks to a variety of information reported through the Directive, it is now possible to have a complete overview of when, how and where animals are being used.

For example, it allows assessment of the impact of scientifically valid alternative methods and to determine where to focus for the development of future alternatives.

By recording the severities of animal uses, it also helps to identify the areas where animals can experience more suffering and where refinement should be prioritised. Lastly, with the provision of detailed numbers of animals used by category, it helps to identify specific areas where the number of animals used could be reduced (see Figure 5.1).

▸▸ Staff working document: *https://ec.europa.eu/environment/chemicals/lab_animals/pdf/SWD_%20part_A_and_B.pdf*

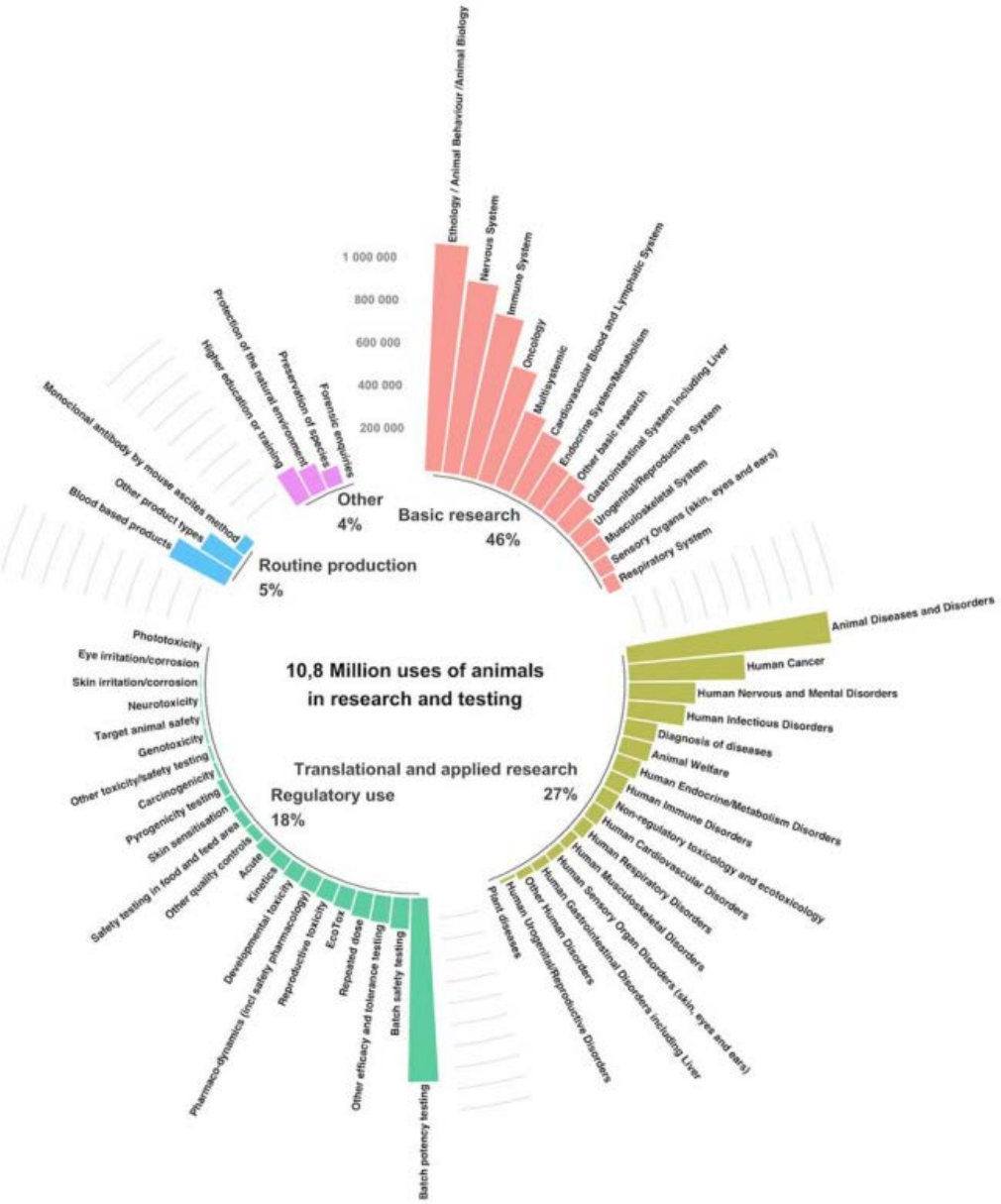


Figure 5.1: Statistics on the use of animals for scientific purposes for EU and Norway, including re-uses, in 2018.

increasing our understanding of why some populations are more vulnerable than others. The modular aspect of AOPs also allows the development of a COVID-19 related AOP network where central biological key events, the interrelation between the outcomes and knowledge gaps become more evident.

In addition, the application of the AOP framework to model a viral disease of high societal relevance is providing lessons and outputs on potential adaptations of the framework itself, relevant not only for regulatory toxicology, but also for further potential large-scale AOP-aligned collaboration in the biomedical field (Carusi *et al.*, 2018).

Currently, more than 65 scientists around the world are participating in the CIAO project and are developing AOPs which model COVID-19 to better understand the disease and ultimately help defeat the virus.

▸▸ Modelling the pathogenesis of COVID-19 using AOPs (CIAO): *https://www.ciao-covid.net*

5.1.3 Gauging the output and impact of biomedical research

To retrospectively monitor the outputs and impact of EU-funded biomedical research over the last 20 years in the fields of Alzheimer's disease, breast cancer and prostate cancer, in collaboration with Directorate-General for Research and Innovation, EURL ECVAM launched a survey (2020) addressed to researchers who participated in projects funded under EU framework programmes FP5, FP6, FP7 and H2020.

In 2021, EURL ECVAM published the results in a factual summary report (Mc Carthy *et al.*, 2021), and more recently, a synopsis report (Pistollato *et al.*, 2021a) providing a more detailed analysis, complemented with insights obtained from 29 structured interviews to survey participants. This analysis showed that several years are generally needed for biomedical research projects to have a concrete impact on public health and that targeted follow-on funding is believed to increase the chance of generating societal impact.

Epidemiology-based research has recognised potential to generate human relevant results, and research projects designing novel diagnostic or prognostic tools often gain higher translational impact. The development and use of complex *in vitro* models, such as 3D models, spheroids, organoids, microfluidic, organ/tissue-on-chips, and computational approaches are also increasing with time.

As pointed out by some survey participants, animal models are still considered unavoidable; however, some shared the opinion that animal models often do not recapitulate the multi-factorial complexity of human diseases and may therefore contribute to translational research failures.

This report represents a useful starting point to explore how to foster human relevance and increase translatability of EU-funded biomedical research, with a view to maximising impact on public health.

▸▸ Synopsis report – executive summary: *https://op.europa.eu/en/publication-detail/-/publication/85a92e8f-271b-11ec-bd8e-01aa75ed71a1/language-en/format-PDF/source-search*

5.1.4 Science in an era of information overload

Numerous issues around more efficient knowledge production and dissemination in science are well known (e.g. the "publish or perish" dilemma), and in order to address them, EURL ECVAM invited a group of scientists from European, US and Canadian institutions to plan, organise, and execute a workshop titled "Science with Impact in an era of information overload". This workshop examined the role the AOP framework could play in knowledge dissemination and also followed up on the recommendation by Carusi *et al.* (2019) to analyse existing policies and initiatives regarding open access, open science, and open data, and see what gaps exist between data and method complementarity.

The workshop was held as an online event 7 to 9 June 2021 with about 40 participants from key life sciences societies, policymakers, and publishing enterprises, who discussed potential ways out of the publication dilemma. A series of concrete problems, possible solutions and priority actions were identified around the topics of publications, cross-disciplinary collaboration, and science impact.

Immediate, quick-win actions after the workshop (e.g. a closer look at the "FAIR"-ness of the AOP-Wiki, possible tweaks to the AOP-Wiki data model to accommodate better searchability, better dissemination of test method information) will be followed by more in-depth initiatives in three strands of activities:

- Strand 1 - communication, collaboration and shared understanding
- Strand 2 - engagement with publishers
- Strand 3 - technical implementation and support

For each of the three strands, both ongoing activities were identified that can be continued under the umbrella of the Science with impact initiative, and promising new activities were suggested that would help to implement the actions discussed during the workshop.

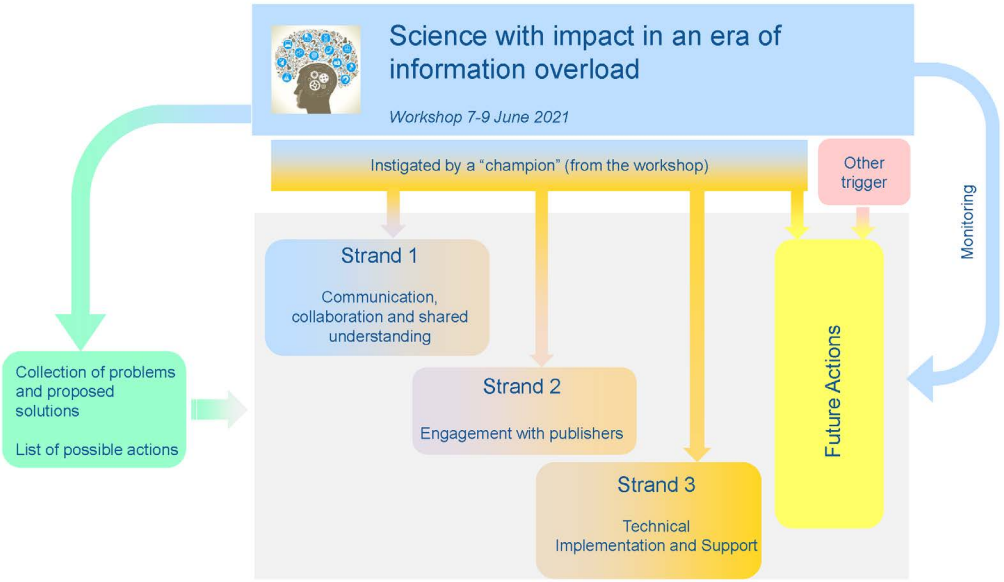


Figure 5.2: Ongoing and planned follow-up activities after the 7-9 June 2021 workshop "Science with impact in an era of information overload".

Activities that are foreseen include:

- pilot studies (e.g. nano publications, ontologies, semantic tagging, knowledge graphs, links between AOP-Wiki and journals, ...)
- training (e.g. awareness of knowledge aggregation systems and technologies, science communication, ...)
- innovative ideation (e.g. conceptualise new modes of peer review, facilitate FAIR data publication, ...)
- removing barriers (e.g. revise copyright laws, socialisation of knowledge as a valuable professional contribution, ...)
- alternative communication streams (e.g. inform scientists about policy/regulation; better communication between science and policymakers/regulators; facilitate dialogue between research and policymakers/regulators to enable them to participate in co-production of knowledge...)

The main recommendations made at the workshop will be presented in an OECD webinar on Adverse Outcome Pathway (AOP) co-operative activities between scientific journals and the OECD on 25 January 2022.

▸▸ AOP-Wiki database: *https://aopwiki.org/*

▸▸ Webinar on Adverse Outcome Pathway (AOP) co-operative activities between scientific journals and the OECD on 25 January 2022: *https://www.oecd.org/chemicalsafety/testing/webinars-on-testing-and-assessment-methodologies.htm*

5.1.5 Methods and models in biomedical sciences: building bridges

As a follow up to the "Bridging Across Methods in the Biosciences" report (Carusi *et al.*, 2019), EURL ECVAM co-organised a workshop with the Champalimaud Foundation, FRESCI, Congento, QuantOCancer and InterChange Research. The workshop aimed at bringing together different communities to facilitate knowledge exchange in an interdisciplinary setting. The event took place on 14 and 15 June 2021 and was designed to provide an open-minded environment allowing constructive discussions on common problems around methods and models.

It was divided into four sessions over two afternoons with the following objectives:

- To share the state of the art of innovative methods in tumour immunology and beyond.
- To connect people, reduce the communication gap, break silos.
- To educate new generations, strengthening future bridges between disciplines.

Each day started with a series of research talks on different methods and models using immuno-oncology as a case study. The remaining sessions aimed to allow more interaction with the public, in one way through breakout groups and the other through a panel discussion on 'building trust through bridges'.

During the breakout group, the participants were challenged to build a super model of a body organ with the

> *"The #MMBridges was a great success, opening my eyes to robust modern biomedical research approaches being used now and, perhaps even more importantly, humanizing ideologies and agendas that can sometimes feel difficult to implement in the "real world".*
>
> *With a strong core centred on ECVAM's accessibility, knowledge and influence, I hope we were able to give animal researchers an equal voice/platform in a not-so-scary-after-all context, seeding a culture of inclusion and transparency.*
>
> *Laura Ward*
> *Project Manager, Champalimaud Foundation*

recommendation to use approaches that they know well. This highlighted the challenges still at stake to build bridges but providing some interesting ideas on how organ models in interdisciplinary groups could be built.

- Conference announcement: *https://joint-research-centre.ec.europa.eu/events/methods-and-models-biomedical-sciences_en*
- Champalimaud Foundation: *https://www.fchampalimaud.org*
- FRESCI: *https://www.fre-sci.com*
- *Congento: https://congento.org*
- *QuantOCancer: https://quantocancer.fchampalimaud.org*
- *Inter-Change Research: https://inter-changeresearch.com*

"Workshops were very interesting and provided me with alternatives to working with animals, although I find it difficult to fit my experimentation with these alternative models, but it has allowed me to reflect."

"It was interesting for someone who works primarily with animals to try algorithms and methodology that minimize their use. The network of expertise was also very interesting."

"It helped me to understand, how to imagine a type of study based on experimental models."

"These types of workshops are very important for the entire scientific community to be active in looking for solutions to reduce the use of animals in research or to make better use of the data obtained from them. In this sense, I found the workshop very interesting, particularly the panel discussion in the second day."

"Absolutely important to leave the own garden plot and short-circuit with other analogue, complementary, consistent, contradictory,... fields, methods, models...; this meeting was a trailblazer."

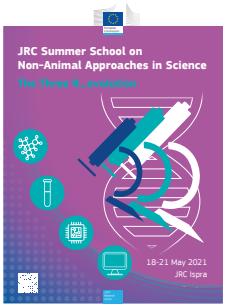
Anonymous feedback from participants

5.2 Education and training

Education and training underpin furthering the development and use of alternative methods and the application of the Three Rs in science, and for a number of years EURL ECVAM has been undertaking education initiatives with this in mind. Following a strategy outlined in an EURL ECVAM Report from 2021, Introducing the Three Rs into secondary schools, universities and continuing education programmes (Holloway *et al.*, 2021), EURL ECVAM continues to build on progress made in introducing the Three Rs into education programmes. This is being done by engaging directly with teachers and young people, in large thanks to further support from the European Parliament spanning 2021-2023, following the success of the European Parliament pilot project 2018-2020 (Zuang *et al.*, 2020; Zuang *et al.*, 2021), and in addition working with teacher trainers, university professors and reaching out to education decision makers.

Finally, an entirely virtual third edition of the popular JRC summer school, aimed at post-graduate students and early career scientists, took place on 17 to 21 May 2021.

5.2.1 JRC virtual summer school on non-animal approaches in science



EURL ECVAM organised the third edition of the JRC Summer School entitled "JRC virtual summer school on non-animal approaches in science: the Three R... evolution", that took place from 17 to 21 May 2021. This edition was accessible to the students online due to the pandemic.

The aim of the JRC summer school was to share knowledge and experience on the latest non-animal approaches and to promote their use in biomedical research, regulatory applications and testing, including cutting-edge technologies such as induced pluripotent stem cells, organ-on-chip, complex *in vitro* methods, computational modelling and artificial intelligence. In addition, the intention was to explore the role of the Three Rs in science today through discussion and debate.

The JRC summer school was specifically tailored for post-graduate students and early-career scientists working in the biosciences and focused on non-animal methods and technologies and the opportunities and challenges associated with their application in various fields such as regulatory toxicology and biomedical research.

The program combined lectures by invited and in-house experts in several key fields through plenary sessions and round tables for discussion on hot topics. The students themselves took the lead in debate sessions and poster presentations. Plenty of interactive and engaging activities encouraged participants to exchange views and facilitated networking.

In 2021 EURL ECVAM celebrated its 30th anniversary. To mark this occasion, a special issue has been published in the journal ATLA and dedicated to EURL ECVAM's mandate and activities with an emphasis on the concept of the JRC summer schools (JRC, 2021).

▸▸ JRC summer school 2021 on non-animal approaches in science: the Three R...evolution: *https://europa.eu/!WgDT8k*

5.2.2 Re-running the Three Rs MOOC – data, stats, overview

EURL ECVAM's Three Rs massive open online course (MOOC) took place between 13 September and 20 October 2021. The MOOC, a revised and updated version of the 2020 edition, and hosted on the European Schoolnet Academy platform, is designed for life sciences teachers in secondary and primary schools. It provides teachers, but also other interested educators and stakeholders with the knowledge and resources to bring the Three Rs into their own classrooms.

With the support of European Schoolnet and a group of assigned teachers, with whom EURL ECVAM is working for the duration of its Three Rs school project, a richer course has been provided. The course had 1,151 registrations, 698 active participants and 311 completions (each receiving a certificate). The course, which took place entirely online, included learning scenarios for engaging Three

Box 5.2

JRC virtual summer school on non-animal approaches in science: the Three R...evolution

The third edition of the JRC summer school entitled "JRC virtual summer school on non-animal approaches in Science: the Three R...evolution", hosted more than 130 international postgraduate students and young professionals to explore non-animal approaches and to promote their use in biomedical research, regulatory applications and testing.

Participants came from 28 countries worldwide and the average age was between 23 and 38 years old. They were selected on the basis of a letter of motivation, and an abstract describing their own work that was presented in poster sessions during the school.

Interactive and engaging activities encouraged participants to exchange views and facilitated networking, also with the speakers, through world café rooms, polls and quizzes. An exhibition hall with thematic booths was also available. Here, students had the possibility to deepen their knowledge of the EURL ECVAM activities, to find out about job opportunities in the field (career session), to virtually visit our lab through virtual reality and participate in an escape room game. Participants were asked to present a poster describing their own studies and interests or work area related to the topics of the JRC summer school. Six prizes were awarded to the best posters. Three awards were given by the JRC and an additional three awards were generously given by supporting organisations: Early Career Researchers Advancing 21st Century Science (ERA21), People for the Ethical Treatment of Animals (PETA) Science Consortium International and the European Society of Toxicology In Vitro (ESTIV).

In 2020, the JRC's EURL ECVAM unit won the first prize of the European Commission's internal competition on sustainable conferences and events for the 2nd Summer School edition held in 2019. Although the 2021 edition of the summer school was held as a virtual event an effort was made to reduce the impact on the environment. Participants were offered engaging virtual and green gifts, in the form of a dedicated training on science communication and the possibility of planting trees in the Amazon forest to help to reduce deforestation and contribute to building agroforestry.

Rs lessons, interviews with the teachers who developed and implemented them, ideas for activities in the classroom, a PC-based 3D interactive experience in the EURL ECVAM *in vitro* lab (see Section 5.2.3), and expert webinars. The MOOC represents one major resource in itself within EURL ECVAM's ever-increasing catalogue of educational material aimed at supporting the introduction of the Three Rs into the classroom (see Section 5.2.3).

The MOOC includes four modules:

- module 1: animal welfare and science
- module 2: human-based science
- module 3: critical thinking
- module 4: your own 3R's learning scenario

In our primary and secondary level Three Rs initiative we are following these steps towards successful integration of the Three Rs in education programmes:

- creating, together with teachers, good resources that show how the Three Rs can be taught (the learning scenarios, resources);

- professional development of teachers to facilitate the use of the learning scenarios and resources (the MOOC);
- dissemination activities targeting teachers (so more are aware of the material); and
- dissemination activities targeting 'teacher trainers' and policy decision makers (e.g., education ministries) to encourage inclusion of the topic on the curriculum.

The first two tasks have been the focus of our work in 2021, and points 3 and 4 will be tackled in 2022, with the finalisation of the project in December 2022.

▸▸ European SchoolNet: *http://www.eun.org/*

▸▸ The Three Rs MOOC Rerun: *https://www.europeanschoolnetacademy.eu/courses/course-v1:ThreeRs+AnimalsInScience+2021/about*

5.2.3 New educational resources

As mentioned in Section 5.2.2, part of the Three Rs MOOC is to demonstrate teaching through learning scenarios and provide those learning scenarios to other teachers interested in using them. As a result of the collaboration between the JRC and European Schoolnet, six learning scenarios for secondary schools were created in 2019 and this collection has been added to the ongoing school education initiative (2021-2022) (Zuang *et al.*, 2021), with a number of them having been adapted for primary school learners.

Adding to the ever-growing list of learning scenarios based on existing courses kindly provided by several university professors, we can now boast a sizeable catalogue of Three Rs learning scenarios with a target range of students of six years to 19 years at school level, and university students from undergraduate level upwards. Discover them at the EURL ECVAM education web page.

In summary here are the resources available which can be accessed from the EURL ECVAM education page:

- eight Podcasts of interviews with scientists talking about their work with replacement methods and approaches;
- a storyboard combined with a slide set - animals in society and animals in science - covering the Three Rs and Directive 2010/63/EU on the protection of animals used for scientific purposes;
- a poster and infographics;
- learning scenarios for primary school, secondary school and university-level education;
- the Three Rs teacher MOOC (including 3D interactive lab);
- two e-learning modules on best practice for developing non-animal methods and searching for non-animal methods; and
- coming in 2022, learning scenarios for primary school, further learning scenarios for secondary school and new resources.

A virtual visit to the EURL ECVAM *in vitro* lab was made possible through a 3D interactive module. The module shows one area of EURL ECVAM's lab work – cell culturing and high throughput screening and can be downloaded on a PC and followed on an individual basis. The virtual EURL ECVAM lab was featured in the MOOC (see Section 5.2.2) and is available to download. As part of EURL

ECVAM's project targeting schools, it will engage with teachers to build on this initial endeavour to bring the lab to the classroom and will develop it further together with the science teachers. The tool however can equally be useful at higher education levels to demonstrate some procedures in our lab.

"The course is well structured – it is not too long and easy to follow at our own pace. The content is clear, and it helped me to pass the knowledge I received to my students. Moreover, I liked the MOOC live events, where we could hear the experts in the field. I also appreciated the quizzes at the end of the various sections: it's a sort of self-evaluation to test the MOOC's content acquisition.

The Padlet pages were a great tool for sharing and feeling part of a learning community. I particularly appreciated the resources of the Society for Humane Science (https://www.forhumanescience.org/) and their non-animal alternatives database: in this webpage I found a lot of useful links to replace in dissections.

Since I participated in the first MOOC, I've already had a chance to introduce Three Rs in the classroom in order to ignite students' activism towards such a meaningful topic and to stimulate their voices and opinions on a very debated ethical question. I like the fact that the Three Rs principles can be included and implemented into not only STEM lessons. This theme is cross-curricular and cross topical, raising various issues: social, political, and economical. Addressing the reduction of animal use in class really can take place in the context of several subjects, also humanistic.

I think that the best way to inspire young people to pursue a scientific career, particularly in this area is through stimulating their activism and letting them be protagonists of the learning process: they should feel that the Three Rs principles concern them too because it opens new scientific scenarios and new approach to testing lab work."

MOOC participant, teacher from Italy

- Scientix platform: *http://www.scientix.eu/home*
- EURL ECVAM education page: *https://europa.eu/!7w8JMh*
- Report: Three Rs into secondary schools, universities and continuing education programmes: *https://publications.jrc.ec.europa.eu/repository/bitstream/JRC123343/jrc123343online_1.pdf*
- The EURL ECVAM 3D interactive lab: *https://jeodpp.jrc.ec.europa.eu/ftp/jrc-opendata/EURL-ECVAM/datasets/LearningScenario/LATEST/20210906-virtual_ECVAM.rar*

5.2.4 The Three Rs in higher education

Education and training are fundamental to driving progress in the development and uptake of the Three Rs, and it is necessary to find common European strategies to involve experts, university professors and students sensitive to this topic. Developing and implementing a curriculum framework can be a

complex process, therefore, a bottom-up approach could be useful since it involves working with educators in universities, and ultimately envisages ways to bring the Three Rs into their lessons using different learning scenarios designed to suit a variety of different learning contexts.

Attempting to influence the curriculum in this way by targeting groups such as teachers, heads of schools or deans of university departments, who are usually not involved in the high-level process of decision-making, has been suggested and adopted in some cases (Nistor *et al.*, 2016). A group of key specialists in teaching Three Rs at university level was invited by the JRC to participate in an online meeting to discuss the needs in this respect and ways to improve Three Rs teaching. During the Three Rs higher education meeting, the participants discussed the feasibility of short, medium and long-term objectives.

They recommended investing in the continuous professional development of teachers and lecturers, to expand the number of teachers through a "train-the-trainer" approach and to support this by sharing content and materials for education. In terms of providing resources for educators, building on the current repository would be helpful, where teachers/educators could go to search for and download ready-made resources, delivered directly or tailored to suit a particular audience. Resources could be for example i) teaching slide-packages in different languages for different audiences, ii) online webinars to watch together with the students iii) EU-Academy seminars to train the teachers not yet implementing Three Rs in their courses.

6. Conclusions

As evidenced by the many activities described in this report, several actions are needed at various levels to progress the acceptance and use of NAMs.

These range from targeted funding and scientific investments into the development of NAMs, over building trust through validation/standardisation of NAMs and their application in case studies, enabling the use of data from NAMs under regulatory assessment frameworks, promotion and dissemination undertakings, to education activities for students and professionals. Importantly, it also needs a general change in mind-set. Shifting to next generation animal-free approaches does not mean less protection for humans and the environment. It rather promises a move from assessments based primarily on observations and extrapolation to those based on better understanding of biological processes and their alterations, ultimately providing levels of protection to humans and the environment that are at least as good if not better than those provided by animal tests.

NAMs should therefore be seen as an opportunity to avoid ethical dilemmas and scientific inadequacies involved in routine regulatory testing in animals.

Public-private partnerships such as the EPAA have discussed opportunities to use non-animal science in regulatory decisions for chemical safety in the EU. They made a range of recommendations on how to deploy NAMs in the most effective and credible way to generate the data required to inform decisions on human and environmental safety. New approaches in toxicology based on *in vitro* methods and computational modelling offer considerable potential to improve the efficiency and effectiveness of chemical hazard and risk assessment in a variety of regulatory contexts. However, this presents challenges both for developers and regulatory assessors because the same level of confidence in a new approach is often not shared between these two communities. Building confidence and trust thus remains an important cornerstone for the uptake of NAMs.

In the research arena, statistics collected from the EU Member States in a standardised and uniform way are essential to monitor when, how and where animals are being used and where to invest for obtaining impactful changes. In biomedical research where most animals are used, reviews of existing non-animal models in specific biomedical fields continued. Ways to facilitate communication and improve collaboration between different scientific communities were identified and the adverse outcome pathways framework, based on a structured representation of biological events leading to adverse effects, continued to be used, not least to model the pathogenesis of COVID-19. EURL ECVAM also continued to invest on further developing education and training resources for students at different education levels. Educating the next generation of scientists and regulators in NAMs and on essential ethical values is an important step towards a fundamental change in society.

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Abbreviations and definitions

2o3	2 out of 3
3D	Three-dimensional
ADME	Absorption, distribution, metabolism and excretion
AF	Application factors
AI	Artificial intelligence
ALI	Air-liquid interface
ANSA	8-anilino-1-naphthalenesulfonic acid ammonium
AOP	Adverse Outcome Pathway
APCRA	Accelerating the Pace of Chemical Risk Assessment
ASPIS	Animal-free Safety assessment of chemicals: Project cluster for Implementation of novel Strategies (H2020)
BfR	German Federal Institute for Risk Assessment
BIAC	Business and Industry Advisory Committee to the OECD
CARACAL	Competent Authorities for REACH and CLP
CEN	European Committee for Standardization
CENELEC	European Committee for Electrotechnical Standardization
CIAO	Modelling the Pathogenesis of COVID19 using the Adverse Outcome Pathway Framework
CLP	Classification, Labelling and Packaging
COVID-19	Coronavirus disease
CSS	Chemicals Strategy for Sustainability (EC)
DA	Defined approach
DASS	Defined approaches on skin sensitisation
DB	Database
DG ENV	Directorate-General for Environment (EC)
DG GROW	Directorate-General for Internal Market, Industry, Entrepreneurship and SMEs (EC)
DIO 1	Diodinase 1
DIP	Data interpretation procedure
DNA	Deoxyribonucleic Acid
DNEL	Derived no-effect levels of exposure
DNT	Developmental neurotoxicity
DRP	Detailed review paper
DTT	Dithiothreitol
EAGMST	Extended Advisory Group for Molecular Screening and Toxicogenomics
EC	European Commission (EU)
ECHA	European Chemicals Agency
ED	Endocrine disruptor
EDQM	European Directorate for the Quality of Medicines & HealthCare (Council of Europe)
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay

EMA	European Medicines Agency
EPA	Environmental Protection Agency
EPAA	European Partnership for Alternatives to Animal Testing
ERA21	Early Career Researchers Advancing 21st Century Science (PCRM)
ESAC	ECVAM Scientific Advisory Committee
ESR	Early Stage Researchers (MSCA-ITN)
ESTIV	European Society of Toxicology In Vitro
EU	European Union
EURION	European Cluster to Improve Identification of Endocrine Disruptors
EUROoCS	European Organ-on-Chip Society
EU-NETVAL	European Union Network of Laboratories for the Validation of Alternative Methods
EU-ToxRisk	An Integrated European 'Flagship' Programme Driving Mechanism-based Toxicity Testing and Risk Assessment for the 21st century
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
EWG	Expert Working Group (OECD)
FAIR	Findability, accessibility, interoperability, and reusability (of data)
FP	Framework programme
GARD	Genomic allergen rapid detection test
GD	Guidance Document
GHS	Globally Harmonised System of Classification and Labelling of chemicals
GIVIMP	Good *In Vitro* Method Practices
GTTC	Genetic Toxicology Technical Committee (HESI)
HBM4EU	European Human Biomonitoring Initiative
HESI	Health and Environmental Sciences Institute (US)
hIPSC	human induced pluripotent stem cells
HPPT	Human patch predictive test
IATA	Integrated Approaches to Testing and Assessment
IC50	Half-maximal inhibitory concentration
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
in3	An integrated interdisciplinary approach to animal-free chemical and nanomaterial safety assessment (MSCA-ITN)
IPCHEM	Information platform for chemical monitoring
ISES	International Society for Exposure Science
IT	Information technology
ITS	Integrated testing strategy
IUCLID	International Uniform Chemical Information Database
IVIVE	*In vitro* to *in vivo* extrapolation

IVT	*In vitro* battery
IWG	Informal working group
JaCVAM	Japanese Center for the Validation of Alternative Methods
JRC	Joint Research Centre (EC)
KB	Knowledge base
KE	Key event (AOP)
KMD	Kinetically derived maximum dose
LLNA	Local Lymph Node Assay
MA	Metabolic activation
MAD	Mutual acceptance of data
MIE	Molecular initiating event
MoA	Mode of action
MOOC	Massive open online course
MSCA-ITN	Marie Skłodowska-Curie Innovative Training Networks
MTD	Maximum tolerated dose
NAM	New approach methodology
NATM	Non-Animal test methods
NGO	Non-governmental organisation
NGRA	Next generation risk assessment
NICEATM	National Toxicology Programme Interagency Center for the Evaluation of Alternative Toxicological Methods (US)
NM	Nanomaterial
OECD	Organisation for Economic Co-operation and Development
ONTOX	Ontology-driven and artificial intelligence-based repeated dose toxicity testing of chemicals for next generation risk assessment (ASPIS cluster)
OoC	Organ-on-chip
OP	Optical density
PARC	European partnership for the assessment of risks from chemicals
PARERE	Preliminary Assessment of Regulatory Relevance network
PATROLS	Physiologically Anchored Tools for Realistic nanOmateriaL hazard aSsessment
PBD	Physiologically based dynamic
PBK	Physiologically based kinetic (also PBPK, PBBK, PBTK)
PC	Personal computer
PISC	PETA Science Consortium International
PNEC	Predicted No Effect Concentration
PoD	Point of departure
POP	Persistent organic pollutants
PrecisionTOX	Toward Precision Toxicology: New Approach Methodologies for Chemical Safety (ASPIS cluster)
PSIS	Putting Science Into Standard
QSAR	Quantitative Structure Activity Relationship

REACH	European Regulation (EC) no 1907/2006 Registration, Evaluation, Authorisation and Restriction of Chemicals
RhE	Reconstructed Human Epidermis
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 (the Netherlands)
RNA	Ribonucleic acid
RS	Reconstructed skin
RSMN	Reconstructed skin micronucleus
SCREENED	Screening for endocrine disruptors (EURION)
SOP	Standard Operating Procedure
SQL	Structured Query Language
STEM	Science, technology, engineering, and mathematics
SVM	Support vector machines
TBEV	Tick borne encephalitis vaccines
TCCD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TG	Test Guideline (OECD)
TK	Toxicokinetics
TM	Test method
TPO	Thyroperoxidase
TSAR	EURL ECVAM Tracking System on Alternative Methods towards Regulatory acceptance
TTR	Thyroxine-binding prealbumin
UISS	Universal immune system simulator
UK	United Kingdom
UN	United Nations
US	United States (of America)
VAC2VAC	Vaccine batch to vaccine batch comparison by consistency testing
VICH	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products
VU	Vrije Universiteit
WG	Working group
WHO	World Health Organization
WNT	Working Party of the National Coordinators of the Test Guidelines Programme (OECD)
WPEA	Working Party on Exposure Assessment (OECD)
WPHA	Working Party on Hazard Assessment (OECD)
XML	Extensible Markup Language

Figures and boxes

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