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EURL ECVAM Status Report on the Development, Validation and Regulatory Acceptance of Alternative Methods and Approaches (2013-April 2014)

A report prepared by EURL ECVAM

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Institute for Health and Consumer Protection

Contact information
Valérie ZUANG
Address: Joint Research Centre, Via Enrico Fermi 2749, 21027 Ispra (VA), Italy
E-mail: Valerie.ZUANG@jrc.ec.europa.eu
Tel.: +39 03332 78 5843
Fax: +39 03332 78 9963

http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam
https://ec.europa.eu/jrc

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Abstract

The EURL ECVAM status report provides an update on the progress made in the development, validation and regulatory acceptance of alternative methods and approaches since the last report published in April 2013. It is informing on ongoing research and development activities, validation studies, peer reviews, recommendations, strategies and international acceptance of alternative methods and approaches.
R&D activities are ongoing for the complex endpoints where the toxicological processes and the mechanistic understanding have not been sufficiently elucidated yet and for which 3Rs solutions are more difficult to find. On the other hand, good progress In the validation and regulatory acceptance is made in areas where non-animal alternative methods have been developed and validated and where the focus lies in an intelligent combination/ integration of the various non-animal approaches.

EURL ECVAM Status Report 2013—April 2014

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Executive Summary

The EURL ECVAM status report provides an update on the development, validation and regulatory acceptance of alternative methods/approaches since the last report prepared by EURL ECVAM in April 2013 in the framework of the Cosmetics Regulation. It covers the year 2013 to April 2014 and primarily, but not exclusively, EURL ECVAM activities. This year the status report also includes updates on alternatives in the areas of vaccines, fish toxicity and fish bioconcentration/bioaccumulation, in addition to the health-effect endpoints which were traditionally described in previous reports[1].

Research and development activities continued for the complex toxicity areas where robust alternative methods and their appropriate integration are still lacking. These projects have led to an improved understanding of toxicological processes and new methodologies and tools for mechanistic-based toxicology are emerging. Targeted R&D projects aiming at improving fish toxicity testing by reducing or avoiding the use of fish were also supported.

The Adverse Outcome Pathway (AOP) framework was further developed and taken up by the OECD to support the collection, organisation and evaluation of relevant chemical, biological and toxicological information for use in human health and environmental risk assessment. The framework provides a tool for a knowledge-based safety assessment that relies on understanding toxicity and helps to identify where methods should be developed and prioritised for validation and how the different approaches should be best integrated to ultimately replace the traditional animal tests.

EURL ECVAM strategies aiming at outlining holistic solutions to achieve reduction, refinement and replacement of animal testing while maintaining or improving human and environmental protection were developed in the areas of genotoxicity, acute systemic toxicity, aquatic toxicicity and bioconcentration/bioaccumulation testing and toxicokinetics.

Test submissions addressing predominantly the endpoints of topical toxicity (skin and eye irritation) and skin sensitisation were received, followed by methods for endocrine disruption, genotoxicity, acute fish toxicity and intestinal permeability testing. In order to explore the availability and the development status of *in vitro* methods in the priority area of toxicokinetics and in view to solicit their submission, EURL ECVAM launched a survey and a call for *in vitro* methods for estimating human hepatic metabolic clearance/stability.

The EURL ECVAM coordinated validation studies on eye irritation (EpiOcular EIT), and CYP-induction were completed in 2013 and in 2014 they entered peer review by the ECVAM Scientific Advisory Committee (ESAC). The ESAC peer review of the h-CLAT method (skin sensitisation) was finalised in 2014. Validation studies on Estrogen Receptor and Androgen Receptor Transactivation Assays (ERTA/ARTA) are either still on-going (ERTA) or were recently started (ARTA). The first validation study making use of the recently established

[1] http://ec.europa.eu/consumers/sectors/cosmetics/animal-testing/index_en.htm

European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) was launched in 2014 for the validation of an ARTA.

Several validation studies on alternative methods for potency and safety testing of various vaccines for human and veterinary use are currently ongoing or planned to start in 2014 within the framework of the Biological Standardisation Programme (BSP) of the European Directorate for the Quality of Medicines & HealthCare (EDQM; Council of Europe) and co-sponsored by the European Commission.

External validation studies (i.e. not co-ordinated by EURL ECVAM) in the areas of genotoxicity, carcinogenicity, endocrine disruption, eye and skin irritation were submitted to EURL ECVAM in view of ESAC peer review. The external validation study on the Bhas 42 CTA was peer reviewed by ESAC and a EURL ECVAM Recommendation was published in 2013. The ESAC peer review of a test method based on a reconstructed human epidermis for skin irritation testing, also externally validated, is currently on-going.

The recently established EURL ECVAM Recommendations are becoming an effective tool to promote and facilitate international regulatory acceptance of validated methods. These Recommendations provide EURL ECVAM views on the validity of a test method including its limitations and proper scientific use, possible regulatory applicability, and possible follow-up activities in view of addressing knowledge gaps. They are developed in close consultation with EURL ECVAM's advisory network of regulators (Preliminary Assessment of Regulatory Relevance (PARERE) network), its stakeholder forum (ESTAF) and international collaboration partners (ICATM).

EURL ECVAM Recommendations on the validated 3T3 Neutral Red Uptake (3T3 NRU) cytotoxicity assay, the Direct Peptide Reactivity assay (DPRA), the KeratinoSens and on the Bhas42 cell transformation assay were published in 2013/2014. The Recommendation on the Zebrafish embryotoxicity test is currently undergoing public commenting. Draft Test Guidelines (TG) on all the validated and peer reviewed methods have been prepared and submitted to the OECD Test Guidelines Programme, apart from the 3T3 NRU cytotoxicity test which is already an OECD Guidance document. A TG based on the Fish embryo acute toxicity (FET) test was already published as OECD TG 236 in 2013.

Further developments were made to content and functionality of web-based communication and dissemination tools on alternative methods hosted by EURL ECVAM, including the database on alternative methods, DB-ALM, and the system for tracking progress of alternative methods through validation and regulatory acceptance (TSAR). A revised version of the EURL ECVAM Search Guide was also published.

The scope of collaboration with partners within the International Cooperation on Alternative Testing Methods (ICATM) working on the validation and acceptance of alternative methods was extended to include increased alignment of respective workflows, earlier consultation and joint evaluation of promising methods, and coordination of post-validation activities to facilitate regulatory acceptance and uptake globally.

1. Introduction

The EURL ECVAM status report has been prepared with a view to respond to the frequent requests of stakeholders on the status of alternative methods and approaches. It is intended to inform many interested parties and serve multiple purposes, including providing input to the annual Commission report prepared by DG SANCO on the progress made in the development, validation and regulatory acceptance of alternative methods/approaches required by Regulation 1223/2009 on cosmetic products. The status report describes primarily, but not exclusively, activities that EURL ECVAM has undertaken or has been involved in during the period from March 2013 to April 2014. The previous EURL ECVAM progress report was published in May 2013 and covered the years 2010 to April 2013[2].

Unlike previous reports that primarily focused on the regulatory toxicity endpoints of relevance to the Cosmetics Regulation, this report also includes updates on the areas of vaccines, fish toxicity and fish bioconcentration/bioaccumulation. Moreover, the layout of the report has been adapted to follow more the lifecycle of alternative toxicity tests and approaches destined for regulatory use, i.e. from research and development activities, through validation, to regulatory uptake.

Testing of vaccines and 3Rs methods

Vaccines play an important role in the prevention of human and animal diseases. They are heterogenous biological products containing components as antigens, adjuvants, excipients, and preservatives. Their composition and manufacturing process varies depending on the type of vaccine and potential use. All vaccines (as other medicinal products) for human or veterinary use have to be authorised and registered either at Member State or Community, i.e. European Medicines Agency, level before they can be placed on the EU market (Directive 2001/82/EC; EC, 2001a; Directive 2001/83/EC; EC, 2001b and amendments). Requirements which have to be met by a given vaccine are laid down in e.g. European Pharmacopoeia monographs, EU guidelines or WHO recommendations. In addition to the tests carried out for obtaining the marketing authorisation, the quality of a vaccine batch must be controlled by the manufacturer and, in Europe, by an Official Medicinal Control Laboratory (OMCL) before it is released for use.

Traditionally, laboratory animals have played a major role in batch release testing of vaccines and still many are required, in particular for potency testing of inactivated vaccines (e.g. tetanus, diphtheria, pertussis, rabies, clostridial, leptospiral vaccines) and safety testing of e.g. acellular pertussis vaccine, tetanus vaccine, and oral polio vaccines.
Over the last two decades, numerous alternative methods to classical animal tests have been developed, validated, incorporated into European Pharmacopoeia monographs (Council of Europe, 2013) and successfully implemented at manufacturer and OMCL level.

[2] see http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-releases-2013-progress-report-development-validation-regulatory-acceptance-alternative-methods.

Moreover, the concept of vaccine quality control is changing as emphasis is being put on ensuring the consistency of production of a vaccine (Hendriksen et al, 2008; De Mattia et al, 2012). This shift of focus from the final product (batch) to the production process by using intensive in-process testing and modern quality systems (consistency approach) is already in place for new generation vaccines, which are better defined and allow the use of *in vitro* and physico-chemical methods for their characterisation and quality control. The challenge will be to implement the consistency approach for established vaccines e.g. DTaP, rabies, clostridial vaccines, which are less defined and therefore more difficult to characterise (see paragraph 5.5.2.2, EPAA vaccine project).

Fish Toxicity and Fish Bioconcentration/Bioaccumulation

Environmental hazard identification is an important component of the safety assessment of all types of chemicals (e.g. industrial chemicals, plant protection products, biocides, pharmaceuticals, feed additives) relevant in the context of EU and international regulations aiming at the protection of the environment. Vertebrate animals used for environmental hazard and risk assessment are fish, amphibia, birds and, on rare occasions, mammals. A recent paper of Scholz et al (2013) summarises possibilities to reduce the use of vertebrates in environmental risk assessment covering aquatic toxicity, avian toxicity, bioaccumulation and endocrine activity.

Over the past ten years, EURL ECVAM's activities have been focused on 3Rs methods for aquatic (fish) toxicity testing and fish bioconcentration/bioaccumulation testing. Work carried out at the JRC resulted in the threshold approach for acute fish toxicity testing (OECD guidance document 126; OECD, 2010), a testing strategy which has the potential to significantly reduce the number of animals used for acute fish toxicity testing. The threshold approach is included in the ITS proposed in the REACH guidance document on aquatic toxicity (ECHA, 2012), in the biocides regulation (EU, 2012) and plant protection products data requirements (EU, 2013a; EU, 2013b). More recently, EURL ECVAM coordinated on behalf of OECD the validation of the zebrafish embryo acute toxicity test which resulted in the OECD TG 236 Fish embryo acute toxicity (FET) test (OECD, 2013; see also XX) EURL ECVAM recommendation).

2. Research and Development Activities Related to Alternative Methods

2.1 SEURAT-1

2.1.1 Overview of SEURAT-1

With the collective aim of ultimately replacing *in vivo* repeated dose systemic toxicity testing with animal-free solutions, the SEURAT-1 cluster is the largest EU initiative ever undertaken on alternative methods to animal testing, comprising 70 European research partners equally co-financed through a unique public-private partnership between the European Commission (FP7 Programme administered by DG Research & Innovation) and Cosmetics Europe. SEURAT-1 combines five research projects (COSMOS, Scr&Tox, DETECTIVE, HeMiBio and NOTOX), a central data and knowledge management project (ToxBank) and a coordination action (COACH). The Systems Toxicology Unit/EURL ECVAM (JRC-IHCP), is heavily involved in three of the five complementary research projects, and manages the coordination efforts of the entire cluster.

Figure 1. Research Projects of SEURAT-1

SEURAT-1 started its activities in 2011 and all the projects are by now well advanced in their work programmes and a wide variety of new methodologies and tools for mechanistic-based toxicology are emerging, such as complex bioreactors for engineering human tissues, innovative 'omics' techniques, differentiation protocols for human induced pluripotent stem cells and modelling tools.

At the centre of all activities there is much emphasis on Proof-of-Concept (PoCs) case studies that will demonstrate the implementation of the SEURAT-1 research strategy, i.e. to develop complementary theoretical, computational and experimental (*in vitro*) models to predict toxicity in humans and apply the predictions in safety assessment of chemicals (see below).

2.1.2 Seurat-1 related collaboration between JRC and Cosmetics Europe

A collaborative arrangement between the JRC and Cosmetics Europe aims to supplement the work programme of the SEURAT-1 cluster. This research initiative, although motivated by the Cosmetic industry, is also relevant to other industrial sectors (e.g. industrial chemicals, pharmaceuticals).

The aims of this project are (a) to facilitate cluster interaction and to demonstrate proof-of-concept at three different levels, namely 1) defining mode-of-action (MoA), 2) predicting toxicity, and 3) supporting safety assessment decisions, (b) to supplement the computational modelling activities of the cluster and (c) to generate large reference datasets to support the establishment of toxicological MoA. In brief, the following work was carried out by EURL ECVAM:

- An assessment framework was proposed that could be used to predict the potential toxicity of a substance, under repeat dose exposure conditions, by using a range of complementary non-animal methods that are integrated according a toxicological MoA framework. This was followed by the cluster-wide definition of predictive toxicology case studies involving collaboration across the six projects within the SEURAT-1 cluster;
- An open source software tool was developed for simulating the long-term (repeat dose) toxic effects of chemicals, including substances in cosmetics and personal care products, in *in vitro* systems. The approach was based on the previously developed Virtual Cell Based Assay (VCBA) (Zaldivar et al, 2010, 2011, 2012), re-coded and re-implemented in the open-source KNIME (– The Konstanz Information Miner) platform. Two case study compounds, paracetamol (acetaminophen) and amiodarone were selected to illustrate the usefulness of this tool in simulating the repeat dose toxicity of compounds;
- An open source approach was developed for predicting the potential of chemicals, which can also be used for substances in cosmetics and personal care products (ingredients and impurities), to bioaccumulate in humans following realistic exposure scenarios. The approach is based on a PBTK model implemented as a KNIME workflow, an open-source application which is freely available on request;
- An illustration of how in silico models describing the kinetics and dynamics of chemicals in cell cultures and the human body can be applied in risk assessment to translate *in vitro* toxicity data into predictions of human toxicity under defined consumer exposure scenarios (*in vitro* to in vivo extrapolation; IVIVE). The consumer exposure scenarios selected were based on the expected use of three caffeine-containing cosmetic products;
- Review of the availability of in silico and *in vitro* methods for assessing dermal bioavailability as being dependent on dermal penetration and first pass dermal metabolism. In the safety assessment of cosmetics, the measurement or simulation of these processes is needed to apply methodology for extrapolating from oral-to-dermal exposure scenarios, and in particular for extrapolating from repeat dose oral toxicity data to repeat dose effects in the skin;
- Approach to design and optimise treatment protocols for repeat dose *in vitro* experiments, based on computational modelling, analytical chemistry tools and mechanistic knowledge. A step-by-step guidance on how to perform repeat dose *in vitro* experiments, consisting of the seven steps, was provided to illustrate the approach;

- Concentration-response data on 100 reference chemicals relevant to hepatotoxicity MoA were provided together with a summary of the scientific/technical work performed to obtain the dataset. The human metabolically competent cell line HepaRG was used for testing reactive oxygen species formation as indicator of oxidative stress and the formation of neutral lipid droplets as indicator of chemical-induced steatosis. Nuclear intensity (an indicator for DNA condensation), nuclear area (an indicator for cell injury) and cell count (an indicator for cell viability) were also analysed and this multi-parametric analysis allowed a categorisation of the tested chemicals. The full raw dataset as well as the protocols used are available for the entire SEURAT-1 cluster for further data analysis through the ToxBank database.

2.1.3 COSMOS project

The COSMOS project (http://www.cosmostox.eu/) is developing publicly available computational workflows based on the integrated use of open-access and open-source models for the prediction of repeated dose toxicity (Anzali et al, 2012). This includes: a) the establishment of an inventory of cosmetic substances (including identifiers and chemical structures) and a repeat dose toxicity database (including oral and dermal data); b) the development of novel ways of establishing thresholds of toxicological concern (TTC), based on innovative chemistry based prediction approaches and biokinetic modelling.

2.1.4 COSMOS database

Although several databases for chronic toxicity data are available, no single repository of chronic toxicity data exists that is open and transparent and includes all aspects of the data such as protocol details. Therefore, the COSMOS project is building a new freely accessible database as a comprehensive and reliable resource for repeated dose toxicity data. The COSMOS database (Rathman et al, 2013) is also linked to high quality and validated chemical structures to facilitate modelling. The first version of the database (v1.0) was released in December 2013 and is accessible via the COSMOS project website. V1.0 of the database contains oral repeat-dose toxicity data (subacute, subchronic, and chronic studies as well as carcinogenicity studies) for over 1000 cosmetics-related chemicals.

2.1.5 PB(T)K modelling

Physiologically-based kinetic (PB(T)K) models can be used to predict *in vivo* toxicokinetics (e.g. time-course of blood or tissue concentrations of a chemical) based on *in vitro* measurements of the underlying processes of absorption, distribution, metabolism and excretion (*in vitro* to in vivo extrapolation; IVIVE), as well as to extrapolate existing animal blood time-courses of a chemical for one exposure route to another exposure route (route-to-route extrapolation; RtR), e.g. oral-to-dermal extrapolation. During 2013-2014, these PB(T)K modelling approaches have been applied for selected compounds within COSMOS (Pery et al, 2013, Gajewska et al, 2014). In addition, a survey with respect to *in vitro*

hepatic metabolic clearance/stability methods has been published on the JRC website. The results of these methods should directly feed into the PB(T)K models. A workshop on this issue will be organised in May 2014.

2.2 Adverse Outcome Pathways (AOPs)

Improved understanding of toxicological processes, together with advances in toxicogenomics, bioinformatics, systems biology and computational toxicology, facilitate the paradigm shift in the approach to regulatory toxicity testing and risk assessment. In this context, the Adverse Outcome Pathway (AOP) framework has been developed to support the collection, organisation and evaluation of relevant chemical, biological and toxicological information for use in human health risk assessment. It provides a tool for a knowledge-based safety assessment that relies on understanding toxicity, rather than simply observing its effects.

The AOP construct is based on the mechanistic understanding of toxicological processes on different biological levels that ultimately lead to an adverse health effect, defined as a series of key events, starting from an initial interaction of a chemical with a biological target (molecular initiating event, MIE) and consecutively showing the key events (KEs) at the molecular, cellular, tissue, organ, and organism levels.

The emphasis moves away from demonstrating the final adversity, to demonstrating the initiating and intermediate events. Among other things, this approach allows the profiling of chemicals according to their potential to trigger such events, instead of the traditional way of associating them with an apical adverse endpoint.

The AOPs elaborated to date are mainly qualitative pathway descriptions, and further research is necessary to develop a more-precise characterisation of the dynamic relationships between actors and events also in quantitative terms. Even though the level of information currently available is not sufficient to perform a comprehensive risk assessment, a well-described AOP may still provide useful information for many purposes, such as priority setting for further testing, hazard identification, classification and labelling, and the development of an integrated test system or read-across for categorisation of chemicals. All this will help to refine, reduce and/or replace conventional in vivo animal testing.

To coordinate and harmonise various international efforts in AOP development, the OECD launched the AOP Development Programme in January 2013. This programme is managed by the Extended Advisory Group on Molecular Screening and Toxicogenomics that the EURL ECVAM is co-chairing together with the US Environmental Protection Agency (EPA).

In this context an AOP Knowledge Base (AOP KB) is being developed in a collaborative effort between EURL ECVAM and US EPA to facilitate the collection, integration, curation

and dissemination of current mechanistic pathway knowledge contributed and evaluated by a wide range of experts. The OECD has launched the AOP-KB project in order to give the global scientific and regulatory communities the possibility to enter, share and discuss their AOP related knowledge within one comprehensive platform. Thanks to this IT system, AOP developers can create an AOP wiki page and then build an AOP by linking related information about Molecular Initiating Events, intermediate Key Events, Adverse Outcomes and Chemical Initiators. Controlled-vocabulary drop-down lists from which to select Methods, Actions, Biological Targets, Life stages, Species etc. related to the AOP simplify the entry of ontology-based information. Information regarding Key Events and Adverse Outcomes shared among multiple AOPs is stored on a single page to eliminate redundant entries and make the collective knowledge about those entities available in all AOPs containing them. This format also facilitates the identification of potential intersections and cross-talk among AOPs.
Phase 1 of the AOP-KB project, the AOP Wiki, is now finished, and some twenty AOPs have been entered into the system, where they are now reviewed, discussed and enhanced - with a view to an eventual adoption at OECD level. The next phase of the project will add quantitative and graphical elements.

An example of an AOP-based research strategy and of how new insights into mechanistic toxicology can be applied for safety assessment, is given by SEURAT-1 in relation to repeat-dose systemic toxicity (see paragraph 2.1 above). In this context, EURL ECVAM took the lead on the development of two AOPs related to liver fibrosis and liver steatosis, following the OECD guidance and template. These are complementing the on-going experiments at EURL ECVAM for evaluating responses to toxicants by combining a relevant human liver cell model (HepaRG cells) with high-content cellular imaging, implemented on an automated platform for high-throughput screening,.

Liver fibrosis is a reversible wound healing response to a variety of chronic injuries including toxic injury from chemicals. It results from an imbalance between the deposition and degradation of extracellular matrix (ECM) and a change of ECM composition. Pathogenic fibrosis typically results from chronic injury with sustained production of growth factors and fibrogenic cytokines in which inflammation, tissue destruction, and repair processes occur simultaneously. The case study with the qualitative description of the AOP from Protein Alkylation to Liver Fibrosis using Allyl Alcohol and Carbon Tetrachloride as reference chemicals reports a plausible but incomplete pathway description and needs further refinement and in-depth analysis with added information on gene expression, quantitative data on dose-response relationships and temporal sequences.

A project to expand this case study to a more widely applicable AOP by looking for data on quantitative and temporal relationships and exploring the chemical space that leads to

fibrosis has been accepted as an OECD AOP development project and included in the work plan of the AOP Development Programme.

The use of nanoparticles in foods and food products and therefore exposure via ingestions is rapidly growing. Nanotoxicology is a complex field with currently many knowledge gaps and a better understanding of the mechanisms of toxicity of nanoparticles is needed. EURL ECVAM is also working on the development of AOPs specific to nanoparticle-induced liver toxicity.

To date there are only few descriptions of AOPs leading to neurotoxicity. This lack of AOPs, and in particular the lack of MIEs, has made it difficult to evaluate the predictive ability of high-throughput chemical testing for neurotoxicity. As a first step in applying the AOP framework to adverse health outcomes associated with exposure to exogenous neurotoxic substances, EURL ECVAM organized a workshop in March 2013 to identify potential AOPs relevant to neurotoxic and developmental neurotoxic outcomes. Although the AOPs outlined during the workshop are not complete, they serve as a basis for further research and identification of MIEs and Key Events (KEs). Following this workshop EURL ECVAM is focussing on the development of an AOP to developmental neurotoxicity via thyroid disruption.

Another project within EURL ECVAM is the AOP development for Peroxisome Proliferator-Activated Receptors (PPAR)-mediated rodent reproductive toxicity. There is substantial evidence for the involvement of PPAR in toxicity to male and female reproductive function. However the underlying mechanisms of these effects still require further investigations.

A further activity is the identification of common mechanisms, like mitochondrial toxicity or cytoskeleton destabilisation, which are associated with different toxicological endpoints.

2.3 Fish Toxicity R&D projects

Three R&D projects related to fish toxicity, which are of specific interest to EURL ECVAM, are described here below.

2.3.1 EPAA scientific award project 2012 – Increasing the predictive capacity of the fish embryo test[3]

EURL ECVAM is on the scientific board of this research project awarded to Nils Kluever (UFZ, Leipzig, Germany). The overall aim of the project is to improve the Zebrafish embryo acute toxicity test (OECD TG236, OECD, 2013a) for predicting fish acute toxicity. For this purpose, UFZ analysed acute fish toxicity data (LC50) derived with juvenile/adult fish (OECD TG 203; OECD, 1992) and fish embryos. In case that fish LC50 values indicate a significantly higher toxicity than fish embryo LC50 values for a given chemical, possible

[3] http://ec.europa.eu/enterprise/epaa/platform-science/award/science_award_en.htm

reasons for this difference will be investigated, e.g. chemical properties, protocol used for the fish embryo test (age of the embryos, exposure duration, etc.), and metabolism. If deemed necessary, chemicals were tested in the Zebrafish embryo acute toxicity test and, in addition to the four lethal endpoint, sublethal endpoints were used to determine toxic effects. Preliminary results indicate that the predictivity of the Zebrafish embryo acute toxicity test for chemicals with neurotoxic mode of action being highly toxic to juvenile and adult fish may be improved by assessing sublethal endpoints. The project was finalised in 2013 and publication of the results is in preparation. It is further foreseen to include the findings into the planned OECD guidance document on the use of the Zebrafish embryo acute toxicity test for acute fish toxicity testing.

2.3.2 Use of a fish cell line-based cytotoxicity assay for acute fish toxicity testing

Within the CEllSens project[4], the RTgill-W1 (rainbow trout gill cell line) cytotoxicity assay was standardised and based on the promising results for the 35 organic chemicals tested, Tanneberger et al (2013) propose its use for acute fish toxicity testing. However, further evaluation of its reproducibility, predictive capacity and applicability domain is needed. As a follow-up of the CEllSens project, EAWAG (K. Schirmer) launched a ring trial assessing the transferability of the assay. In addition, the method was submitted to EURL ECVAM in early 2014 and the assessment of the test submission is ongoing (see paragraph 3.8).

2.3.3 Development of AOPs for chronic fish toxicity testing

Several research groups are working on the identification and description of potential AOPs relevant to chronic fish toxicity, which is currently assessed with fish early life-stage (FELS) test (OECD TG 210; OECD 2013b). In 2013, the CEFIC LRI-funded project (LRI-ECO20-UA) *Development of an alternative testing strategy for the fish early life-stage test for predicting chronic toxicity* started. It aims to map FELS-relevant AOPs, develop an *in vitro* toolbox for screening FELS-relevant AOPs (Tier 1) and Zebrafish embryo based assays (Tier 2). The report of a recent ILSI HESI workshop (Villeneuve et al., 2014) discusses AOPs relevant to FELS toxicity and how they could be discovered and annotated. The authors define key events in the development of a fish embryo as development of the central nervous system, cardiovascular system, liver, kidney, etc. They give examples of hypothesised AOPs focusing on impaired swim bladder inflation and reduced survival of young fish.

[4] ECO8; http://www.cefic-lri.org/projects

3. Test Method Submissions

3.1 Introduction to Test Method Submissions

New *in vitro* methods usually enter the EURL ECVAM validation workflow through a two-step test submission process that target very broadly all human health(-related) and environmental effects and biologicals. A tracking system of alternative methods towards regulatory acceptance provides an overview of all test methods that were submitted to EURL ECVAM for validation and/or peer review from 2008 up to now[5]. This tracking system is currently under revision to add information on the validation and regulatory acceptance process of submitted test methods.

Ideally, test submissions are assessed in the context of EURL ECVAM strategy papers that were, or are being defined in various toxicological areas such as e.g. skin sensitisation, genotoxicity, acute systemic toxicity, fish toxicity, (developmental) neurotoxicity and toxicokinetics (TK, see paragraph 8.5).

The toxicokinetic strategy paper for example, identified hepatic metabolic clearance as a fundamental piece of necessary information since it plays a key role in the transformation and the elimination of chemicals from the human body. As various *in vitro* methods for human hepatic metabolic clearance/stability have been developed in recent times and in order to solicit the submission of the most promising ones, EURL ECVAM has recently launched a survey and a call for *in vitro* methods for estimating human hepatic metabolic clearance/stability[6].

In future, beside the normal test submission process mentioned above, EURL ECVAM may launch other similar calls for *in vitro* methods in areas that have been identified as regulatory priority areas and where *in vitro* methods may have been sufficiently developed and optimised to enter the EURL ECVAM validation workflow.

During the time period covered by this report, in total, 9 test methods have been submitted to EURL ECVAM, of which 4 were pre-submissions, reporting information in the *Test Pre-Submission Form (TPF)*, and 5 were full submissions, reporting information in the *Test Submission Template (TST)*, normally complemented by several annexes. Noteworthy, out of the 5 full submissions, 4 were on external validation studies submitted to EURL ECVAM in view of ESAC peer review. Beside the assessment of the test submissions received from 2013 to April 2014, assessments of test submissions received in late 2012 were also conducted and are reported here below. The majority of test submissions received by EURL ECVAM in the time period covered by this report address the skin sensitisation endpoint followed by similar test methods (so-called "me-too's") for skin irritation testing. One method was submitted in the area of eye irritation, genotoxicity, acute fish toxicity intestinal permeability and endocrine disruption, respectively.

[5] see http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/test-submission

[6] see http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eu-netval/in-vitro-methods-survey-March-2014

3.2 Test method submissions related to skin sensitisation

The Genomic Allergen Rapid Detection (GARD) test method is a transcriptomics-based *in vitro* assay proposed to discriminate between skin sensitising and non-sensitising chemicals on the basis of the expression level of mRNA transcripts for a panel of 200 genes analysed using a whole genome array (Johansson et al., 2013). The cell line used is the human myeloid leukemia-derived cell line MUTZ-3, as a model for in vivo dendritic cells (DC) (Johansson et al., 2011, 2013). Optimisation work is ongoing to further optimise the prediction model for potency prediction and to transfer the assay to a real-time quantitative polymerase chain reaction (qRT-PCR) platform. EURL ECVAM is monitoring these developments and the test developer has been invited to resubmit the test method once this work has been finalised.

The RHE IL18 Potency Test represents an update of the two-tiered approach, previously submitted to EURL-ECVAM. In the RHE IL18 Potency Test the two readouts, intracellular IL-18 released used to discriminate between sensitisers and non-sensitisers, and viability as a measure of sensitising potency are combined in a single test which uses reconstructed human epidermis (RhE) as test system (Gibbs et al., 2013). The test method is proposed as an improvement to the two-tiered approach since it more closely resembles the architecture and the barrier function of the normal human epidermis and allows testing low water soluble chemicals since the protocol foresees the use of solvents and solvent mixtures with different polarities.

The updated Myeloid U937 Skin Sensitisation Test (MUSST), which distinguishes sensitisers from non-sensitisers on the basis of CD86 membrane protein enhanced expression in U937 cells, includes an addition to the prediction model, consisting of a score derived from 6 rules that allows classifying a chemical, which would have been considered inconclusive on the basis of the original prediction model, as a sensitiser or non-sensitiser. The MUSST prediction model and its addition have been incorporated in an application called “MUSST PREDICTOR” which allows automated data analysis and derivation of individual run conclusions and the final chemical's classification. The chemical solubilisation procedure was also revised to minimise variability in vehicle selection.

The SENS-IS is a gene expression-based test method proposed to discriminate between sensitisers, non-sensitisers and irritants by analysing the expression of a panel of 65 genes grouped in one gene set for irritancy and two (SENS-IS and ARE) for sensitisation. A test substance is classified as sensitizer on the basis of the number of overexpressed genes (compared to the solvent control) measured by qRT-PCR in Episkin tissues (SkinEthic, France). 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.

The RHE IL 18 Potency Test, the updated MUSST and the SENS-IS are being evaluated in external multi-laboratories studies (i.e. not coordinated by EURL ECVAM). In case the validation of these test methods will be successfully finalised, the information generated will be submitted to EURL ECVAM for evaluation and eventual ESAC peer review.

The Skin Sensitizer Predictor is a test method proposed for skin sensitisation hazard assessment. The expression levels of mRNA transcripts for a set of four genes and proteins involved in three different intracellular signalling pathways are quantified following treatment of a mouse foetal skin-derived DC cell line (FSDC) with test chemicals. Readouts are then used in a prediction model based on linear discriminant analysis to derive a Prediction Score for distinguishing between sensitising and non-sensitising chemicals (Neves et al., 2013). Following the evaluation of the submitted information EURL ECVAM decided not to pursue this submission further since it was felt that additional optimization work on the test method was needed.

3.3 Test method submission related to intestinal permeability

The Caco-2 permeability assay was received in 2013. It is an *in vitro* assay that measures the permeability of compounds through a human intestinal epithelial cell barrier. The intestinal permeability measured is expressed as apparent permeability (Papp value). The test method was proposed for bioequivalence testing of pharmaceuticals, while its relevance to chemicals testing was uncertain. Some shortcomings were identified during the evaluation process. For instance, there was no clear evidence on the improvements offered by the Caco-2 permeability assay proposed in comparison to other available assays. It was also unclear how the apparent permeability's cut off values proposed for classification were derived. The test method was not prioritised since its potential 3Rs impact was unclear.

3.4 Test method submission related to genotoxicity

A full test submission was received in 2013 on a method intended to predict the *in vivo* genotoxic potential of chemicals: the Green Screen HC™ (GSHC) assay. The GSHC is a microplate format genotoxicity plus cytotoxicity screening assay which uses the DNA damage-inducible "Growth Arrest and DNA Damage 45 alpha" (GADD45a) - Green Fluorescent Protein (GFP) reporter gene, expressed in the p53-competent human lymphoblastoid TK6 cell line (Hastwell et al., 2006; Jagger et al., 2009). Upon DNA damaging agent treatment, GADD45a is highly induced mostly in a p53-dependent manner. When GADD45a transcription is increased over its constitutive level, cells accumulate GFP, the fluorescence of which can be measured as a proportional assessment of genome damage and genotoxic stress.

Based on the information provided, the method appears to be mechanistically and biologically relevant in relation to genotoxicity. A well standardised protocol is already published in the EURL ECVAM DB-ALM (http://ecvam-dbalm.jrc.ec.europa.eu/). The data provided in this submission are convincing enough to support the use of the test method as a screening tool in early phases of compound discovery and development. However, due to the properties of the method as a general indicator of genotoxicity that does not allow the identification of the specific mode of genotoxic action, the actual role of the GreenScreen HC™ method within current regulatory testing approaches needs to be further elucidated. In the light of this, and before considering peer review by the ECVAM Scientific Advisory

Committee (ESAC), the EURL ECVAM network of regulators (PARERE - Preliminary Assessment of Regulatory Relevance) will be consulted to further the assessment of regulatory relevance of the test method.

3.5 Test method submissions related to skin irritation

EURL ECVAM regularly receive requests and inquiries on technical matters relating to the development, optimisation and validation of in *vitro* skin irritation test methods based on tissue engineered Reconstructed human Epidermis (RhE) models. Moreover, all formal test submissions received in the period covered by this report concern RhE-based test methods seeking formal evaluation and validation by EURL ECVAM in reference to the Performance Standards for *in vitro* skin irritation testing (EURL ECVAM 2009; OECD TG 439, 2010). The submissions concern requests for the evaluation of similarity of test methods prior to the execution of external PS-based validation studies. In response to such submissions, EURL ECVAM conducts an assessment of the compliance of the submitted test methods with the criteria as outlined in the 'essential test method components' of the PS. These assessments may be subject to scientific judgements since the PS do not provide a simple "tick-box approach" and are thus reviewed and, if appropriate, confirmed by ESAC. Other submissions concern finalised PS-based validation studies conducted by industry and submitted for evaluation by EURL ECVAM and eventual ESAC peer review.

Importantly, so far all internationally accepted test methods for skin irritation testing (apart from the traditional animal test) are *in vitro* test methods based on the Reconstructed Human Epidermis (RhE) and the majority of these test methods have been validated on the basis of the EURL ECVAM PS for *in vitro* skin irritation testing). As outlined above, all test methods submitted to EURL ECVAM are based on this technology, with minor variations relating to specifications of the test system or the way the test system (i.e. RhE) is being produced.

The epiCS® test method concerns an external validation study based on the Performance Standards for *in vitro* skin irritation testing (EURL ECVAM, 2009; OECD TG439). As for other RhE-based methods, the same test system (i.e. RhE skin model) is used both for skin irritation and skin corrosion testing: depending on the health effect to be assessed the appropriate SOP (either for corrosion or for irritation assessment) needs to be chosen. The test system formerly marketed as "EST-1000" has been recently renamed to epiCS®. The test system and associated SOP have already undergone external PS-based validation for skin corrosion testing and the epiCS test method has been included in OECD TG431 on *in vitro* skin corrosion testing based on RhE (OECD TG431). The test method for skin irritation testing had been submitted to EURL ECVAM already in 2009 in view of assessing compliance of the test system / protocol with regard to the specifications outlined in the PS (essential test method components). After confirmation of compliance by EURL ECVAM, the submitter organised a ring trial involving three laboratories from industry, including one from overseas (US) and one naïve one (i.e. no prior experience with using RhE-based test methods). The study, submitted in 2011, showed that the test method fulfilled the acceptance criteria of the PS with regard to predictive capacity. However, problems were

noted with regard to between-laboratory and, mainly, within laboratory reproducibility, in particular in the naïve laboratory. These were probably related to the fact that no training/transfer had been organised prior to conducting the ring trial. Additionally, in case of the US laboratory, issues with the shipment of the tissue kits may have impacted on the results. Following additional testing to address these issues (submitted in 2013), the test method has entered ESAC peer review in early 2014. The review is expected to be finalised in 2Q 2014.

The OsREP test method employs an "open source" concept. The protocol underlying OsREP was originally developed by Poumay et al. and has been published without any restrictions (Poumay et al., 2004). The "open source" concept is intended to utilise this protocol in conjunction with information on the evaluation / validated of the protocol: all necessary information on both the production and maintenance of OS-Rep (i.e. the relevant SOPs) will be free of any restrictions (e.g. patents or other intellectual property rights). Thus, all interested users (e.g. in academia, in research or contract-research organisations) will be able to reconstruct the RhE test system in their facilities and use the test system either in association with the relevant protocol for skin irritation testing (i.e. as skin irritation test method) or for other purposes (e.g. research and development). This approach is novel to an area of complex tissue-engineered test systems which, so far, are all manufactured and quality-controlled by the original test method producers selling batch-controlled tissue kits with elements that are protected by intellectual property rights. EURL ECVAM is monitoring this development. In particular the issues of (1) *transferability* of the reconstructed model and the implementation of appropriate (2) *batch quality control procedures* for tissue models reconstructed in individual user laboratories need to be carefully evaluated, in particular if data are being used for regulatory decision making. This has been communicated to the test method developer following evaluation of the test method submission. Further the submitter of OsREP intends to produce the test method on an automated platform. As epidermal models are still rather costly and could, in addition for skin corrosion/irritation purposes possibly also be employed for other endpoints (e.g. genotoxicity, percutaneous absorption, skin sensitisation), economical ways of producing these tissues may support employing human based tissue models in a variety of toxicological assessments. An automated production facility has been installed by Fraunhofer Gesellschaft in Germany (see link below) collaborating with the test submitter. Important parameters such as batch-release quality and stability as well as robustness and relevance of this tissue model remain to be established and independently evaluated. At present the submitter, in collaboration with Fraunhofer Gesellschaft[7], is carrying out a PS-based validation study employing RhE tissue kits produced by automation. EURL ECVAM is expecting to receive the results of this study in the near future.

[7] http://www.igb.fraunhofer.de/en/competences/tissue-engineering/tissue-models/skin-from-the-factory.html

3.6 Test method submission related to eye irritation

In December 2013, EURL ECVAM received a full submission on the Ocular Irritection® test method. The Ocular Irritection® represents a refinement of the former Eytex® method (Kelly, 1989; Gordon, 1992) following recommendations made by Balls et al. (1995). It predicts the ocular hazard effects of chemicals based on the premise that corneal opacity may result from the disruptive effects ocular irritants may have on the highly organized structure of corneal proteins and carbohydrates. This assay mimics the biochemical phenomena of corneal protein denaturation and disruption caused by irritant chemicals acting on the cornea. One of the components of the test method is a macromolecular reagent composed of a mixture of proteins, glycoproteins, carbohydrates, lipids and low molecular weight components. The constituents of the macromolecular reagent combine with each other to form a complex macromolecular matrix that mimics the highly ordered structure of the transparent cornea. It is believed that irritant chemicals produce a turbidity of the macromolecular reagent by promoting protein denaturation, protein unfold and change in conformation, which then results in the disruption and disaggregation of the highly organized matrix macromolecular reagent matrix. This mechanism is believed to mimic the disruptive effects ocular irritants can have on the highly organized structure of corneal proteins and carbohydrates, which result in corneal cloudiness / opacity in the *in vivo* Draize eye test.

The Ocular Irritection® test method underwent an external validation study and is being proposed for identifying chemicals not requiring classification for serious eye damage/eye irritation (No Category) as well as chemicals inducing serious eye damage (Category 1), according to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (UN, 2013) and in the framework of a Bottom-Up/Top-Down test strategy (Scott et al., 2010). If, after evaluation by EURL ECVAM, the submission is considered to be complete and ready to enter peer-review, it will be submitted to the EURL ECVAM Scientific Advisory Committee (ESAC). Peer-review of the Ocular Irritection® test method by ESAC is expected to occur in Q3-Q4 2014.

3.7 Test method submission related to endocrine disruption

The Yeast Androgen Screen (YAS) assay was submitted to EURL ECVAM towards the end of 2013 to be considered for an ESAC peer review. A full validation study, addressing the modular approach, had been carried out with 4 laboratories. This assay uses yeast cells that are transformed with a human androgen receptor and a lacZ reporter gene. It measures the response towards chemicals with (anti) androgenic potential. Such assay may have potential to be annexed to a future Performance Based Test Guideline (PBTG) for Androgen Transactivation Assay (ARTAs). The submission dossier has been assessed by EURL ECVAM in early 2014 and feedback was requested from the submitter for some critical procedural aspects prior to continuation of the evaluation.

3.8 Test method submission related to acute fish toxicity

EURL ECVAM received a pre-submission on the RTgill-W1 cell line assay in March 2014.The method uses the fish cell line RTgill-W1 derived from rainbow trout gills. Cells are exposed to a series of test concentration for 24 h. Cytotoxic effects (EC50 values) are determined by measuring the cell viability using three fluorescent dyes (AlamarBlue for cellular metabolic activity, 5-carboxyfluorescein diacetate acetoxymethyl ester for cell membrane integrity and Neutral Red for lysosomal membrane integrity testing). Cell viability of the exposed cells is expressed in % of the control cells. The assessment of the pre-submission is ongoing; more information on the method is available in Tanneberger et al (2013).

4. Validation of Alternative Methods

4.1 On-going and finalised validation studies

4.1.1 Endocrine Disruption

At OECD level, there is an ongoing activity towards OECD performance based test guidelines (PBTG) for estrogen receptor transactivation assays (ERTAs) and androgen receptor transactivation assays (ARTAs). PBTG 455 for the detection of estrogen receptor agonists and PBTG 457 for the detection of estrogen agonists and antagonists were both adopted in 2012 while a proposal for a PBTG for the detection of androgen receptor agonists and antagonists was submitted to the OECD and included in the OECD work plan in 2013.

The AR-CALUX method is an androgen receptor transactivation assay (ARTA) for the detection of substances with androgenic properties. Such substances can bind to the androgen receptors and activate (agonist) or block (anti-agonist) cellular responses within the endocrine system. Once validated, this assay in conjunction with other similar assays (e.g. the Japanese EcoScreen assay) will contribute towards an OECD PBTG for androgen receptor transactivation assays. It is based on the use of osteosarcoma cells transfected with the cDNA of a human androgen receptor and the androgen responsive elements (AREs) coupled to a luciferase reporter gene (AR-CALUX®). Response towards substances with androgenic activity is hence easily evaluated via the measurement of emitted light.

The method was accepted to enter a EURL ECVAM validation study in 2012. A multi-study validation trial is scheduled for 2014 and the European Union Network of laboratories for the Validation of Alternative Methods (EU-NETVAL) will be involved in this exercise for the first time. Three test facilities will be selected and after approval by the Member States via the National Contact Points (NCPs), they will participate in the multi-study validation trial.

A validation study on a transactivation assay that detects chemicals with (anti-) estrogenic potential using MELN cells is still ongoing. The cell line used is the MCF-7 human breast cancer cell line, that has been stably transfected with an estrogen responsive reporter system. The MCF-7 cells express endogenously the estrogen receptor. The first part of the validation study was completed in 2012 with an assessment of the reliability (transferability and within and between laboratory reproducibility) for agonist and antagonist manual protocols in three laboratories for 16 chemicals. A second step to assess relevance (predictive capacity) on a wider set of chemicals in one laboratory by transferring the protocol to an inter-plate format on the robotic platform was supported by the OECD's non-animal Validation Management Group. Transfer of the agonist protocol to inter-plate HTS format (robotic platform) for 16 non-coded chemicals followed by the testing of 22 coded chemicals from the PBTG OECD 455 Performance Standards has been completed and good accuracy (i.e. good prediction of negatives and positives) was obtained but the induction factor was low. The next step is to proceed in the same manner for the antagonist

protocol using the reference chemicals from the performance standards of the OECD TG 457 (ERTA for antagonist based on BG1 Luc cells), provided that a new batch of cells can be obtained which can respond with a suitably high induction factor.

4.1.2 Cytochrome P450 Induction

Cytochrome P450s (CYP) are Phase I biotransformation enzymes and have a high prevalence in biotransformation of both endogenous and exogenous xenobiotics. Exposure to xenobiotics can lead to the induction of CYP enzymes. EURL ECVAM has carried out a multi-study validation trial in order to assess reliability and relevance of two CYP induction *in vitro* methods, each of them evaluating the induction of enzymatic activity of four CYP enzymes (CYP1A2, CYP2B6, CYP2C9 and CYP3A4). The human CYP induction method project was included in the OECD work plan in 2013.

The human *in vitro* CYP validation project had two main objectives:

A. Assess the transferability, the reproducibility (within and between-laboratory) of two human CYP induction-*in vitro* methods, by evaluating the induction of four CYP enzyme activities (CYP1A2, CYP2B6, CYP2C9, CYP3A4). The two *in vitro* methods use the metabolically competent test systems:

- cryopreserved human HepaRG cells (cryoHepaRG) (1)
- cryopreserved human primary hepatocytes (cryoheps) (2, 3)

B. Assess the predictive capacity using human CYP induction in vivo reference data.

The CYP induction validation project compares two human derived metabolically competent test systems using test items for which in vivo human CYP induction data are available. The *in vitro* method simulates the human in vivo method, by means of using a cocktail approach. The Karolinska cocktail was indeed developed to investigate CYP activities of different CYPs in vivo in humans (4). Later, a cocktail approach was developed to determine, in the same experiment, on *in vitro* human hepatic test systems, the induction of different important human CYP isoforms. In this project, this *in vitro* methodology was used to assess the potential of 13 blind-coded test items to induce CYP1A2,CYP2B6,CYP2C9,CYP3A4, using four CYP selective probes (phenacetin, midazolam, diclofenac and bupropion). The biotransformed CYP products have been measured with analytical method (LC/MS-MS),

During the validation project, the two human CYP induction *in vitro* methods have been successfully transferred by the lead laboratories to the participating laboratories. The *in vitro* methods can be performed in any modern analytical laboratory and with minimum standards in cell culture (Good Cell Culture Practice). Experienced personnel can readily be trained in the test methods. The test definition/description aspects of the SOPs are clearly written, the execution of the experimental steps and the data analysis can be performed without difficulties. All apparatus and reagents needed for the execution of the two human

CYP induction *in vitro* methods are readily commercially available. The cryoHepaRG® cells are nowadays available from different suppliers in Europe, USA, Japan and Brazil.

Based on the data generated during the project, all the different laboratories produced the same induction classification (potent/weak inducer, non-inducer) of blind-coded test items when performing the experiment with the same batch. 66 % of the experiments with the human CYP cryoHepaRG cell line induction *in vitro* method and 55% of the experiments with human CYP cryoheps induction *in vitro* method were judged to give the same induction class in all laboratories and at least 2 out of three laboratories for both human CYP induction *in vitro* methods were concordant in >90% of the experiments.

The cryoHepaRG CYP induction *in vitro* method showed higher reproducibility for the 4 CYPS under investigation compared to cryoheps CYP induction *in vitro* method when using for the evaluation the same batches. The highest reproducibility value was observed for CYP3A4 (all batches ≥ 90%).

At least qualitatively, CYP1A2-, 2B6- and 3A4- selective probe activities performed as expected in both *in vitro* methods both for the data generated with model inducers (rifampicin, phenobarbital and beta-napthoflavone at defined concentration) and with the blind-coded test items evaluated at different concentrations. CYP2C9- selective probe activity was relative high in both test systems and overall induction responses remained quite low. The weak induction of CYP2C9 in all conducted experiments reflects the clinical situation. In clinical studies CYP2C9 induction by rifampicin is much lower than CYP3A4 induction. For this reason FDA, EMA and the pharmaceutical industry excluded CYP2C9 induction assessment from the induction battery. Furthermore, it is considered to be a minor problem and always secondary to induction of CYP3A4.

Data generated during the project give clear indications that the human CYP cryoHepaRG cell line induction *in vitro* method and the human cryopreserved primary hepatocytes induction *in vitro* method are relatively similar in their ability to detect and classify substances in terms of CYP1A2, CYP2B6, CYP3A4 and CYP2C9 induction. The overall classifications of the blind-coded test items on the basis of the two *in vitro* methods are in line with the in vivo knowledge on classification of these test items.

The results indicated that human cryoHepaRG cells CYP induction *in vitro* method showed 100% sensitivity and specificity for the prediction of CYP1A2, CYP2B6 and CYP3A induction based on the results from the blind-coded test items used in the present study. The human cryoheps CYP induction *in vitro* method also showed 100% sensitivity and specificity for CYP2B6 and CYP3A induction. However, the human cryoheps CYP induction *in vitro* method showed only 25% sensitivity for prediction of CYP1A2 induction since the method failed to predict induction of three test items known to induce CYP1A2 in vivo. The human cryoheps CYP induction *in vitro* method showed 100% specificity since this method predicted all CYP1A2 non-inducers to be negative.

Because blind-coded test items and reference inducers (except for the positive CYP1A2 control beta-naphthoflavone) and non-inducers were pharmaceuticals, the described study design provides direct evidence on the applicability domain (pharmaceuticals). However, since the CYP induction method is based on xenobiotic-nuclear receptor binding, dimerization, activation of DNA binding domain and enhanced transcription of the target gene, any class of compounds that can interact with such receptors is predicted to be qualified to be used in the *in vitro* human CYP induction methods.

4.1.3 Eye Irritation

A prospective validation study of two *in vitro* test methods using Reconstructed human Tissue (RhT) models (MatTek EpiOcular™ and SkinEthic™ Human Corneal Epithelium (HCE)) for the detection of chemical induced serious eye damage/eye irritation, has been conducted by EURL ECVAM and Cosmetics Europe - The Personal Care Association. Pre-validation studies with both test methods have served to optimise protocols and refine prediction models, and were able to show that both test methods are able to predict ocular toxicity properties of test substances with a high degree of accuracy, approximately 80% overall. The Eye Irritation Validation Study (EIVS), co-sponsored by EURL ECVAM and Cosmetics Europe, evaluated the validity (relevance and reliability) of these two RhT test methods to discriminate chemicals not requiring classification for serious eye damage/eye irritancy (No Category) from chemicals requiring classification and labelling (Category 1 and Category 2) according to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) and as implemented by the EU Classification, Labelling and Packaging regulation (EU CLP) (UN, 2013; EC, 2008). These RhT test methods are not intended to differentiate between UN GHS/EU CLP Category 1 (serious eye damage) and UN GHS/EU CLP Category 2 (eye irritation). This differentiation would be left to another tier of a test strategy as described e.g., by Scott *et al.* (2010). The EIVS has been undertaken in accordance with the principles and criteria documented in the OECD Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (No. 34, OECD, 2005) and according to the Modular Approach to validation (Hartung *et al.*, 2004).

The protocols assessed were the original EpiOcular™ Eye Irritation Test (EIT) protocol for liquid chemicals, the original EpiOcular™ EIT protocol for solid chemicals, an EpiOcular™ EIT optimised protocol for solid chemicals, the SkinEthic™ HCE Short-time Exposure (SE) protocol, the SkinEthic™ HCE Long-time Exposure (LE) protocol, and the SkinEthic™ HCE test strategy combining the SE and LE protocols as well as the Eye irritation Peptide Reactivity Assay (EPRA). Two prediction models, using 50% or 60% mean tissue viability as the threshold differentiating classified (UN GHS Cat. 1 and Cat. 2) chemicals (mean tissue viability ≤ 50% or 60%) from non-classified (UN GHS No Cat.) chemicals (mean tissue viability > 50% or 60%), were evaluated with each of the EpiOcular™ EIT protocols, while a single prediction model using a 50% mean tissue viability cut-off was evaluated with the SkinEthic™ HCE SE, LE and test strategy.

EIVS included a statistically sufficient number of chemicals, supported by complete and quality assured *in vivo* Draize eye test data, for comparative evaluation of results. A total of 104 selected test chemicals (52 liquids and 52 solids) were distributed as identity coded aliquots for blind ring trial testing as three runs in three laboratories for both test methods. Following the ring trial, the 52 solid chemicals were re-tested, with an additional 8 others newly selected (all identity coded i.e., blind testing) in three runs in one laboratory, for validation of an optimised EpiOcular™ EIT solid chemicals protocol.

EpiOcular™ EIT

Three independent laboratories participated in the validation of EpiOcular™ EIT, two European, i.e. the lead laboratory and a naïve laboratory, and one in the US. All the participating laboratories were able to demonstrate their proficiency in performing the EpiOcular™ EIT and readiness to enter the formal validation study following training and transferability studies. The validation study of EpiOcular™ EIT proved very effective and efficient due to a near complete dataset being generated with negligible re-testing necessary.

The EpiOcular™ EIT test method was found to be highly reproducible. The within-laboratory reproducibility (WLR) (93.6% and 95.2% concordance of classifications for the 50% and 60% cut-offs analysed in this study, respectively) and the between-laboratory reproducibility (BLR) (91.3% and 93.3% concordance of classifications for the 50% and 60% cut-offs analysed in this study, respectively) were significantly above the acceptance criteria set by the Validation Management Group (VMG) (WLR ≥ 85% and BLR ≥ 80%).

Taking 60% mean viability as the prediction model threshold differentiating classified (UN GHS Cat. 1 and Cat. 2) from non-classified (UN GHS No Cat.) chemicals, the overall accuracy (79.0%) and specificity (70.5%) were 'definitely acceptable' according to the acceptance criteria as defined by the VMG (overall accuracy ≥ 75%; specificity ≥ 60%), whereas the sensitivity (87.6%) was between the limits of 'definitely unacceptable' (< 80%) and 'definitely acceptable' (≥ 90%). Considering only the liquid chemicals, the test method fulfilled all of the 'definitely acceptable' criteria (overall accuracy of 81.9%; sensitivity of 98.3%; specificity of 66.7%). For the solid chemicals both the overall accuracy (75.9%) and the specificity (74.8%) were 'definitely acceptable', whereas the sensitivity (76.9%) was 'definitely unacceptable'. Six chemicals were under-predicted based on the mode of all predictions, of which one was classified *in vivo* as Category 1. Taking 50% mean viability as the prediction model threshold differentiating classified (UN GHS Cat. 1 and Cat. 2) from non-classified (UN GHS No Cat.) chemicals, the overall accuracy (77.9%) and specificity (74.5%) were 'definitely acceptable' according to the acceptance criteria defined by the VMG (overall accuracy ≥ 75%; specificity ≥ 60%), whereas the sensitivity (81.4%) was still between the limits of 'definitely unacceptable' (< 80%) and 'definitely acceptable' (≥ 90%). Again, considering only the liquid chemicals, the test method fulfilled all of the 'definitely acceptable' criteria (overall accuracy of 82.5%; sensitivity of 96.2%; specificity of 69.8%), while for the solid chemicals only the specificity (79.7%) was 'definitely acceptable'. The overall accuracy (73.0%) fell short of 'definitely

acceptable' (≥ 75%) but surpassed 'definitely unacceptable' (< 65%), while the sensitivity (66.7%) was 'definitely unacceptable'.

Analysis of the EIVS data for solid chemicals indicated scope for improvement through a balanced increase in sensitivity with decrease in specificity to attain a compromise of sensitivity ≥ 90% with specificity maintained ≥ 60%. Optimisation of the EpiOcular™ EIT solids protocol was therefore conducted and follow-up validation of an optimised protocol with increased exposure time was performed. The validation of the EpiOcular™ EIT optimised solids protocol was conducted with the original 52 EIVS solid chemicals plus an extra 8. The post-optimisation validation of the EpiOcular™ EIT optimised solid chemicals protocol took place in the lead laboratory for EpiOcular™ EIT in the original validation study.

The EpiOcular™ EIT optimised solid chemicals protocol was found to be at least as reproducible as the original solid chemicals protocol, with 93.2% and 96.6% concordance of classifications (based on 59 chemicals) being obtained by the lead laboratory with the optimised protocol for the 50% and 60% cut-offs analysed in this study, respectively, as compared to 92.0% and 94.0% obtained by the same laboratory with the original protocol (based on 50 chemicals). Forty nine (49) chemicals are common to the two datasets. If only these are considered in the calculations, the concordance of classifications obtained were 91.8% (50% cut-off) and 95.9% (60% cut-off) for the optimised protocol and 91.8% (50% cut-off) and 93.9% (60% cut-off) for the original protocol. The within-laboratory reproducibility (WLR) of the EpiOcular™ EIT optimised solid chemicals protocol was thus significantly above the acceptance criterion set by the VMG (WLR ≥ 85%). Further BLR evaluation was identified, by the core VMG, to be unnecessary given the previous good reproducibility of the EpiOcular™ EIT test method, and a similar (or even slightly better) WLR observed for the optimised solids protocol as compared to the original protocol. With the increased exposure time in the optimised solid chemicals protocol, a stronger separation between irritants and non-irritants in the viability scale was observed as compared to the original protocol, which is expected to improve the reproducibility of the test method.

Taking 60% mean viability as the prediction model threshold differentiating classified (UN GHS Cat. 1 and Cat. 2) from non-classified (UN GHS No Cat.) chemicals, the overall accuracy (78.0%), the specificity (60.7%) and the sensitivity (93.5%) were all 'definitely acceptable' according to the acceptance criteria as defined by the VMG (overall accuracy ≥ 75%; specificity ≥ 60%; sensitivity ≥ 90%).

Taking 50% mean viability as the prediction model threshold differentiating classified (UN GHS Cat. 1 and Cat. 2) from non-classified (UN GHS No Cat.) chemicals, the overall accuracy (76.8%) and the specificity (64.3%) were 'definitely acceptable' according to the acceptance criteria defined by the VMG, whereas the sensitivity (88.2%) was between the limits of 'definitely unacceptable' (< 80%) and 'definitely acceptable' (≥ 90%), but very close to being 'definitely acceptable'.

The 60% cut-off was considered to be better than the 50% cut-off because it resulted in a better sensitivity and generated less false negatives based on the mode of all predictions, with similar overall accuracy. The overall predictive capacity of EpiOcular™ EIT considering a combination of the data obtained for the liquid chemicals protocol with the data obtained using the optimised solid chemicals protocol, and a cut-off of 60%, consists of a sensitivity of 95.7%, a specificity of 63.0% and an overall accuracy of 79.7%. On this basis, all of the acceptance criteria defined by the VMG are met. Two out of 57 chemicals (2 solid Cat. 2B chemicals) were under-predicted (false negatives) and 20 out of 54 chemicals (9 liquids and 11 solids) were over-predicted (false positives) based on the mode of all predictions.

SkinEthic™ HCE

Three independent laboratories participated in the validation of SkinEthic™ HCE, two European, i.e. the test method developer and the lead laboratory and a naïve laboratory, and one in the US, a second naïve laboratory. All the participating laboratories were able to demonstrate their proficiency in performing the SkinEthic™ HCE and readiness to enter the formal validation study following training and transferability studies. The validation study of the SkinEthic™ HCE proved to be very straightforward due to a near complete dataset being generated with negligible re-testing performed.

The SkinEthic™ HCE test method was found to be highly reproducible. The WLR (93.9% and 95.5% concordance of classifications for the SE and LE, respectively) and the BLR (92.3% concordance of classifications for both the SE and the LE protocols) were significantly above the acceptance criteria set by the VMG (WLR ≥ 85% and BLR ≥ 80%).

The only prediction model that was evaluated used a mean viability of 50% as the threshold differentiating classified (UN GHS Cat. 1 and Cat. 2) from non-classified (UN GHS No Cat.) chemicals. The specificity of this prediction model was found to be 'definitely acceptable' according to the acceptance criterion defined by the VMG (≥ 60%), regardless of the protocol or strategy (SE: 88.5%; LE: 65.5%; test strategy: 77.1%). The sensitivity was on the other hand 'definitely unacceptable' (< 80%) according to the same acceptance criteria (SE: 42.7%; LE: 71.6%; test strategy: 54.5%). The overall accuracy was between the limits of 'definitely unacceptable' (< 65%) and 'definitely acceptable' (≥ 75%) (SE: 65.6%; LE: 68.6%; test strategy: 65.8%). Moreover, of the 30 chemicals that were under-predicted by SE and of the 15 that were under-predicted by LE based on the mode of all predictions, 14 and 5, respectively, were classified *in vivo* as Category 1, which was also 'definitely unacceptable'. Use of the SkinEthic™ HCE test strategy also led to 10 Category 1 chemicals being under-predicted as non-irritants (based on the mode of all predictions). Based on these values neither of the two SkinEthic™ HCE protocols (SE or LE) was considered valid. The use of EPRA to orient chemicals to the LE (non-reactive) or SE (reactive) protocol (SkinEthic™ HCE test strategy) was also considered not valid.
Nevertheless, analysis of the data for the SkinEthic™ HCE indicated scope for improvement. Further optimisation has therefore been recommended for the SkinEthic™ HCE test method considering different protocols for liquid chemicals and solid chemicals, as with EpiOcular™

EIT. Optimisation of the SkinEthic™ HCE test method is currently being conducted by the test method developer and further validation is foreseen in the near future.

4.1.4 Genotoxicity

Micronucleus test and comet assay in reconstructed skin models

The validation of methods for genotoxicity testing in reconstructed human 3D skin models, i.e. the micronucleus test and the comet assay, is still ongoing (Aardema et al., 2010; Reus et al., 2013). The validation of the micronucleus test in 3D epidermis model, led by Cosmetics Europe, has entered in its final experimental phase; while the between-laboratory reproducibility of the comet assay in full-thickness skin models, a joint effort between Cosmetics Europe and the German Federal Institute for Risk Assessment (BfR), is under evaluation.

Hen's egg test for micronucleus induction (HET-MN)

The hen's egg test for micronucleus induction (HET-MN; Wolf et al., 2008) has been proposed as a follow-up test method for *in vitro* positives. The HET-MN combines the use of the commonly accepted genetic endpoint "formation of micronuclei" with the well-characterised and complex model of the incubated hen's egg, which enables metabolic activation, elimination and excretion of xenobiotics, including those that are mutagens or pro-mutagens. The transferability and within and between laboratory reproducibility are currently being evaluated by a German consortium (Greywe et al., 2012).

4.1.5 Ongoing Validation Studies for Vaccine Testing - Biological Standardisation Programme

Most of the validation studies on alternative methods for vaccine testing are carried out within the framework of the Biological Standardisation Programme (BSP) of the European Directorate for the Quality of Medicines & HealthCare (EDQM; Council of Europe) and co-sponsored by the European Commission. Several validation studies on alternative methods for potency and safety testing of various vaccines for human and veterinary use are currently ongoing or planned to start in 2014 (e.g. a serological assay for the potency testing of whole-cell pertussis vaccines, a serological assay for the potency testing of rabies vaccines; an *in vitro* method for the safety testing of tetanus vaccines; *in vitro* methods for potency testing of diphtheria and tetanus vaccines). More information of the BSP, its background and work programme is available at http://www.edqm.eu/en/Biological-Standardisation-Programme-mission-60.html.

In this context, three projects should be highlighted which are currently ongoing and aim at standardising methods to become ready for full validation within the BSP: *in vitro* methods for safety testing of acellular pertussis vaccines (Bache et al, 2012; Isbrucker et al, 2014), *in vitro* methods for in process control of clostridial vaccines and *in vitro* methods for the potency testing of rabies vaccines (*see paragraph 5.5.2.2 EPAA projects*). These are joint projects involving manufacturers and Official Medicines Control Laboratories.

4.2 Peer reviews by the EURL ECVAM Scientific Advisory Committee

4.2.1 Renewal of ESAC in 2013

The ESAC is managed and coordinated in agreement principles and practices of the Commission relating to external expert advice for policy conception, monitoring and update. A key document is the Commission communication on "The collection & use of expertise by the Commission: Principles and Guidelines. Improving the knowledge base for better policies" (EC, 2002). These guidelines have been taken up in ESAC's Rules of Procedure which describe the principles and mode of operation of the committee. An important element of ESAC is that members should not represent their employing organisations, but participate in their personal capacity as scientists. Moreover, members have to declare their interests, and sign declarations of commitment (to the Committee's work in the interest of the public good) and confidentiality (to protect legitimate interests of test method submitters). Moreover, EURL ECVAM uses a consistent workflow based on formal "EURL ECVAM requests for ESAC advice" outlining the background to the topic, the objective and detailed charge questions as well as timelines and deliverables. One important element of ESAC's functioning is the use of specialised *ESAC Working Groups*, which draw also on the expertise of external scientist proposed by ESAC members, EURL ECVAM or nominated by ICATM partners.

Another important element is the principle of 'plurality', i.e. the need to listen to as wide as possible a variety of experts. Since the ESAC for logistical and budgetary reasons needs to have a limited number of members (n=15), EURL ECVAM is implementing this principle via a regular renewal of the committee: according to the Rules of Procedure, the term of office of the ESAC is three years. Since ESAC had been last renewed in 2009, EURL ECVAM published an open call for the expression of interest from December 2012 to January 2013. This call was modelled closely along the one from 2009 but addressing further aspects of ECVAM's work (now referred to as EURL ECVAM) such as test method development and modern chemical safety assessment science. The open call was directed at scientists active in the fields of life sciences, medicine, toxicology, chemistry and various technical competences. Applications from 42 scientists were received. These were pooled with candidatures on the reserve list of the 2009 call (n=32) as well as expressions of interest of current ESAC members regarding a second term (n=11). Therefore, a total of 85 applications / expressions of interest had to be evaluated.

A Selection Committee (SC) consisting of two external scientists with profound knowledge of the field plus two staff members of EURL ECVAM had been set up, charged with evaluating all eligible applications and expressions of interest in view of developing a shortlist of about 30 candidates. The SC was asked to identify, based on this shortlist, 15 candidates particularly suited for nomination by the Director of the Institute. The SC worked on the basis of a guidance document outlining objective and transparent eligibility and selection criteria and providing guidance on how to apply these in a semi-quantitative manner based on the intrinsic weighing of six selection criteria. Following assessment of the

applications against the pre-defined criteria, the committee shortlisted 15 scientists. The shortlist was based predominantly on considerations of scientific excellence (as for the shortlist) but weight was also given to aspects of gender and geographical balance as well as the right blend of experience versus fresh ideas that potential members would bring to the table. The 15 shortlisted candidates were appointed for membership of ESAC by the Director of the JRC's Institute for Health and Consumer Protection (hosting EURL ECVAM) in summer 2013.

The first meeting of the renewed ESAC was held in June 2013 and served to kick-off the activities of the new committee and introduce its members with the necessary information on EURL ECVAM's mission, projects and activities as well as the principles and processes of ESAC Scientific Peer Reviews. During this first meeting, the ESAC decided, following a proposal of EURL ECVAM, to set up three ESAC Working Groups (WGs) dedicated with preparing detailed scientific peer reviews for consideration by ESAC.

4.2.2 ESAC Scientific Peer Reviews

In 2012 ESAC finalised four scientific peer reviews of validation studies which led to EURL ECVAM recommendations in 2013 and 2014 (see 4.3). These concerned:

(1) The EURL ECVAM-coordinated follow-up study on the 3T3 NRU assay to support identification of substances not requiring classification as acute oral toxicants.

(2) The Direct Peptide Reactivity Assay (DPRA), an *in chemico* method for assessing the skin sensitisation potential of chemicals by determining their reactivity towards synthetic Lysin and Cystein-containing peptides.

(3) The Bhas Cell Transformation Assay (Bhas CTA) for assessing the potential carcinogenicity of substances by assessing the downstream morphological phenomena associated with the malignant transformation of cells.

(4) The KeratinoSens™ assay for assessing the skin sensitisation potential of chemicals by measuring the activation of the Keap1-Nrf2-ARE cytoprotective pathway in a human keratinocytes-derived cell line (HaCaT).

In 2013 ESAC finalised the scientific peer review on the Zebrafish Embryo Toxicity Test (ZFET) for acute fish toxicity testing. The ESAC found that the ZFET is a reproducible method that, based on a retrospective data set evaluating through orthogonal regression the correlation between (Z)FET data and data from acute and juvenile fish of various species, appears to provide information equivalent to that generated by the standardised acute fish toxicity test (i.e. OECD TG 203). At its spring meeting in 2014 ESAC finalised the scientific peer review of the human Cell Line Activation Test (h-CLAT) for skin sensitisation testing. The EURL ECVAM Recommendation on the test method is currently in preparation and will undergo a restricted consultation round (i.e. by PARERE, ESTAF and ICATM) in summer 2014.

Further, in 2014, ESAC has started with the scientific peer review of EURL ECVAM-coordinated validation studies on Eye Irritation and on two human hepatic test systems for measuring the induction of Cytochrome P 450 enzymes (CYP). The reviews are expected to be finalised by summer 2014. The review of the MELN assay (an Estrogen Receptor Transactivation Assay, ERTA) had to be postponed as the validation data could not be generated as planned due to problems with the test system.

4.3 EURL ECVAM Recommendations

Since 2011, the final product of the EURL ECVAM validation process are the EURL ECVAM recommendations[8]. These are based on the validation reports, the ESAC peer review and the input received during consultation rounds with EURL ECVAM's network of regulators (PARERE-Preliminary Assessment of Regulatory Relevance), the ECVAM Stakeholder Forum (ESTAF), the International Cooperation on Alternative Test Method (ICATM) and the general public. Also the test method submitter has a right to comment on the EURL Recommendation prior to its publication.

4.3.1 EURL ECVAM recommendation on the 3T3 Neutral Red Uptake (3T3 NRU) Cytotoxicity Assay

In 2013, EURL ECVAM published a recommendation on the 3T3 NRU test method for the identification of substances not requiring classification for acute oral toxicity (EC EURL ECVAM 2013). EURL ECVAM fully endorsed the ESAC opinion and concluded that the 3T3 NRU test method may prove a valuable component of a Weight of Evidence approaches (WoE) or Integrated Testing Strategies (ITS) for supporting hazard identification and safety assessment in agreement with the EU CLP Regulation and international regulatory schemes implementing the upper threshold of UN GHS Category 4 as the cut-off for non-classification of substances (i.e. oral LD50 > 2000 mg/kg b.w). In particular, data from the 3T3 NRU assay may constitute an information source within a WoE approach under the provisions of the REACH Regulation (Annex XI, 1.2) potentially supporting conclusions on absence of acute oral toxicity of industrial chemicals. Considering its limitations, namely lack of metabolic competence associated with Phase I and Phase II biotransformation and mechanistic relevance limited to basal cytotoxicity, EURL ECVAM recommended that results derived from the 3T3 NRU test method should always be used in combination with other information sources to build confidence in the decision not to classify a substance for acute oral toxicity. Thus, efforts need to be invested in the identification of relevant and complementary information as well as gathering and organising mode-of-action knowledge related to this endpoint since this would be very valuable in the design and validation of integrated prediction methods.

[8] for more information: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvams-validation-process)

4.3.2 EURL ECVAM recommendation on the DPRA

EURL ECVAM fully endorsed the ESAC opinion on the EURL ECVAM-coordinated validation study of the DPRA and concluded that the DPRA is a mechanistically relevant test method that can contribute to the assessment of the skin sensitisation potential of chemicals when used within integrated approaches such as WOE approaches or ITS (EC EURL ECVAM 2013a). The EURL ECVAM study showed that the DPRA is a transferable test method and sufficiently reproducible within and between laboratories. A complete evaluation of the predictive capacity of the DPRA was outside the scope of the ECVAM validation study since the test method is not proposed as a stand-alone full replacement, nevertheless the accuracy in discriminating between sensitisers and non-sensitisers calculated with the set of chemicals tested in the validation study was 86% which is in agreement with published information from previous studies. The Recommendation acknowledged that in addition to supporting the discrimination between sensitisers and non-sensitisers, DPRA information may also have the potential to contribute to potency assessment. However, additional work is still required to determine how DPRA results can be exploited within integrated approaches for potency prediction using preferably human data. EURL ECVAM recommended that in consideration of the fact that the DPRA is covering only one single biological mechanism of the skin sensitisation pathway and because of DPRA's known limitations such as the lack of metabolic capacity, DPRA information should always be used in combination with other information sources. EURL ECVAM recommended the development of an OECD Test Guideline for the DPRA.

4.3.3 EURL ECVAM recommendation on the KeratinoSens™

EURL ECVAM fully agreed with the ESAC opinion on the Givaudan-coordinated validation study of the KeratinoSens™ test method and supplementary information submitted to EURL ECVAM and concluded that the test method is mechanistically relevant to be used as a valuable component within integrated approaches for skin sensitisation testing (EC EURL ECVAM 2014). According to the validation study data the test method has shown to be transferable and reproducible within and between laboratories. In the recommendation EURL ECVAM acknowledged that the 75% accuracy of the KeratinoSens™ in discriminating between sensitisers and non-sensitisers calculated with the data submitted to EURL ECVAM is in line with the accuracy values published in the scientific literature indicating the usefulness of the method to contribute to the assessment of the skin sensitisation potential of chemicals. EURL ECVAM also acknowledged that concentration-response information generated with the KeratinoSens™ may play a role in integrated approaches for potency prediction. As in the case of the DPRA, also for the KeratinoSens™, follow up work was recommended to better understand how KeratinoSens™ information can support potency prediction preferably making use of human data. Given the fact that the test method addresses only one single biological mechanism of skin sensitisation and considering its known limitations such as the limited metabolic capacity and ability to detect only cysteine-reactive chemicals EURL ECVAM recommended the use of the method

in combination with other information sources. EURL ECVAM in its recommendation supported the development of an OECD Test Guideline for the KeratinoSens™.

4.3.4 EURL ECVAM recommendation on Bhas42 cell transformation assay (CTA)

Similar to previously validated *in vitro* cell transformation assays (CTAs), the CTA in Bhas 42 cells aims at predicting carcinogenic potential. Based on the results of a validation study coordinated by Hadano Research Institute (HRI) Food and Drug Safety Center (FDSC) and other published data, the Bhas 42 CTA protocol (including the 6-well and 96-well plate versions) was considered to be sufficiently standardised, transferable, reproducible between laboratories and relevant to support the identification of potential carcinogenicity of substances. Following independent scientific peer review by ESAC and having considered the input from regulators, stakeholders, international partners and the general public, EURL ECVAM published a recommendation on the Bhas 42 CTA, which concluded that the CTA in Bhas 42 cells shows promise for inclusion within weight of evidence or integrated testing strategy approaches to assess carcinogenic potential or to support chemical category formation and read-across. Thus EURL ECVAM recommends that an OECD Test Guideline be developed. In addition, further investigations on the capability of the assay to detect tumour promoters would provide useful information on mode of action of carcinogens for risk assessment purposes.

4.3.5 Draft EURL ECVAM recommendation on the Zebrafish Embryo Acute Toxicity Test Method (ZFET)

The draft EURL ECVAM recommendation on the Zebrafish Embryo Acute Toxicity Test Method (ZFET) is being finalised and is available for public comments. It concludes that the ZFET is transferable and reproducible within and between laboratories as shown in the OECD validation study. The comparison of data on 144 chemicals (Belanger et al, 2013) demonstrated a strong correlation between fish embryo acute toxicity data (24-120 h exposure; mainly Zebrafish) and fish acute toxicity data (96 h; five freshwater species recommended in OECD TG 203). Notably, the chemicals covered a broad range of physico-chemical properties, toxicological modes of action, and sectorial use, e.g. industrial chemicals, plant protection products, biocides, and pharmaceuticals. It is therefore concluded that the ZFET provides information on acute fish toxicity that can be considered comparable to that derived from standard acute fish toxicity tests (e.g. OECD TG203; OECD 1992) and regulators should consider its use for acute fish toxicity testing whenever possible. The use of the ZFET will result in an overall reduction of the numbers of juvenile and adult fish required for aquatic toxicity testing.

As per Article 1(3)(a)(i) of Directive 2010/63/EU (EU 2010) on the protection of animals used for scientific purposes, live non-human vertebrate animals including independently feeding larval forms are covered by its scope. Zebrafish is generally not considered as being capable of independent feeding until 5 days post fertilisation. This is confirmed by the Commission Implementing Decision 2012/707/EU (EU 2012) on a common format on collection of information on the use of animals for scientific purposes in the EU that states

that "Fish should be counted from the stage of being capable of independent feeding onward. Zebrafish kept in optimal breeding conditions (approximately + 28°C) should be counted 5 days post fertilisation". Considering the foregoing, the embryos in question should not be considered as "independently feeding larval forms" within the meaning of the Directive 2010/63/EU and therefore the procedure, as far as the embryos are concerned, does not fall within its scope.

4.4 The European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL)

4.4.1 Background and context of EU NETVAL

The European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) mission is to provide support for EURL ECVAM validation studies that serve to assess the reliability and relevance of alternative methods that have a potential to replace, reduce or refine the use of animals for scientific purposes.

EU-NETVAL has been established to address some of the provisions of Directive 2010/63/EU on the protection of animals used for scientific purposes. Article 47 of the Directive provides that;

> *"1. The Commission and the Member States shall contribute to the development and validation of alternative approaches which could provide the same or higher levels of information as those obtained in procedures using animals, but which do not involve the use of animals or use fewer animals or which entail less painful procedures, and they shall take such other steps as they consider appropriate to encourage research in this field.*
>
> *2. Member States shall assist the Commission in identifying and nominating suitable specialised and qualified laboratories to carry out such validation studies.*
>
> *3. After consulting the Member States, the Commission shall set the priorities for those validation studies and allocate the tasks between the laboratories for carrying out those studies.*
>
> *4. Member States shall, at national level, ensure the promotion of alternative approaches and the dissemination of information thereon."*

In line with Article 48 of the Directive, Annex VII lists the duties and tasks of the EU Reference Laboratory, covering *inter alia*:

> *"(a) coordinating and promoting the development and use of alternatives to procedures including in the areas of basic and applied research and regulatory testing;*
>
> *(b) coordinating the validation of alternative approaches at Union level;*
>
> *(c) acting as a focal point for the exchange of information on the development of alternative approaches;*

(d) setting up, maintaining and managing public databases and information systems on alternative approaches and their state of development;

(e) promoting dialogue between legislators, regulators, and all relevant stakeholders, in particular, industry, biomedical scientists, consumer organisations and animal-welfare groups, with a view to the development, validation, regulatory acceptance, international recognition, and application of alternative approaches."

EU-NETVAL will facilitate the EU Reference Laboratory to meet its objectives under Article 48. Furthermore, membership in EU NETVAL provides one channel, among others, for both the Commission and the Member States to actively contribute to the development and validation of alternative approaches as required by the Directive.

EU-NETVAL is coordinated and supported by EURL ECVAM, in close collaboration with Directorate-General for Environment and the National Contact Points (NCPs) for implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes. Also Directorate-General for Industry and Enterprise who is chair of the Good Laboratory Practice EU work group is involved in the activity.

A Terms of Reference (ToR) of EU-NETVAL has been developed. The ToR outlines the legislative anchor, the establishment of the network and the maintenance of its membership, tasks of the network members and of EURL ECVAM in support to validation studies, the allocation of tasks to the members, and the financing of network activities (approved by the National Contact Points of Directive 2010/63/EU on 26/11/2013). The ToR was formally approved by the NCPs on 26 November 2013.

4.4.2 EU-NETVAL establishment

In 2012, the NCPs for implementation of Directive 2010/63/EU were requested to provide a list of candidate laboratories to be assessed for inclusion in EU-NETVAL. In January 2013 EU-NETVAL drew up a list of Eligibility Criteria previously agreed with NCPs for the assessment of candidate members for inclusion in EU-NETVAL. An on-line questionnaire, based on these Eligibility Criteria was developed and launched in July 2013. One of the eligibility criteria requests the availability of a quality system, preferable Good laboratory Practice GLP. Fourty candidate laboratories were provided by the NCPs, who were then requested to complete the on-line questionnaire, exclusively made available in March 2013 for the candidate laboratories proposed by the NCPs, so to assess the suitability of the laboratories to be selected as EU-NETVAL members. The replies to the on-line questionnaire were then evaluated for their completeness and suitability in accordance with the Eligibility Criteria. Of the original 40 laboratories, 14 facilities were selected for inclusion in EU-NETVAL. These 14 laboratories were formally appointed to EU-NETVAL on the 3rd July 2013. After the selection of the first EU-NETVAL member laboratories, a public EURL ECVAM web-call was launched to broaden the competence base and covering more EU Member States. Any suitably qualified test laboratories residing in either an EU country, EU candidate country or EFTA member country could apply for EU-NETVAL membership. Based on this process today there are a total of 26 test facilities within EU-NETVAL (25

test facilities from EU Member States plus the European Commission's own *in vitro* GLP test facility operated by EURL ECVAM, who also coordinates the network), selected against the pre-defined eligibility criteria and approved by the National Contact Points[9]. The EU-NETVAL member test facilities are getting proposals from EURL ECVAM for participation in multi-study validation trials.

The first project that started in 2014 was the generation of experimental data using the *in vitro* AR-CALUX method to support the development of an OECD performance-based test guideline and associated performance standards for Androgen Receptor Transactivation Assays (ARTA) for the detection of compounds with (anti)androgenic potential (see 4.1.1).

[9] http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eu-netval.

5. Promoting the Regulatory Acceptance of Alternative Methods and Approaches

5.1 OECD Test Guideline Programme

5.1.1 Draft OECD Test Guidelines on DPRA and Keratinosens™

EURL ECVAM is leading at the OECD the development of the DPRA test guideline and is co-leading, together with the Swiss Federal Office of Public Health, the development of the KeratinoSens™ test guideline. Draft test guidelines have been submitted to the OECD in November 2013 and underwent revisions following the first commenting round and an OECD expert meeting held in February 2014. The DPRA and KeratinoSens™ test methods are proposed in the TGs to be used for supporting the discrimination between sensitisers and non-sensitisers within Integrated Approaches for Testing and Assessment (IATA). Their potential to inform potency prediction will also need to be evaluated in the context of an IATA. The development of an OECD draft test guideline for the h-CLAT is foreseen to be undertaken in 2014 under the co-leadership of JaCVAM and EURL ECVAM.

5.1.2 Draft OECD Test Guideline on SHE and Bhas CTA

In support to the draft OECD Test Guideline (TG) on cell transformation assays (CTA) in Syrian Hamster embryo (SHE) cells, some additional analyses were undertaken in order to address comments raised by a few WNT representatives on the modification of data interpretation criteria, the equivalence of the SHE CTA performed at pH 6.7 and pH 7.0 and the relative sensitivities of the assay to non-genotoxic carcinogens at the two pH conditions.

To address these questions, the following analyses were undertaken:

Comparison of the results obtained for chemicals tested at both pH 6.7 and 7.0, using re-analysed data; re-analysis of the results of the Detailed Review Paper (DRP) 31, going back to the published literature and applying the draft TG interpretation criteria; determination of the performance of the assay based on the old and new interpretation criteria**,** including information regarding non-genotoxic chemicals; categorization of chemicals into genotoxic and non-genotoxic; identification of target tumour and carcinogenic mechanism of action of non-genotoxic carcinogens.

A revised TG has been submitted for adoption at the 2014 WNT. There was no consensus to approve the revised TG but it was agreed to make the SHE CTA test method available in a guidance document (GD), after further revision (including a couple of introductory paragraphs on the caveats related to the method) and subsequent WNT review.

GDs are not covered by Mutual Acceptance of Data (MAD), but it will allow test method users to perform the test method in a standardised manner.

The need for the development of an IATA (Integrated Approach to Testing and Assessment) was also expressed and it was proposed that a scoping exercise be started and a short

paper developed in preparation of a workshop on IATAs to be held in Washington DC in November 2014.

A TG was drafted on the BHAS 42 CTA, which underwent a first commenting round. Further analyses will need to be undertaken as suggested at the CTA expert meeting held in January 2013. After revision of the draft TG, a second commenting round will take place in 2014. The fate of the draft TG on the Bhas 42 is currently unclear, i.e. it has not yet been decided if the draft TG will become a guidance document like the SHE CTA TG. The validation report of the test method was endorsed by the WNT in April 2014 and it will be declassified and published in June or July 2014 in the OECD Series on Testing and Assessment.

5.1.3 Revision of OECD Test guidelines related to genotoxicity testing

Existing OECD TGs for *in vitro* and *in vivo* genotoxicity testing (TGs 473, 474, 475, 487) have been revised according to recent developments and have been adopted (with amendments for some of them) by the WNT in 2014. Moreover a TG on the *in vivo* comet assay validated by JaCVAM has also been adopted in 2014.

5.1.4 Test Guideline on Aquatic Toxicity

The OECD TG 236 "Fish embryo acute toxicity (FET) test" was adopted by the OECD WNT in April 2013 and published in July 2013 (OECD, 2013). EURL ECVAM contributed to the development of this TG since 2006 as a member of the relevant OECD expert group and by coordinating the validation of the Zebrafish embryo acute toxicity test on behalf of OECD (OECD, 2011; OECD, 2012). The standard operating procedure used in the validation study formed the basis for the OECD TG 236.
Other TGs or GDs based on alternative methods were approved at the OECD WNT 26 held on 8 to 11 April 2014. They can be found in Annex 1.

5.1.5 New Projects Submitted to the OECD

5.1.5.1 EpiOcular™ EIT

Based on the outcome of the Eye Irritation validation study described in paragraph 4.1.3, a Standard Project Submission Form (SPSF) was submitted by the European Commission to the OECD for the development of a new OECD Test Guideline, with lead by the European Commission Joint Research Centre (EURL ECVAM). The SPSF was discussed and approved by the OECD WNT at its 26th meeting on April 8-11, 2014. A new draft Test Guideline on the EpiOcular™ EIT for the identification of chemicals not requiring classification for serious eye damage/eye irritation will be prepared and submitted to the OECD during Q2-Q3 2014.

5.1.5.2 In vitro Fish Hepatic Metabolism Test

Information on accumulation in aquatic organisms is important for understanding the behaviour of a compound in the environment. This information is used for hazard classification and for the assessment of persistent, bioaccumulative and toxic (PBT)

substances. In general, a bioconcentration factor (BCF) is estimated based on various prediction techniques as log Kow, (Q)SARs or other computer models, or, if deemed necessary, derived from experimental data measured with aquatic species, preferably fish. The standard test guideline (OECD TG 305; OECD, 2013) requires at least 108 fish, is expensive and time-consuming. Since in silico BCF models are often neglecting the contribution of metabolism as a clearance mechanism, they might overestimate the bioaccumulative potential of a chemical and, in consequence, might trigger unnecessary in vivo tests. Inclusion of biotransformation rates would enhance the reliability of the in silico models for BCF prediction.

The project (proposed by the USA and European Commission) aims at developing a test guideline for *in vitro* methods to determine information on possible biotransformation of chemicals in fish. The *in vitro* fish intrinsic hepatic clearance rates are derived using rainbow trout S9 fraction (Johanning et al, 2012) or cryopreserved hepatocytes (Fay et al, submitted) and extrapolated to whole-body metabolism rate constant (k_{MET}; 1/h; Nichols et al, 2013). It is planned to carry out a multi-laboratory ring trial to assess the reliability, transferability, and predictive value of the two *in vitro* systems within the framework of the ILSI HESI project committee "Bioaccumulation" of which EURL ECVAM is a member (http://www.hesiglobal.org/i4a/pages/index.cfm?pageid=3319). The project was approved in 2014 and included in the OECD work plan.

5.1.5.3 Good In Vitro Method Practice (GIVIMP): Guidance on the implementation of in vitro methods within a GLP environment to support regulatory human safety assessment of chemicals

In vitro methods often based on the use of human cells and tissues are submitted to international validation bodies (Rispin A. *et al* 2004; Gupta K. *et al*, 2005; OECD, 2004). Well-designed, robust, reliable *in vitro* methods that can run in a GLP environment for generating data sets are becoming more and more instrumental for supporting regulatory decisions.

GIVIMP will contribute to increased standardisation and international harmonisation in the generation of *in vitro* information on test item safety and will give guidance to obtain a high level of data quality based on sound scientific principles to support regulatory human safety assessment of chemicals using *in vitro* methods.

GIVIMP will also further facilitate the application of the OECD Mutual Acceptance of Data Agreement (MAD) for data generated by *in vitro* methods avoiding as such unnecessary duplication of testing by MAD-adherent countries. GIVIMP will take into account the requirements of the existing OECD guidelines and advisory documents to ensure that the guidance is complementary and 100% in line with these issued documents (OECD, 2004 and 2005).

The objectives of this guidance document are:

(1) To provide a detailed update on today's state-of-the-art of good practices when applying *in vitro* methods in regulatory human safety assessment of chemicals of various kinds;

(2) To provide guidance to users and implementers of *in vitro* methods to help to ensure that the Standard Operating Procedures (SOPs) of such methods are well-designed, robust, well-defined and described and can run in a GLP environment, which is essential for use in a regulatory environment;
(3) To provide guidance on minimum SOP requirements and reporting features to strive for more harmonised approaches for today's regulatory needs in the field of human safety assessment;
(4) To describe the key importance of applying Good Cell Culture Practice (Coecke *et al*., (2005)), essential in the identification, authentication and characterisation of the *in vitro* biological model (e.g. test systems such as cell lines, stem cells, primary cells and tissues) used in *in vitro* methods;
(5) To describe the key importance of applying good test item handling procedures and clarify the importance of a clear definition of the *in vitro* environment that hosts the *in vitro* test system, which is essential for the correct dosing of the test system, and for the assessment of test item compatibility with the specific *in vitro* environment;
(6) To describe the key importance of applying good experimental design, establishing acceptance criteria for *in vitro* methods, describing equipment requirements (including also those based on new technologies and any scientific progress in the field of detection methods) and performance standards based on scientific evidence from the generated *in vitro* data sets;
(7) To describe how International collaborations and networks can help in disseminating GIVIMP and the use of the generated data sets for specific regulatory applications. GIVIMP will contribute to the use of *in vitro* method data to support regulatory human safety assessment of chemicals by striving that such data are being generated in compliance with high quality standards and based on current good scientific practices.

EURL ECVAM will pursue this project with the involvement of EU-NETVAL and other relevant European experts with competences in both GLP and good scientific practices in *in vitro* methods. Eventual inclusion of the project within a programme of the OECD in order to produce an internationally recognised Guidance Document is under discussion.

5.2 Guidelines on vaccines

VICH guidelines - Harmonisation of criteria for waiving of target animal batch safety testing of vaccines for veterinary use

The requirements on batch safety testing differ between the various geographic regions. For example, general safety tests for batch release of human and veterinary vaccines are no longer required in Europe and have been deleted from European Pharmacopoeia monographs several years ago (abnormal toxicity test; Schwanig et al, 1997) or recently (target animal batch safety test; EDQM 2012). Since these tests may still be required outside of Europe, European manufacturers may need to carry out these tests when exporting to third countries.

In 2013, the Committee for Medicinal Products for Veterinary Use of the European Medicines Agency (EMA, 2013) adopted the guideline on "Harmonization of criteria to waive the target animal batch safety testing for inactivated vaccines for veterinary use" (VICH GL50). Its implementation (from 1st March 2014 onwards) is a major step towards international harmonisation and allows European manufacturers to apply for a waiver of the TABST when exporting to the other VICH regions (Japan, North America) or countries following the VICH guidelines.
Since 2008, EURL ECVAM has been working on behalf of EMA with VICH experts on the development of this guideline thus following up a project dating back to 1997 (more information is available on the EURL ECVAM website; AGAATI, 2002).
The work on a comparable VICH guideline for live veterinary vaccines started in 2013 with EURL ECVAM as topic leader.

5.3 EURL ECVAM support to ECHA

During 2013, EURL ECVAM carried out a project for the European Chemicals Agency (ECHA) under the terms of a Service Level Agreement (SLA/ECHA-JRC/2012-2). The aim of the project was to develop a reference report on the state of the science of non-standard methods that are available for assessing the toxicological and ecotoxicological properties of chemicals. Non-standard methods refer to alternatives to animal experiments, such as *in vitro* tests and computational models, as well as animal methods that are not covered by current regulatory guidelines.
ECHA needs to have up-to-date information on non-standard methods since the Agency is responsible for implementing different regulatory processes in which there is an obligation or an opportunity to use non-standard methods, depending on the context. These processes relate to the REACH regulation, the Biocidal Products Regulation (BPR), as well as the Classification and Labelling and Packaging (CLP) Regulation.
To support ECHA in examining cases where non-standard data are used or proposed under the above-mentioned regulations, the reference report covers the current scientific status, with commentary on the mechanistic basis and regulatory applicability, of methods for a range of human health and ecotoxicological endpoints. In particular, the following human health endpoints are covered: a) skin irritation and corrosion; b) serious eye damage and eye irritation; c) skin sensitisation; d) acute systemic toxicity; e) repeat dose toxicity; f) genotoxicity and mutagenicity; g) carcinogenicity; h) reproductive toxicity (including effects on development and fertility); i) endocrine disruption relevant to human health; and j) toxicokinetics. In relation to ecotoxicological endpoints, the reference report focuses on non-standard methods for acute and chronic fish toxicity.
The reference report will be published as a JRC Science and Policy report during 2014.

5.4 European Partnership for Alternative Approaches to Animal Testing (EPAA)

5.4.1 EPAA Carcinogenicity Workshop

To compare regulatory requirements in different sectors and to look for opportunities for cross-sector learning that could lead to appreciable 3Rs benefits, a questionnaire was designed and sent to The European Partnership for Alternative Approaches to Animal Testing (EPAA) partners, involved in human medicine (European Federation of Pharmaceutical Industries and Associations (EFPIA)), veterinary medicine (International Federation for Animal Health Europe (IFAH-Europe)) and crop protection (European Crop Protection Association (ECPA)). Based on the outcome of this survey, it became evident that especially in the field of carcinogenicity, different schemes operating in the European Union regulate different types of chemical products. A conference was held in Brussels in 2013 where representatives of the pharmaceutical, animal health, chemical and crop protection industries, together with representatives of other stakeholders, met under the auspices of the EPAA to discuss the varying requirements for carcinogenicity testing, and how these studies might be refined to improve hazard evaluation and risk assessment while implementing principles of the 3Rs. Whilst there are some similarities, the regulatory approaches in all four sectors have varying degrees of flexibility in requirements for carcinogenicity testing, to an extent reflecting concerns over the magnitude and duration of human exposure, either directly as in therapeutic exposure to pharmaceuticals, or indirectly through the ingestion of residues of veterinary drugs or crop protection chemicals (Annys et al., 2014). It was also recognised that there is significant scope for the harmonisation of testing requirements while extending the interests of the 3Rs in terms of replacement, refinement and reduction in the design and conduct of carcinogenicity studies. It was recommended that consideration of alternative methodologies should be data-driven, and should involve industry and regulators from the sector involved.

5.4.2 EPAA Platform on Regulation

5.4.2.1 EPAA Project on "Harmonisation on Biologicals"

The project aims at progressing harmonisation of requirements for batch testing of vaccines and other biological products at global level. Due to evident differences in the current regional requirements, manufacturers may need to carry out animal tests which are no longer required in Europe, if they want to market their products outside of Europe.
In a first step, regulatory bodies, key requirements and differences in the various regions are mapped and possible areas for harmonisation defined. It is planned to organise a workshop and discuss the findings and possible ways forward with regulatory bodies in late 2014. EURL ECVAM is a member of the project team. More information is available on the EPAA website[10].

[10] http://ec.europa.eu/enterprise/epaa/platform-regulation/biologicals/biologicals-project_en.htm

5.4.2.2 EPAA Project on "The Vaccines Consistency Approach"

In order to facilitate the introduction of the consistency approach for the quality control of established human and veterinary vaccines, EPAA has initiated a project aiming at developing and validating non-animal methods with the support of stakeholders from academia, regulators, Official Medicines Control Laboratories (OMCLs), European Directorate for the Quality of Medicines & HealthCare (EDQM), European Commission and vaccine manufacturers. The project's Technical Committee agreed on four priority vaccines/vaccine groups (diphtheria/tetanus/acellular pertussis vaccines; human rabies vaccines; veterinary rabies vaccines; clostridial vaccines) and established expert working groups to explore ways to implement the consistency approach. For this purpose, workshops were organised during 2012 and 2013[11]. Two collaborative studies have been launched in 2013 and will be finalised in 2014. The clostridial vaccines group is validating cell culture based methods to replace the Minimum Lethal Dose and Total Combining Power assays required for in-process control of *Clostridium septicum* vaccines. The experimental work started early in 2014 and a workshop will be held later in the year to discuss the results. The human rabies vaccines group aims at replacing the current in vivo method for potency testing of rabies vaccines with an *in vitro* antigen quantification assay. Since several methods are in use, the group launched a study for selecting the most suitable ELISA for quantitation of glycoprotein-G, involving reagents (vaccines and standards) from three manufacturers and testing in five laboratories. The European Commission (via EURL ECVAM) is providing funding for carrying out independent statistical data analysis. EPAA will host a meeting later in 2014 to discuss the findings and make recommendations for a formal collaborative study to the Biological Standardisation Programme of EDQM. The meeting will be shared with the veterinary rabies group.

5.5 Development of Integrated Approaches to Testing and Assessment

5.5.1 Guidance Document on IATA on skin sensitisation

In order to replace animal testing for the assessment of skin sensitisation, it is proposed that a combination of alternative methods addressing key mechanisms of the skin sensitisation pathway will be needed. Efforts are underway to develop integrated approaches for predicting skin sensitisation potential and potency based on the use of various information including predictions from in silico, in chemico, and *in vitro* methods. Following an initial proposal for integrating information from alternative methods by Jowsey et al. (2006), a number of different approaches for data integration have emerged since then, most of which foresee the use of data generated with the validated test methods mentioned above. These integration approaches range from more simple sequential testing strategies (van der Veen et al., 2014; Nukada et al., 2013) and statistical/weight-of-evidence based solutions (e.g. Natsch et al., 2009; Bauch et al., 2012,) to Bayesian Networks which encode probabilistic relationships among the input variables

[11] see flash reports on the project website http://ec.europa.eu/enterprise/epaa/platform-regulation/vaccines/vaccines-consistency_en.htm

(Jaworska et al., 2013) and artificial neural network analysis of data from multiple *in vitro* assays (Tsujita-Inoue et al., 2014). In addition, the use of mathematical models is being explored to quantify the relationship between the dose of sensitiser applied to the skin and the extent of the hapten-specific T cell response that would result in humans (Maxwell et al., 2014).

As described in its strategy paper (EC EURL ECVAM, 2013b), EURL ECVAM is contributing to the development of non-animal integrated approaches, underpinned by physico-chemical properties, in silico predictions and data from mechanistically relevant in chemico and *in vitro* methods. Such approaches are envisaged to have the capability not only to discriminate between sensitisers and non-sensitisers but also to categorise sensitisers into sub-categories 1A and 1B of UN GHS (UN GHS; UN 2013). EURL ECVAM is collaborating with ECHA in making sure that the developed approaches will fulfil information requirements under the 2018 REACH registration deadline.

A complementary project is pursued by the Cosmetics Europe Skin Tolerance Task Force consisting of a multiple-phases evaluation program for prioritising test methods for assessing their contribution within integrated approaches, with the ultimate goal to deliver non-animal testing strategies for potency prediction. The US National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) is evaluating high-throughput screening assays for skin sensitisation in coordination with the US National Institute of Environmental Health Sciences (NIEHS) Tox21activities. In addition, the US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recently communicated their intention to start activities aimed at designing integrated decision strategies based on the use of data from EURL ECVAM validated methods (DPRA, KeratinoSens™ and h-CLAT) and in silico predictions. In the light of the above it is foreseen that different integrated approaches for skin sensitisation that will cover different regulatory goals (i.e. hazard identification and/or potency prediction) will become available in the near future. It will be therefore important to have a consistent approach for their assessment and application. To this end EURL ECVAM is playing a prominent role at the OECD in the definition of guidance on how to document and evaluate IATA in view of their intended applications in order to promote their international acceptance. This project foresees the development of a general framework for skin sensitisation IATA suitable for all chemicals and regulatory applications. Different integrated approaches, targeting various regulatory needs, will be described in a consistent manner to help identifying strengths and limitations of each ultimately to facilitate interpretation of predictions by end-users.

5.5.2 Guidance Document on IATA on skin irritation/corrosion

In the past two years, EURL ECVAM has been involved, through participation in the OECD expert group on skin corrosion/irritation, in the development and drafting of a Guidance Document (GD) on an *Integrated Assessment and Testing Approach* (IATA) for skin corrosion and irritation testing, a project led by Germany. The impartiality of JRC scientists, acknowledged by all participants of the OECD expert groupwhich contains also

stakeholders with vested interests, helped in resolving some contentious issues and allowed finalising the GD so that it could be adopted by the WNT in 2014.

Briefly, the GD builds on the Integrated Testing Strategy developed for REACH in 2006/2007 under ECVAM coordination, but provides, as its major innovation, a more systematic approach towards the performance characterisation of individual information sources. The IATA is composed of well described and characterised "Modules" (i.e. distinct information sources such as testing and non-testing methods). The strengths and limitations as well as the potential role and contribution of each Module and their individual components in the IATA for skin irritation and corrosion are described with the purpose of minimizing the use of animals to the extent possible. Furthermore, the IATA includes, in line with provisions of the REACH Regulation (e.g. Annex XI) already implemented in 2007, reference to test methods that have not (yet) been validated and are not (yet) internationally recognized. Moreover, the GD's guidance on how to integrate the information from various sources exploits recent activities (e.g. subcategorisation by RhE-based assays for skin corrosion) and experiences and is more prescriptive than the current ITS related to REACH (ECHA, 2013).

The rationale for developing the GD was as follows: Since 2002, the OECD TG 404 on *in vivo* acute dermal irritation and corrosion testing (OECD, 2002) contains a supplement describing a sequential testing and evaluation strategy for skin corrosion and irritation. While this supplement is not covered by the OECD Council decision on Mutual Acceptance of Data (MAD), it has nevertheless provided valuable guidance on how to consider existing information and organise the generation of new testing data on skin corrosion/irritation. Steps 5 and 6 of this sequential testing and evaluation strategy refer to validated and accepted *in vitro* or *ex vivo* test methods for skin corrosion and skin irritation, respectively, before the use of the *in vivo* test (i.e. TG 404) in step 7. This was intended to minimise animal use. However there was some ambiguity within this strategy as it does not foresee the use of *negative results* from validated and accepted *in vitro* skin irritation assays, which can be understood as requiring confirmatory *in vivo* testing in such situations. Since publication of the supplement in 2002, several Test Guidelines on *in vitro* methods for skin corrosion or irritation have been published and/or updated, notably TG 439 (OECD, 2013, first released in 2010) on *in vitro* skin irritation and TGs 430 (OECD 2013b), 431 (OECD, 2013c) and 435 (OECD, 2006) on *in vitro* skin corrosion. Depending on country requirements, the now available validated and OECD accepted *in vitro* methods may satisfy all information requirements for skin corrosion and irritation. In addition, test methods not yet covered by regulatory guidelines at national, supranational or international level may provide further information required by some authorities, e.g. on sub-categorisation of corrosives or information on Category 3 irritants. Although the suitability of such data for regulatory purposes needs to be judged case-by-case, they should be considered before conducting animal tests. Thus, the supplement to TG 404 required update in view of amending the possible use and usefulness of individual test methods described within this

strategy and in order to avoid contradiction between the provisions of individual TGs on *in vitro* methods and the provisions of the TG 404 supplement. Moreover, in view of growing experience with the composition and use of IATAs, in particular for this specific health endpoint, a revision in view of incorporating current scientific and regulatory considerations and practices seemed timely.

6. Dissemination of Information and Alternatives[12]

6.1 In vitro methods

6.1.1 DB-ALM—EURL ECVAM's DataBase service on ALternative Methods to animal experimentation

The readily access to comprehensively described methods is a prerequisite for their use within decision making processes by regulators and scientists or any end-user in biomedical sciences and toxicology. The DB-ALM[13] provides standardised descriptions of methods that are at all stages of development, validation or regulatory acceptance in the different policy areas and for different purposes. Information at various level of detail is provided and defined according to pre-determined criteria for data content by experts in the field. Current focus is given to *in vitro* methods used for safety assessments of chemicals and/or formulations, but it is not limited to it.

The method reporting formats have evolved to the state-of-the-art of science and costumer needs so as to capture all information elements necessary to allow judgments of its usefulness, that now cover: information on the *potential of a method* including its intended objectives and applications, the *scientific principle and need for it,* a summary description of *study results* obtained so far including *performance and reliability evaluations* as available and appropriate, discussions on *strengths* and *eventual limitations* completed with their *status of development, validation or regulatory acceptance*.

Status

- *Contents*

To date the DB-ALM provides the following information:

Information Sector	Data Sheet Number
Topic Summaries	5
Method Summaries	157
Protocols	147
Evaluations, EU projects, Validation studies	85
Test Results (individual investigations)	9231
Persons & Institutions active in the field of alternative methods	238
Bibliographic References	7003

[12] The EURL ECVAM databases on alternative methods originate from the Communication of the Commission to Council and European Parliament SEC(91)1794, further reinforced by Directive 2010/63.
[13] DB-ALM: http://ecvam-dbalm.jrc.ec.europa.eu

During 2013 the online information content has been enhanced with particular focus on methods submitted for validation and those originating form EU Integrated Projects, such as ACuteTox. In total 17 methods in the form of summary descriptions or protocols and related information have been revised or newly published; further 14 are close to finalisation. In total, 5 topic areas in the form of thematic reviews are covered providing method summary descriptions and related information, while individual protocols are made available for 25 topic areas addressing human health and ecotoxicological effects of chemical substances, mechanistic information, quality control of biological products, and biocompatibility and safety testing of medical devices.

Over 150 biological endpoints are moreover addressed referring to biological processes, responses or effects that can be measured at various levels of biological organisation, such as:

- interactions on the molecular level (including biochemistry and bio-kinetics)
- basal cytotoxicity testing
- functional parameters of organs and tissues
- model organism responses

The methods can be consulted at the address provided.

- *Usage*

The year 2013 has seen a duplication in the number of new registrations compared to the same period of the year before and a download of medially 300 documents/months representing an increase by 65 % compared to the same period of 2012. In total, the DB-ALM can refer to over 3500 registered users from 82 countries covering representatives from academia (45%), industry (33%) and regulators (13%), the animal welfare movement and others (9%).

Further in 2013, the OECD Advisory Group on Molecular Screening and Toxicogenomics has set up a drafting group to develop guidance for characterising non-guideline *in vitro* methods used for regulatory purposes as a supplement to the existing guidance for development and assessment of Adverse Outcome Pathways. The DB-ALM was considered as a potential public resource for storing those methods in a standardised manner.

6.1.2 TSAR (Tracking System for Alternative test methods towards Regulatory acceptance)

A first version of TSAR[14] has been set up by the Joint Research Centre to track progress, in a transparent manner, from proposal of an alternative method for validation through to its final adoption by its inclusion into the regulatory framework (EU, OECD and related standards). An entirely revised version will be made available by the end of 2014. It will also address the needs of the individual partners participating in the International

[14] TSAR: http://tsar.jrc.ec.europa.eu/index.php?process=1&stage=1

Collaboration on Alternative Testing Methods (ICATM) providing in this way an overall view of the methods under evaluation by all ICATM partners from one public access point.

6.2 In silico methods

JRC QSAR Model Database

The JRC QSAR Model Database[15] is a freely accessible web application that enables users to submit, publish, and search QSAR Model Reporting Format (QMRF) reports. Developers and users of QSAR models can submit to the dedicated mailbox information on QSARs by using the QMRF. A downloadable QMRF editor is used for this purpose. The JRC then performs a quality control (i.e. adequacy and completeness of the documentation) of the QMRF submitted. Properly documented QMRFs are included in the JRC QSAR Model Database. Inclusion of the model does not imply acceptance or endorsement by the JRC or the European Commission, and responsibility for use of the models lies with the end-users.

Status of QMRFs in the JRC QSAR Model Database

At the time of writing (March 2014), the JRC QSAR Model Database contains 70 QMRFs, including two for physical chemical properties, 12 for environmental fate parameters, 23 for ecotoxic effects, 30 for human health effects, two for toxicokinetic properties and one for chromosome damage. A number of additional QMRFs will also be uploaded in a new version of the database that will be launched in 2014 from the same webpage.

[15] http://qsardb.jrc.ec.europa.eu/qmrf/

To date the QSAR database provides the following information content:

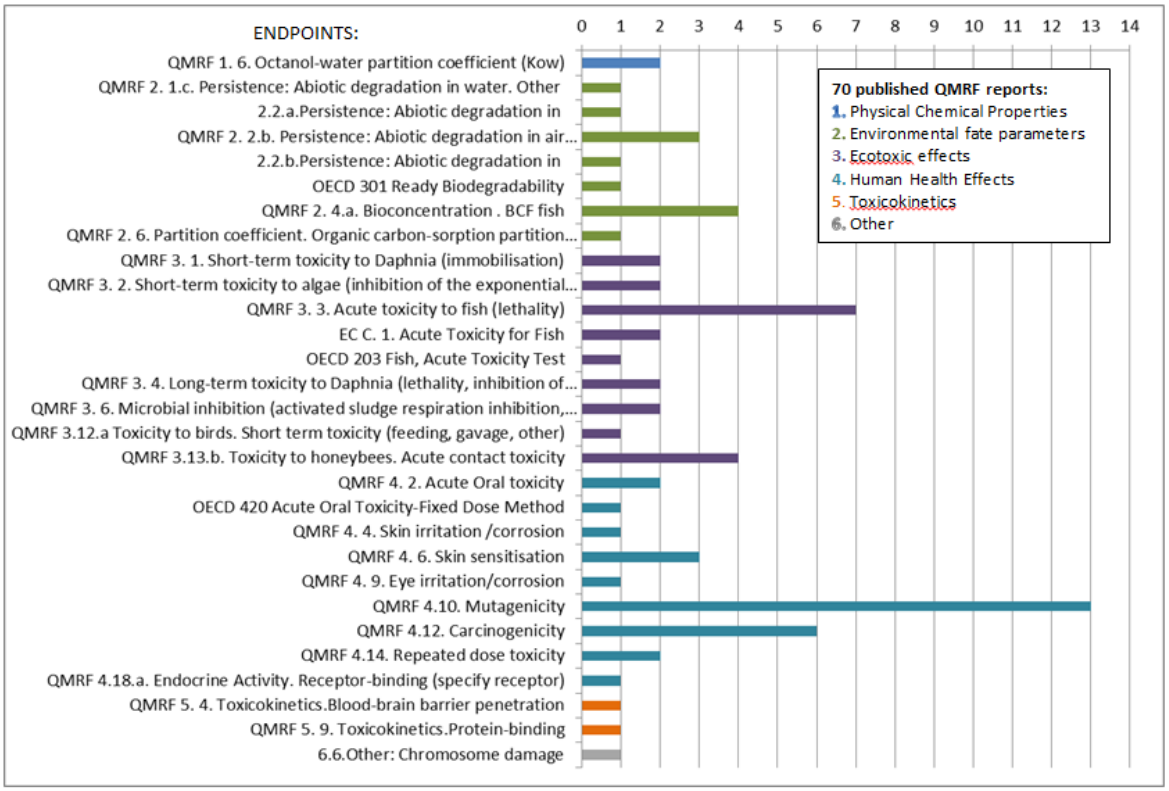

Figure 2. Information content provided by the QSAR database

6.3 Information Retrieval Guidance

EURL ECVAM Search Guide

After the success that the EURL ECVAM Search Guide[16] reached in 2012, the JRC has decided to re-publish an entirely updated version which is available as a printed version (handbook) or as an E-Book from the EU Bookshop since August 2013. It is continuing to be used as a resource for higher education in academic institutions and by national authorities for project authorisations.

The Guide has specifically been developed to inform and support untrained database users to find high quality information on relevant alternative methods and strategies in the large amount of available information resources as an easy, yet systematic, and efficient way.

[16] The Guide: http://bookshop.europa.eu/en/the-eurl-ecvam-search-guide-pbLBN124391

7. International Cooperation on Alternative Methods (ICATM)

7.1 Context of ICATM Establishment

The International Cooperation on Alternative Methods was formally established on April 27th, 2009 through the signature of a Memorandum of Cooperation (MoC). Through this MoC, ICATM partners recognized that their purpose is to promote consistent and enhanced voluntary international cooperation, collaboration, and communication among national validation organizations. This purpose may be achieved through five objectives:

1. further the optimal design and conduct of validation studies to support national and international regulatory decisions on the usefulness and limitations of alternative methods;
2. further high quality independent scientific peer reviews of alternative test methods that incorporate transparency and the opportunity for stakeholder involvement;
3. enhance the likelihood of harmonized recommendations by validation organizations on the usefulness and limitations of alternative test methods for regulatory testing purposes;
4. achieve greater efficiency and effectiveness by avoiding duplication of effort and leveraging limited resources;
5. support the timely international adoption of alternative methods.

Four organizations originally signed this MoC on April 27th, 2009:

1. the Japanese Center for the Validation of Alternative Methods (JaCVAM), within the National Institute of Health Sciences;
2. the US National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), a component of the National Institute of Environmental Health Sciences, administers the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM);
3. the European Centre for the Validation of Alternative Methods (ECVAM), within the Institute for Health and Consumer Protection, Joint Research Centre, European Commission;
4. the Environmental Health Science and Research Bureau within Health Canada.

On March 8th, 2011 a fifth partner organization, the Korean Center for the Validation of Alternative Methods joined the ICATM and an updated MoC was signed by all partners for this purpose.

Since the establishment of ICATM, all partners met several times a year in order to reinforce their cooperation, address further the terms and practicalities of this collaboration and present updates on their activities.

Annex II presents a table summarizing ICATM activities on alternative test methods validation and regulatory acceptance.

7.2 ICATM Meetings in 2013

In 2013, the ICATM partners met three times, in Tokyo (Japan) on February 12th, 2013; in Seoul (Korea) on July 3rd, 2013 and in Ispra Joint Research Centre (Italy) on November 26-27th, 2013. ICATM partners received a letter of interest from the Brazilian Center for Validation of Alternative Methods (BraCVAM) on May 31st, 2013 to join ICATM.

During these meetings ICATM partners presented updates of their respective activities, progresses achieved towards the validation of alternative methods, and presented their in house specific procedures. In that respect, they re-evaluated the scope of the MoC and agreed that it was describing correctly, and within a broad extent, their collaboration. They recognized that ICATM collaboration practices would ideally be agreed upon in consensus. In these meetings, the partners addressed five main areas of cooperation for the validation of alternative methods that are:

1. Conduct of validation studies;
2. Conduct of peer reviews;
3. Harmonized approaches;
4. Communication and dissemination;
5. Strategic considerations with regard to international regulatory acceptance.

7.3 ICATM Achievements in 2013

At their latest meeting in November 2013, ICATM partners discussed their activities and set up a more sustainable cooperation. During this two-days meeting they widely exchanged information on their mandates, objectives, priorities, functioning, and interaction with other parties—such as e.g. national/international regulators and stakeholders. They addressed further opportunities for cooperation with regard to a broad range of topics related to alternative methods.

- Regarding the selection and prioritisation of test methods, ICATM partners agreed on key criteria to be met: regulatory relevance, impact on the 3Rs and on human health & environmental protection; scientific value; scientific and regulatory gaps addressed and costs involved.
- Regarding the conduct of validation studies, ICATM partners agreed to strengthen their collaboration on a voluntary basis and to create guidance on how validation studies should be conducted. They examined technical aspects encountered in validation studies of *in vitro* methods especially for the chemicals selection; the establishment of validation management groups (VMGs); the selection of participating laboratories; the potential harmonisation of study designs. They also discussed thoroughly the expertise that needed to be covered by Validation

Management Group (VMG) members as well as their respective contribution to and involvement in the VMGs of the other ICATM partners.

- Regarding the conduct of peer-reviews, ICATM partners converged in their views. They agreed that peer reviews should be independent—i.e. carried out by persons without any vested interest in the evaluated test method/approach—and performed through a scientific approach that thoroughly evaluates quality of studies and their results. The peer review process should deliver opinions and recommendations on the scientific validity of a test method. ICATM partners recognized the interest of sharing peer review outputs and getting mutually informed about their recommendations. After the conduct of a peer review, the ICATM partner to whom a method has been submitted will then communicate a position to regulators and relevant stakeholders, which will serve as a basis for regulatory acceptance at the international level. ICATM decided to further compare peer review processes in which they are involved in order to extend their future collaboration and enable reciprocally reference to each other peer reviews.
- For the communication and dissemination, they agreed to facilitate communication amongst themselves and towards regulators and stakeholders. ICATM partners aim to promote transparency and disseminating information to the public on alternative test methods that are being evaluated for regulatory purposes. For this purpose, they agreed to use the EURL ECVAM Tracking System for Alternative methods towards Regulatory acceptance (TSAR) currently under development, and analysed implications of potential dissemination through this common platform.
- They decided that their cooperative activities should encompass both validation and research/development with a regulatory objective. They also agreed to work together on topical toxicity, skin sensitisation, endocrine disruptors, biokinetics and genotoxicity.
- ICATM partners widely addressed opportunities for cooperation with regards to their involvement in international programmes, and concurred to develop sustained activities. They decided to expand their upstream collaboration on activities regarding the Organisation for Economic Cooperation and Development (OECD), and to support each other at the level of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and any other international programmes, when relevant. This includes getting reciprocally informed whenever possible on submission of projects in order to efficiently cooperate on test methods and approaches addressing the 3Rs and on their regulatory acceptance and impact.

7.4 ICATM involvement in Validation Management Teams (VMTs).

In 2013, EURL ECVAM was involved in ICATM collaborations as liaisons in validation management teams in the areas of eye irritation and reproductive and developmental toxicity. Currently, EURL ECVAM activities in VMTs include the cooperation in three different

validation studies led by JaCVAM i.e., the Hand1-Luc Stem cell test, the SIRC CVS and the Vitrigel-EIT.

The Hand1-Luc stem cell based test, designed to evaluate developmental toxicity of chemicals is undergoing a JaCVAM coordinated validation study. Currently, between-laboratory reproducibility and predictive capacity of the test method are assessed.
Another JaCVAM coordinated validation study is currently conducted with the SIRC-CVS cytotoxicity test assay in the area of eye irritation. The SIRC-CVS assay is related to the Short term exposure (STE) test, that is currently under review at the OECD. The SIRC-CVS assay is intended to be used in an initial step of a bottom-up approach for eye irritation testing. Currently the predictive capacity of the test method using blinded test chemicals is evaluated.

The Vitrigel-Eye Irritancy Test (EIT) method is based on a human corneal epithelial cell line, grown on a scaffold of a collagen vitrigel membrane, containing high-density collagen fibrils equivalent to connective tissue as a three-dimensional cell culture model. Changes in the transepithelial electrical resistance (TEER) in response to the exposure of a test chemical, using the barrier function of the epithelium as an indicator allows the estimation of its irritancy potential. In an initial study, 30 Chemicals were tested for their correlation between the GHS classification and Vitrigel EIT. Data from this study suggests that eye irritation potential can be determined by this test method with a very low false negative rate. A JaCVAM coordinated validation study according to the conceptual framework of the "modular approach" was launched in 2013.

EURL ECVAM is also liaising with JaCVAM on the validation of the IL-8 Luc assay, a test method based on the use of a stable THP-1-derived IL-8 reporter cell line and proposed for the discrimination between sensitisers and non-sensitisers (Takahashi et a., 2011).

8. EURL ECVAM Strategies

8.1 EURL ECVAM strategy on how to avoid and reduce animal use for assessing chemicals for genotoxicity

The EURL ECVAM strategy on how to avoid and reduce animal use for assessing chemicals for genotoxicity[17] was published in 2013.Although several *in vitro* tests are available at different stages of development and acceptance, they cannot at present be considered to fully replace animal tests needed to evaluate the safety of substances. Based on an analysis of regulatory requirements for this endpoint within different pieces of EU legislation, EURL ECVAM proposes a pragmatic approach to improve the traditional genotoxicity testing paradigm that offers solutions in both the short- and medium-term and that draws on the considerable experience of 40 years of regulatory toxicology testing in this area. EURL ECVAM considers that efforts should be directed towards the overall improvement of the current testing strategy for better hazard and risk assessment approaches, which either avoids or minimises the use of animals, whilst satisfying regulatory information requirements, irrespective of regulatory context. Several opportunities for the improvement of the testing strategy have been identified which aim to enhance the performance of the *in vitro* testing battery so that fewer in vivo follow-up tests are necessary, and guide more intelligent in vivo follow-up testing to reduce unnecessary use of animals. The strategy went through consultation with EURL ECVAM's advisory and stakeholder bodies and aims to satisfy regulatory requirements within various pieces of EU legislation. The implementation of this strategic plan will rely on the cooperation of EURL ECVAM with other existing initiatives and the coordinated contribution from various stakeholders.

8.2 EURL ECVAM Genotoxicity and Carcinogenicity Database of positive Ames test results

The Ames test conducted in bacteria is the most commonly used genotoxicity test within the *in vitro* battery as it is considered able to reveal DNA reactivity and DNA reactive compounds. It is used to assess almost all types of substances including impurities, low production volume chemicals, etc. Therefore, knowing whether *in vitro* positive results are accurate indicators of *in vivo* mutagenic potential or carcinogenicity is extremely important in determining whether follow-up *in vivo* tests are needed or whether substances should be further tested. Despite the many activities on false positive results in *in vitro* mammalian cell tests, positive results in the Ames test have not been analysed in the same way as for mammalian cell tests. In 2013 EURL ECVAM held a workshop and initiated a project with the aim of 1) evaluating the predictivity of the Ames for *in vivo* genotoxicity and carcinogenicity when considered alone or in association with a mammalian cell assay for

[17] http://publications.jrc.ec.europa.eu/repository/bitstream/111111111/30088/1/jrc_report_en_34844_online.pdf

the detection of chromosome damage and/or gene mutations, and 2) better characterising the cases where the Ames test seems to lead to irrelevant (false positive) results (e.g. chemical classes, type of bacterial strains, magnitude of effects). As data presented at the workshop were from different sources (ECHA dissemination database, EFSA pesticides, carcinogens of the Carcinogenicity and Genotoxicity eXperience database (CGX), US NTP, US FDA, NTP, FDA, Japanese CSCL and ISHL, SCCS and other industry databases), and some of the chemicals were duplicated across different databases, EURL ECVAM constructed a consolidated database (manuscript in preparation) that will represent a powerful resource for data analysis and can be used to guide a thorough evaluation of genotoxicity and carcinogenicity.

8.3 EURL ECVAM Strategy to avoid, reduce and refine the use of animals in the assessment of acute systemic toxicity

The EURL ECVAM strategy is based on the assessment of the regulatory needs for acute systemic toxicity and the current state of the science in the area of acute systemic toxicity, including recent and ongoing efforts. Currently, only in vivo tests are accepted by regulatory bodies and most of them use lethality as an apical endpoint. One of the main drivers for conducting these standard tests is classification and labelling of substances. The development of mechanistically-based alternative methods and strategies for acute systemic toxicity is desirable; however it is still hampered by the limited understanding of the key acute toxicity pathways in humans. Although to date, *in vitro* cytotoxicity assays are recognised only as additional tests that can be used for estimating the initial doses for acute oral systemic toxicity tests in vivo (OECD GD N°129), the evidence indicates that the 3T3 NRU basal cytotoxicity assay can be used to support the identification of negatives (non-classified substances – i.e. oral LD50 value > 2000 mg/kg b.w.), with the caveat that, due to the limitations of this test method, results should always be used in combination with other information sources to build confidence in the decision not to classify a substance for acute oral toxicity (EC ECVAM, 2013). With this in mind, EURL ECVAM proposes to explore options to make better use of existing alternative methods, such as the validated 3T3 NRU cytotoxicity assay, since the information provided is expected to contribute to the weight-of-evidence in future IATA for acute systemic toxicity. Information on repeat dose toxicity, if available, might also be informative and useful to support classification and labelling. Special consideration should be given to collecting and organising mechanistic knowledge of acute systemic toxicity in order to improve the design and validation of integrated prediction models. In addition, EURL ECVAM will continue to support activities aimed at the refinement of relevant in vivo studies. The strategy is now under consultation with EURL ECVAM's advisory and stakeholder bodies. After its publication, the implementation will rely on the cooperation of EURL ECVAM with other related initiatives and the contribution from various stakeholders.

8.4 EURL ECVAM Strategy to avoid, reduce and refine the use of fish in aquatic toxicity and bioconcentration/bioaccumulation testing

EURL ECVAM is currently drafting its strategic plan on activities that are expected to have a 3Rs impact in the area of aquatic toxicity and bioaccumulation. It is based on an assessment of the regulatory needs for these endpoints and on the scientific state-of-the art in the areas, including recent and ongoing activities. The strategy will also provide a framework for the prioritisation of alternative test methods submitted to EURL ECVAM for validation.

8.5 Update on the EURL ECVAM Strategy on Toxicokinetics

At the time of writing, a draft for the EURL ECVAM Strategy on Toxicokinetics (TK) was under preparation. In order to improve risk assessment methodology for human health related to exposure to chemicals and create a basis to address some prominent public health issues in this field, The Toxicokinetics Strategy document has as a single overarching aim to strive for a transition from the traditional approach based on external exposure dose metrics to an approach based on internal dose metrics. . An important topic is the necessity to develop standards necessary to characterise *in vitro* and *in silico* methods that measure individual ADME parameters. *In vitro* methods standards will be instrumental to determine the performance of an *in vitro* ADME method. Developing a series of *in vitro* ADME methods with an acceptable level of performance (relevance and reliability) and a toolbox of standards (standard methods) to measure that performance for each method in relation to its use case seems to become a challenging need. Furthermore, good physiologically-based toxicokinetic (PBTK) modelling practices will be necessary to facilitate the use of PBTK modelling in a risk assessment environment. As such the issuing of guidance on the development and use of toxicokinetic data in testing and assessment strategies using case studies approaches will be an action that will be necessary. In summary, five key topics are defined: standards for *in vitro* and *in silico* ADME methods, GMP for good PBTK modelling practices, databases for ADME parameters, databases for measured TK data and guidance for the use of ADME and TK in IATA.

9. Conclusion

EURL ECVAM's activities in 2013 to April 2014 covered a broad range of topics, from R&D in areas still lacking 3Rs solutions (e.g. repeated dose toxicity testing) to leading efforts towards the international adoption of Test Guidelines and Guidance Documents in areas which are more advanced (e.g. skin irritation/corrosion, eye irritation and skin sensitisation).

Research and development activities undertaken to tackle the complex endpoints have yielded a wide variety of new methodologies and tools for mechanistic-based toxicology, such as complex bioreactors for engineering of human tissues, innovative 'omics' technics, differentiation protocols for human induced pluripotent stem cells and computational modelling tools. It is currently being assessed how this range of complementary non-animal methods can be integrated according to a toxicological MoA/AOP framework.

EURL ECVAM strategy papers were developed in the areas of genotoxicity, acute systemic toxicity, aquatic toxicicity and bioconcentration/bioaccumulation testing and toxicokinetics. These strategies address different regulatory areas (e.g. chemicals, cosmetics, biocidal products, pharmaceuticals) and their related needs. They review the progress made so far, identify gaps and opportunities in relation to method development and validation and outline what actions should be taken to deliver solutions with 3Rs impact. They are developed in close consultation with EURL ECVAM's network of regulators, stakeholders and international partners.

The Adverse Outcome Pathways framework, guiding the design of Integrated Approaches to Assessment and Testing (IATA), provides a knowledge-based safety assessment framework that enables the selection of the most relevant test methods for validation. The availability of a toolbox of validated alternative methods addressing key events of an AOP and incorporated in IATAs facilitates the regulatory acceptance/adoption process of alternative methods at both international and European level and their global use. It is anticipated therefore that as various AOP-related initiatives gain momentum in the years ahead, more clarity and opportunity will emerge on where to target efforts in the development and validation of alternative methods to maximum effect.

International cooperation between multiple entities active in the3Rs is an essential element to progress the field. EURL ECVAM continues therefore to engage proactively on an international stage endeavouring to guide the integration of efforts and to provide leadership in key areas where it has competence.

In conclusion, considerable progress has been made in many aspects of the development, validation and regulatory acceptance of alternative methods and approaches during the period March 2013 to April 2014. This rate of progress can be maintained with continuing commitment from the 3Rs community coupled with sustained investment and support from a variety of key actors and stakeholders.

Annex I—Summary status of the adoption of Test Guidelines based on alternative methods in the OECD TG programme (2012-2014)

Table 1 summarises the status of adoption of OECD test guidelines on alternative methods from 2012 to 2014. It should be noted that beside TGs, also Guidance Documents and new projects on alternative methods were respectively adopted and included on the OECD Work programme during that period. For additional information, please consult the OECD website of the Test Guideline Programme: http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicalsandrelateddocuments.htm

Table 1. Status of adoption of OECD Test Guidelines based on alternative methods 2012-2014

Nr.	Toxicity area	Test method description	Acceptance status
1	Skin corrosion	Reconstructed human Epidermis test methods (RhE) as included in OECD TG 431/EU TM B.40 bis	Adopted in 2004; updated version (sub-categorisation, inclusion of performance standards, inclusion of SkinEthic™ RHE and epiCS®) adopted in 2013. Revised version including sub-categorisation with the epiCS® test method adopted in 2014
2		Transcutaneous Electrical Resistance (TER) test as included in OECD TG 430/EU TM B.40	Adopted in 2004; updated version (inclusion of performance standards) adopted in 2013
3	Skin irritation	Reconstructed human Epidermis test methods (RhE) as included in OECD TG 439/EU B.46	Adopted in 2010; updated version (inclusion of LabCyte EPI-model24 SIT) adopted in 2013
4	Serious eye damage/eye irritation	Fluorescein Leakage (FL) test method as included in OECD TG 460	Adopted in 2012
5		Bovine Corneal Opacity and Permeability (BCOP) test method as included in OECD TG 437/EU TM B.47	Adopted in 2009; updated version (revision of positive controls, use to identify non-classified chemicals) adopted in 2013

Nr.	Toxicity area	Test method description	Acceptance status
6		Isolated Chicken Eye (ICE) test method as included in OECD TG 438/EU TM B.48	Adopted in 2009, updated version (use to identify non-classified chemicals) adopted in 2013
7		Cytosensor Microphysiometer (CM) test method	New draft TG discussed at WNT in 2013 but not yet adopted, pending further clarification on its use to identify non-classified chemicals
8		Short Time Exposure (STE) test	Draft TG underwent one commenting round in November 2013 and will undergo a second commenting round in view to be considered for adoption in April 2015
9	Skin sensitisation	Direct Peptide Reactivity Assay (DPRA)	New draft TG underwent one commenting round in November 2013 Revised TG circulated to the WNT in May 2014 for review and approval by written procedure
10		KeratinoSens	New draft TG underwent one commenting round in November 2013 Revised TG circulated to the WNT in May 2014 for review and approval by written procedure
11	Carcinogenicity	Cell Transformation Assay (CTA) SHE	New draft TG discussed at WNT in 2013 but not yet adopted. Draft TG was not adopted by WNT in 2014 either, but will be considered for adoption as Guidance Document after revisions
12		Cell Transformation Assay (CTA) Bhas 42	New draft TG underwent one

Nr.	Toxicity area	Test method description	Acceptance status
			commenting round. Draft TG will undergo a second commenting round in view to be considered for adoption in April 2015
13	Genotoxicity	Existing OECD TGs under revision	Revised OECD TG 473 (*in vitro* chromosome aberration assay; originally adopted in 1997) and OECD TG 487 (*in vitro* mammalian cell micronucleus test; originally adopted in 2010) adopted in 2014
14	Endocrine disruption	Estrogen receptor transactivation assay (BG1Luc ER TA; agonist and antagonist protocols) as included in OECD TG 457	Adopted in 2012
15		Performance-Based Test Guideline for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists OECD TG 455	Adopted in 2009 (STTA assay using the hERα-HeLa-9903 cell line); updated version (PBTG, inclusion of BG1Luc ER TA assay using the BG1Luc-4E2 cell line) adopted in 2012
16	Acute fish toxicity	Fish Embryo Acute Toxicity (FET) Test as included in OECD TG 236	Adopted in 2013

Annex II—ICATM Alternative Test Methods Validation and Regulatory Acceptance

Table 2. ICATM Alternative Test Methods Validation and Regulatory Acceptance

Method	Current Status	Lead Action Organization	International Acceptance
Dermal Corrosivity Test Methods			
CORROSITEX Skin Corrosivity Test	**Completed**		**OECD TG 435 (2006)**
EpiSkin™, EpiDerm™, SkinEthic™, epiCS® Skin Corrosivity Tests	**Completed**		**OECD TG 431 (2004), updated version (sub-categorization, inclusion of performance standards, inclusion of SkinEthic™ RHE and epiCS™) adopted in 2013. Revised version including the sub-categorization with the epiCS™ test method adopted in 2014**
Rat TER Skin Corrosivity Test	**Completed**		**OECD TG 430 (2004), updated version (inclusion of performance standards) adopted in 2013**
Dermal Irritation Test Methods			
In vitro* reconstructed human epidermis (RhE) test methods: EpiDerm™, EpiSkin™, SkinEthic™ RHE and LabCyte EPI-MODEL24 *SIT	**Completed**		**OECD TG 439 (2010), updated version (inclusion of LabCyte™ EPI-model) adopted in 2013**
In vitro reconstructed human epidermis (RhE) test methods: Korean epidermis	KoCVAM sponsored validation study is on-going	KoCVAM; EURL ECVAM, NICEATM-ICCVAM, Health	

Method	Current Status	Lead Action Organization	International Acceptance
model		Canada and JaCVAM VMT liaisons	
Phototoxicity Test Methods			
3T3 NRU Phototoxicity Test	**Completed**		**OECD TG 432 (2004)**
Test method battery to predict phototoxicity (yeast growth inhibition phototoxicity assay and red blood cell photohemolysis assay)	Japanese Regulatory Acceptance Board recommended additional work be performed	JaCVAM	
In vitro test method based on reactive oxygen species (ROS) and photostability	Peer review of the JaCVAM-sponsored validation study finalized in 2013.	JaCVAM; EURL ECVAM, NICEATM-ICCVAM, Health Canada and KoCVAM VMT liaisons	ICH S10 including the ROS assay and the 3T3 NRU test method was completed in 2014
Ocular Toxicity Test Methods			
Bovine Corneal Opacity and Permeability (BCOP) Test Method	**Completed**		**OECD TG 437 (2009), updated version (positive control, use in a bottom-up approach to identify non-classified chemicals) adopted in 2013**
Isolated Chicken Eye (ICE) Test Method	**Completed**		**OECD TG 438 (2009), updated version (use in a bottom-up approach to identify non-classified chemicals) adopted at WNT in 2013**

Method	Current Status	Lead Action Organization	International Acceptance
Use of Histopathology as an additional endpoint in Ocular Safety Testing	**Completed**		**OECD GD 160 (2011)**
Cytotoxicity test: SIRC CVS	JaCVAM-sponsored validation study is ongoing	JaCVAM; EURL ECVAM, NICEATM-ICCVAM, and Health Canada VMT	
Cytotoxicity test: three-dimensional dermal model (MATREX)	JaCVAM-sponsored validation study in the planning stage	JaCVAM; EURL ECVAM, NICEATM-ICCVAM, and Health Canada VMT	
Cytotoxicity test: Short Time Exposure (STE) test	Peer review coordinated by NICEATM-ICCVAM of the JaCVAM-sponsored validation study completed. The draft TG was submitted to OECD for comments.	JaCVAM; EURL ECVAM, NICEATM-ICCVAM, and Health Canada VMT liaisons	Draft TG underwent one commenting round. Draft TG will undergo a second commenting round in view to be considered for adoption in April 2015
Use of anaesthetics, analgesics, and humane endpoints for routine use in TG 405	**Completed**		**OECD updated TG 405 (2012)**
Low volume eye test; recommendation for no future use.	Completed		
In vitro approach for categorization of anti-microbial cleaning products: recommendations for further studies	Completed. EPA/OPP has concluded from submission and review of alternative eye irritation tests conducted on antimicrobial pesticide products with cleaning claims (AMCPs) that the proposed testing	NICEATM-ICCVAM	

Method	Current Status	Lead Action Organization	International Acceptance
	approach is acceptable for determining the appropriate eye hazard classification and labelling for AMCPs (see http://www.epa.gov/pesticides/regulating/eye-policy.pdf for the details of the scope of the policy).		
Cytosensor Microphysiometer® (CM) Test method	The draft TG was submitted to OECD for comments including a set of Performance Standards	EURL ECVAM; NICEATM-ICCVAM	New draft TG discussed at WNT in 2013 but not yet adopted, pending further clarification on its use in a bottom-up approach
Fluorescein Leakage (FL) test method	**Completed**		**OECD TG 460 (2012)**
Human reconstructed tissue models for eye irritation EpiOcular™ EIT SkinEthic™ HCE	EURL ECVAM validation study finalized (experimental part started in 2010 and ended in April 2013; the validation of an optimized EpiOcular™ solids protocol was completed in June 2013). Peer review of EpiOcular™ EIT anticipated for 2014.	EURL ECVAM; JaCVAM, NICEATM-ICCVAM, and Health Canada VMT liaisons	SPSF for EpiOcular™ EIT was approved by WNT in 2014
Vitrigel-EIT	MAFF-sponsored validation study is on-going	JaCVAM; EURL ECVAM, NICEATM-ICCVAM, and Health Canada VMT liaisons	
Immunotoxicity (Allergic Contact Dermatitis) Test Methods			

Method	Current Status	Lead Action Organization	International Acceptance
Murine local lymph node assay (LLNA) for skin sensitization	**Completed**		**OECD TG 429 (2002)** **ISO (2002)**
Updated Murine local lymph node assay (LLNA) for skin sensitization (20% reduction)	**Completed**		**Update to TG 429 OECD (2010)** **ISO (2010)**
Reduced LLNA (rLLNA)	**Completed**		**Update to TG 429 OECD (2010)**
Nonradioactive LLNA protocol (LLNA: BrdU-ELISA)	**Completed**		**OECD TG 442B OECD (2010)**
Nonradioactive LLNA protocol, LLNA:DA	**Completed**		**OECD TG 442A OECD (2010)**
Harmonized performance standards for the LLNA	**Completed**		**Update to TG 429 OECD (2010)**
Nonradioactive LLNA protocol (LLNA: BrdU-Flow Cytometry)	KoCVAM validation study is on-going	KoCVAM	
In vitro skin sensitization assays (h-CLAT; DPRA; MUSST)	Multi-laboratory validation ended in August 2012 (h-CLAT and MUSST). DPRA peer review finalized. h-CLAT peer review on-going.. EURL ECVAM recommendation on DPRA published.	EURL ECVAM; JaCVAM and NICEATM-ICCVAM VMT liaison members	SPSFs for TGs on the DPRA, and hCLAT approved in 2012 and 2013, respectively. Draft TG on DPRA under discussion at OECD, underwent one commenting round. Second commenting round will take place in 2014, with a view to have approval by WNT via written procedure in 2014

Method	Current Status	Lead Action Organization	International Acceptance
In vitro skin sensitization assay KeratinoSens™	External Validation Study, peer review finalized. EURL ECVAM recommendation published	EURL ECVAM	SPSF for a TG on the Keratinosens approved in 2012. Draft TG under discussion at OECD, underwent one commenting round. Second commenting round will take place in 2014, with a view to have approval by WNT via written procedure in 2014
In vitro skin sensitization assay IL-8 Luc assay	METI-sponsored validation study is on-going	JaCVAM; EURL ECVAM, NICEATM-ICCVAM, KoCVAM and Health Canada VMT liaisons	
Vitrigel-SST	MAFF-sponsored validation study is on-going	JaCVAM; EURL ECVAM, NICEATM-ICCVAM, and Health Canada VMT liaisons	
Acute Toxicity Test Methods			
Up and Down Procedure (UDP)	**Completed**		**OECD TG 425 (2008)**
***In vitro* cytotoxicity test methods for estimating starting doses for acute oral systemic toxicity tests**	**Completed**		**OECD GD 129 (2010)**
In vitro cytotoxicity test (3T3 Neutral Red Uptake) for identifying substances with acute oral LD50 > 2000 mg/kg b.w.	EURL ECVAM ESAC peer review completed, and EURL ECVAM Recommendation published in 2013.	EURL ECVAM and ICATM organisations	
Zebrafish Embryo Toxicity test (ZFET)	ESAC peer review finalized. EURL ECVAM recommendation pending	EURL ECVAM	Adoption of OECD TG 236 in April 2013

Method	Current Status	Lead Action Organization	International Acceptance
	publication.		
Toxicokinetic test methods			
In vitro hepatic biotransformation – CYP induction: Hepa RG and cryopreserved human hepatocytes	ESAC peer review foreseen in 2014	EURL ECVAM; NICEATM-ICCVAM, and JaCVAM VMT liaisons	SPSF for a PBTG approved in April 2013
In vitro Fish Hepatic Metabolism - *in vitro* system for deriving information on biotransformation and improving reliability of bio-concentration & bio-accumulation factors (BCF & BAF) and avoiding use of fish bio-concentration tests	Ring trial to be conducted in 2014 under the auspices of the OECD	United States and European Commission	SPSF for a TG on in vitro Fish Hepatic Metabolism approved in April 2014
Endocrine Disruptor Test Methods			
Stably transfected human estrogen receptor-α transcriptional activation assay for detection of estrogenic agonist-activity of chemicals	**Completed**		**OECD TG 455 (2009), updated 2012**
Stably transfected human estrogen receptor-α transcriptional activation assay for detection of estrogenic antagonist-activity of chemicals	International validation study in progress	JaCVAM and VMG NA liaisons	To be added to TG 455 when validated and peer reviewed
LUMI-CELL® human estrogen	**Completed**		**OECD TG 457 (2012)**

Method	Current Status	Lead Action Organization	International Acceptance
receptor transcriptional activation assay: agonist and antagonist protocols			
CertiChem MCF-7 cell proliferation assay for the detection of human estrogen receptor agonists and antagonists	International validation study completed. Protocol must be revised for adequate reproducibility	NICEATM-ICCVAM; EURL ECVAM, JaCVAM and KoCVAM VMT liaisons	
Stably transfected CHO Androgen receptor-α transcriptional activation assay for detection of androgenic agonist and antagonist activity of chemicals.	METI-sponsored validation is on-going	JaCVAM and VMG NA liaisons	
MELN® human estrogen receptor transcriptional activation assay: agonist and antagonist protocols	Validation study ongoing (EURL ECVAM). Peer review foreseen in 2014.	EURL ECVAM (lead), NICEATM-ICCVAM, JaCVAM	To be added to the PBTG when validated and peer reviewed
Stably Transfected Transactivation *in vitro* Assay to detect Androgen Receptor Agonists and Antagonists	Validation study starts in 2014	EURL ECVAM (lead), NICEATM-ICCVAM, JaCVAM	SPSF to develop a PBTG on ARTA approved in April 2013
Genetic Toxicity Test Methods			
***In vitro* micronucleus test**	**Completed**		**OECD TG 487 (2010) adopted in 2014**
***In vitro* chromosome aberration assay**	**Completed**		**OECD TG 473 (1997) adopted in 2014**
In vivo/in vitro comet assay	The peer review of the *in vivo* Comet	JaCVAM (lead); EURL	Adoption of draft OECD TG on the *in*

Method	Current Status	Lead Action Organization	International Acceptance
	assay by the OECD Comet assay expert group has been finalised. Validation of *in vitro* study ongoing	ECVAM, NICEATM-ICCVAM, KoCVAM and Health Canada VMT liaisons	*vivo* Comet assay expected in 2014
Genotoxicity assays (micronucleus and comet) in 3D skin models	Validation study ongoing	Cosmetics Europe (lead); EURL ECVAM support	
Transgenic rodent *in vivo* gene mutation assays. OECD TG 488 (2011)	Draft TG to be updated.	Health Canada	SPSF approved in April 2013.
Carcinogenicity Test Methods			
Bhas cell transformation assay (CTA)	Peer review coordinated by EURL ECVAM completed EURL ECVAM Recommendation published in 2013	JaCVAM (lead); EURL ECVAM, NICEATM-ICCVAM, and Health Canada VMT liaisons	SPSF approved in 2010. New draft TG underwent one commenting round. Draft TG will undergo a second commenting round in view to be considered for adoption in April 2015
SHE pH 6.7, SHE pH 7 and Balb/c 3T3 cell transformation assays (CTAs)	Pre-validation study and ESAC peer review completed Feb 2011; EURL ECVAM recommendation published.	EURL ECVAM	Draft TG was not adopted by WNT 26 in 2014 but to be considered for adoption as Guidance Document after revisions
Reproductive Test Methods			
Hand-1 Luc assay	METI-sponsored validation is on-going	JaCVAM (lead); EURL ECVAM, NICEATM-ICCVAM, and Health Canada VMT liaisons	

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