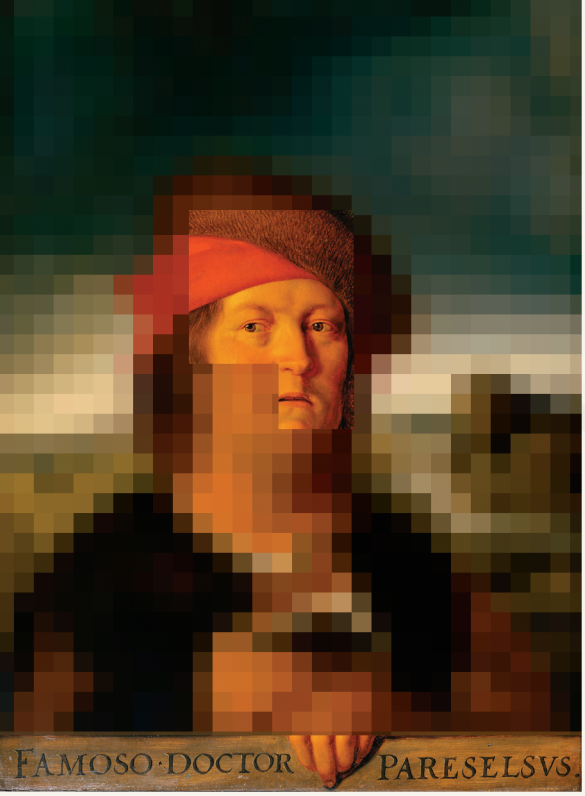


EUROPEAN COMMISSION
Research & Innovation

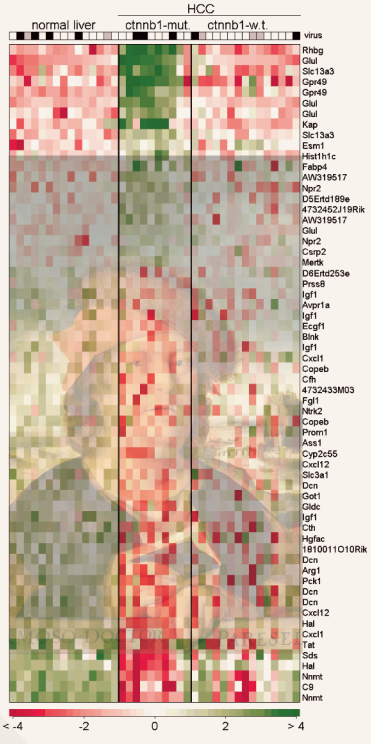
Cosmetics Europe
the personal care association

SEURAT-1

Paracelsus (1493-1541):
"The dose makes the poison"

Towards the Replacement of *in vivo* Repeated Dose Systemic Toxicity Testing

Toxicology in the 21st century:
Mechanism-driven Toxicology
defines the safe dose



Volume 6
2016

SEVENTH FRAMEWORK PROGRAMME

Health Programme: Advanced therapies and systems medicine (F4)
Coordination Action COACH: Grant Agreement N°: 26 7044
DG Research & Innovation

Paracelsus (1493 - 1541)
Portrait by Quentin Massys

« The dose makes the poison »

Paracelsus was a 16th century physician and alchemist who made significant progress in the field of medicine. Pioneer in chemistry, he made revolutionary advances in understanding and treating wounds and diseases.

Vol. 1 Vol. 2 Vol. 3 Vol. 4 Vol. 5 Vol. 6

This is the final volume of a series of six annual reports describing scientific progress, strategic development and evolution of the legislative and regulatory context in the field of repeated dose systemic toxicity.

The picture series illustrates the phase-out and replacement of the classical concepts of toxicology.

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This book is prepared by the Coordinating Action COACH Grant agreement N° 267044

"Coordination of projects on new approaches to replace current repeated dose systemic toxicity testing of cosmetics and chemicals"

The Blueprint for Future Safety Assessment of Chemicals

Edited by:
Tilman Gocht, Michael Schwarz

volume 6

SEURAT was announced as a strategy of the FP7 Health Theme by director Dr. Manuel Hallen on the occasion of the EPAA Annual Conference in 2008 in line with Commissioners G. Verheugen and J. Potocnik. The long term strategic target is defined as "Safety Evaluation Ultimately Replacing Animal Testing" (SEURAT).

SEURAT-1 is the Research Initiative launched by the European Commission and the European Cosmetics Association Colipa (funding: EUR 50 million from 2011 to 2015). It is called "SEURAT-1", indicating that more steps have to be taken before the final strategic target will be reached. **SEURAT-1** develops a long term research strategy and building blocks needed for the development of new non-animal test systems in the field of repeated dose systemic toxicity for the innovative assessment of human safety.

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ISBN : 978-2-9539634-5-8

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Printed in France - Imprimerie Mouzet

Foreword

This is the final volume of a series of six Annual Reports describing the achievements of the largest European Research Initiative focusing on the replacement of *in vivo* repeated dose systemic toxicity testing. As the editors of this book series and members of the coordination action project COACH, we are taking this opportunity to look back on the 5 years of **SEURAT-1** and into the near future in its field of research. **SEURAT-1** is coming to an end, but the SEURAT approach, defined as the strategic target 'safety evaluation ultimately replacing animal testing', is now in a transition phase. This specific feel of transition was also tangible during the final **SEURAT-1** symposium entitled 'Painting the future animal-free safety assessment of chemical substances: Achievements of **SEURAT-1**', which took place in Brussels on 4 December 2015. **SEURAT-1** success stories were presented in an accessible manner, and an exhibition showcased how the tools developed can be used today to improve safety assessment approaches. The end of **SEURAT-1** marks the beginning of follow-up activities and, consequently, the next major European project focussing on the development of mechanism-based, animal-free toxicity testing methods, called EU-ToxRisk, was outlined at this final symposium. This project, funded under Horizon 2020, the EU Framework Programme for Research and Innovation, will build on **SEURAT-1**'s findings, showing that **SEURAT-1** is over but the journey continues. The main activities of finalising **SEURAT-1** are now to hand over our achievements, prepare the legacy of **SEURAT-1,** and hand the baton on to the next consortium to keep the momentum going. That is also the purpose of this final **SEURAT-1** Annual Report.

We worked together for almost six years in this consortium, sharing ideas and stimulating cooperation between the various projects of the **SEURAT-1** Research Initiative and with other international consortia such as those organised under the umbrella of the US Tox21 research programme. As we are still major steps away from being able to replace animal testing in chemical safety assessment, our main conclusion is that there is a strong need to unify forces working towards knowledge-based safety assessment procedures based on a comprehensive understanding of how chemicals disturb biological systems. The challenge is still to overcome sectoral thinking, in particular to overcome barriers between the academic world and the regulatory community and to find ways to carry forward scientific developments into applications operating at the industrial scale. In this respect, the collaborative spirit of **SEURAT-1** may be the most important legacy for future activities in this field.

We wish our collaborators, in particular the EU-ToxRisk consortium but also the whole community involved in the development of alternative methods to replace animal testing, all the best to transform the 'SEURAT' vision into reality.

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Executive Summary

The **SEURAT-1** Research Initiative presented its achievements to the stakeholders from industry, policy, science and regulatory authorities during the final **SEURAT-1** Symposium on 4 December 2015 in Brussels, Belgium. The impact in the field of human safety assessment was highlighted at this Symposium demonstrating how the extensive, five-year research efforts of **SEURAT-1** can be translated into solutions for safety assessment ultimately replacing animal testing (see Text Box 1).

Major Achievements of the SEURAT-1 Research Initiative

Development of a research strategy based on generating and applying knowledge of mode-of-action

Development of highly innovative tools and methodology that can ultimately support regulatory safety assessment

Sustainable collection of data and standard operating procedures in a Data Warehouse

Development of a conceptual framework to combine evidence derived from predictive tools to support a safety assessment decision in a biologically rational manner

Application of the tools and concept developed in case studies addressing three scenarios:

- A Threshold of Toxicological Concern (TCC) case study, expanding the applicability domain of the established TTC approach towards cosmetic ingredients applied dermally
- A read-across case study demonstrating use of *in vitro* and *in silico* tools in regulatory toxicology
- An *ab initio* case study, illustrating proof of concept of how risk assessment for a cosmetic ingredient might be carried out without animal testing

The history of the **SEURAT-1** Research Initiative is strongly influenced by the full ban on animal testing for cosmetic products within the European Union, which finally entered into force on 11 March 2013. This deadline was set by the Seventh Amendment to the Council Directive on the approximation of the laws of the Member States relating to cosmetic products (76/768/EEC, 'Cosmetics Directive') and triggered the need to establish a European Research Initiative in the field of repeated dose systemic toxicity.

This publication is the final volume of a series of six Annual Reports that summarise the activities and main results of the **SEURAT-1** Research Initiative. **SEURAT-1** worked towards the long-term chemical safety testing goal of 'Safety Evaluation Ultimately Replacing Animal Testing' (SEURAT), which was presented by the HEALTH programme of the Seventh European Framework Programme for Research and Innovation (FP7) in 2008. The framework for this Research Initiative was created in June 2009, following the FP7 Call for Proposals 'Alternative Testing Strategies: Towards the replacement of *in vivo* repeated dose systemic toxicity testing' with total funding of EUR 50 million. It was called '**SEURAT-1**', indicating that this was the first step in the specific area of repeated dose systemic toxicity addressing the global long-term strategic target of SEURAT. The **SEURAT-1** Research Initiative started on 1 January 2011 and was co-funded by the European Commission Directorate-General for Research & Innovation through the HEALTH programme of FP7, and Cosmetics Europe.

The aim of the **SEURAT-1** Research Initiative was to develop a long-term research strategy leading to pathway-based human safety assessments in the field of repeated dose systemic toxicity testing of chemicals. The overall goal was to establish animal-free innovative toxicity testing methods, enabling robust safety assessments that are more predictive than existing testing procedures. In order to achieve this, a cluster of five research projects spread over 70 European universities, public research institutes and private companies was created, supported by a 'data handling and servicing' project and a 'coordination and support' project. The Scientific Expert Panel, made up of the **SEURAT-1** project coordinators and external international experts in the field of repeated dose systemic toxicity, provided scientific advice regarding the research work and future direction of the **SEURAT-1** Research Initiative, and thus played a key role in its scientific coordination.

The **SEURAT-1** Research Initiative combined expertise in cell culture for the preparation of stable human cell lines with the establishment of sophisticated experimental systems such as organ-simulating devices. This experimental work was linked with advanced methods of computational modelling and estimation techniques, taking innovative systems biology approaches into consideration; this required a coordinated effort from the **SEURAT-1** Research Initiative. The focal point of these joint activities was given by proof-of-concept studies (case studies) on three levels, demonstrating that: (i) mode-of-action theory provides a solid foundation for mechanistic understanding of adverse effects at the subcellular scale (theoretical level), which (ii) can be converted into the development of integrated animal-free

prediction methods (product level) that will (iii) ultimately support regulatory safety assessment (application level). The achievements of these proof-of-concept studies formed the backbone for the **SEURAT-1** roadmap (*Figure 1*), which was developed based on key contributions from each of the projects addressing the cluster-level objectives. It is as yet impossible to cover all toxicological endpoints with such a strategy, but the mechanism-based **SEURAT-1** case study approach was designed to provide a cornerstone for the transition from descriptive to predictive toxicology.

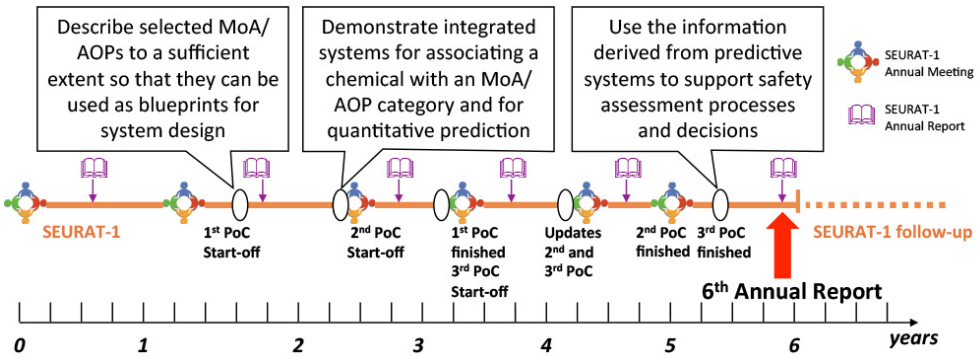

Figure 1 *Roadmap illustrating the timing of the proof-of-concept (PoC) at three conceptual levels as the backbone for interactions between* ***SEURAT-1*** *projects.*

The infrastructure for such a collaborative, interactive task was established through the organisation of cross-cluster working groups focusing on: (i) selection of standard reference compounds to be used for toxicity testing (Gold Compounds Working Group); (ii) data exchange between projects and standardisation of data analysis (Data Analysis Working Group); (iii) identification of modes-of-action relevant for repeated dose systemic toxicity (Mode-of-Action Working Group); (iv) *in vitro* to *in vivo* extrapolation and calculation of appropriate concentration ranges to be tested in *in vitro* experiments (Biokinetics Working Group); (v) standardisation of quality control issues of the cells used by the various partners and projects (Stem Cells Working Group); and (vi) bridging the gap between non-animal toxicity testing and safety assessment decision-making needs (Safety Assessment Working Group).

This final Annual Report, prepared by COACH, presents: (i) a comprehensive overview of the achievements of the different projects of the **SEURAT-1** Research Initiative; and (ii) final reports from the proof-of-concept case studies at the application level. As shown in *Figure 1*, the focus has moved from the level of test system development based on pre-existing mode-of-action descriptions towards the application level. Three different scenarios were developed at the application level, to which methods developed within **SEURAT-1** contributed (see Text Box 2 on page 13). The aim of this case study approach was also to provide guidance towards further development of mechanism-based integrated toxicity testing strategies and modern safety assessment approaches beyond **SEURAT-1**.

The Annual Report is organised in five chapters: Chapter 1 provides a general introduction to the **SEURAT-1** Research Initiative. It describes the model of **SEURAT-1** as a cluster of projects in the context of the call for research proposals under FP7. Furthermore, it introduces the cluster-level objectives as well as the structure and organisation of the **SEURAT-1** Research Initiative. Finally, some key elements of the new European Framework Programme for Research and Innovation (Horizon 2020) are highlighted with a focus on **SEURAT-1** follow-up activities.

Chapter 2 outlines the context of the **SEURAT-1** Research Initiative from the following perspectives:

(i) Legislation (Cosmetics Directive): On 11 March 2013, the full ban on animal testing for cosmetic products in the EU came into force, despite the fact that alternative methods to animal testing were not available for a number of endpoints. The history and rationale of the testing ban on cosmetic products, as well as the consequences of its implementation, were thoroughly discussed in the first three volumes of this Annual Report and is not further addressed in this volume.

(ii) Regulation: Now that animal testing for cosmetic ingredients is banned, pressure has increased on both scientific efforts to develop animal-free testing strategies and regulatory implementation of such methods in safety assessment. The latter was the focus of a workshop organised by the European Chemicals Agency (ECHA) in Helsinki in April 2016, and a summary report from that workshop is given in this Annual Report. Read-across in the context of the REACH regulations, for which ECHA developed a Read-across Assessment Framework, seems to be the most obvious way to implement animal-free testing strategies (e.g. in order to reduce uncertainty in the risk assessment procedure), and **SEURAT-1** had the opportunity to contribute to the workshop with relevant case studies. A second theme of the workshop was screening and priority setting, for which non-animal test methods are already used, mostly in the United States and Canada. A clear drawback in the application of non-animal test methods in the context of safety assessment is the lack of tools to address toxicokinetics, in particular regarding metabolism of parent compounds. However, it is apparent that both the validity and regulatory acceptability of alternative approaches will continue to develop over the next few years.

However, in the absence of accepted methods for many endpoints, there is an increasing demand for the development of frameworks that incorporate existing knowledge and complement it with new data, preferably based on non-animal methods. **SEURAT-1** developed such a conceptual framework, and other initiatives were also active in this field. The **SEURAT-1** approach is outlined together with the framework developed by the Health and Environmental Sciences Institute (HESI), called RISK21 roadmap, which has a clear focus on problem formulation as a starting point and suggests a tiered

approach for both exposure and hazard assessment. A third, data-driven framework also suggests a tiered approach that takes into account the scientific progress in, as well as the uncertainty of, *in vitro* testing methods and use of non-standard data for the reduction of animal testing. All of these frameworks are flexible in the sense that progress in the related fields is expected and can be considered. They can, therefore, combine the greater understanding of modes of action, adverse outcome pathways, toxicokinetics, high throughput assays, exposure models, databases, *in silico* algorithms and other advances being made by many groups.

(i) Science: The achievements of the **SEURAT-1** Research Initiative are discussed in the context of the Cosmetics Europe Long Range Science Strategy from an industry practitioner's point of view. Even though **SEURAT-1** pushed forward the scientific agenda in the field of repeated dose systemic toxicity significantly, there is still a long way to go and the scientific basis for dealing with this endpoint needs to be further reinforced. In the near term, the Cosmetics Europe Long Range Science Strategy addresses regulatory acceptance in the fields of eye irritation and genotoxicity, respectively.

The SEURAT-1 Proof-of-Concept Case Studies
Knowledge Level: Level 1 Proof-of-Concept Case Studies Challenging the predictive power and robustness of an Adverse Outcome Pathway construct Available Mode-of-Action Descriptions: From protein alkylation to liver fibrosis From Liver X Receptor activation to liver steatosis From inhibition of the bile salt export pump to cholestasis
Methodological Level: Level 2 Proof-of-Concept Case Studies Investigation of the fibrotic response induced by methotrexate and acetaminophen in the *HeMiBio* bioreactor Evaluation of valproic acid-induced steatosis in HepaRG cells Use of molecular modelling approaches to predict potential binding to nuclear receptors involved in the development of liver steatosis Development of biomarker based on a read-across use case on valproic acid analogues Screening of perturbed toxicity pathways by transcriptomics fingerprinting of data-poor substances Developing chemotypes for mitochondrial toxicity Mode-of-action-based classification model for repeated dose liver toxicity
Application Level: Level 3 Proof-of-Concept Case Studies Threshold of Toxicological Concern Read-across using **SEURAT-1** evidence *Ab initio* case study

In the first three volumes of this Annual Report series, Chapter 3 focused on the development of a long-term research strategy and its implementation within the cluster. The research strategy was to adopt a toxicological mode-of-action framework to describe how any given substance may adversely affect human health, and to use this knowledge to develop complementary theoretical, computational and experimental (*in vitro*) models that predict quantitative points of departure needed for safety assessment. Based on this strategy, the proof-of-concept case studies emerged on the respective three levels, and the first proof-of-concept level was achieved by the development of the three theoretical adverse outcome pathways (AOPs) for the three major liver adverse outcomes, which are fibrosis, steatosis and cholestasis. These were taken as the foundation for the development of integrated testing strategies, i.e. the level 2 proof-of-concept case studies, and the fourth Annual Report marked a transition to

the concrete demonstration of how these mode-of-action descriptions triggered development of tools for toxicity testing through the formulation of case studies on the methodological level. Consequently, the test system developments focus on certain key events and their sensitivity and specificity were assessed by a sophisticated selection of standard reference compounds, demonstrating that the test system is indeed predictive for the mechanism addressed (which follows a strategy of 'mechanistic validation'). Alternatively, AOP knowledge can also be applied when choosing a key event common for many pathways, and then predict general toxicity affecting many organs simultaneously (e.g. mitochondrial toxicity). This is also reflected in the level 2 proof-of-concept case studies.

The fifth Annual Report presented first results from the case studies operating on the methodological level and presented the consolidated plans to use them in the context of safety assessment considering the three different application scenarios outlined in Text Box 2. As a guiding tool for combining the available methods for regulatory purposes, a flexible 'conceptual framework' has emerged that can be used as a basis for the rational combination of information derived from predictive tools to support a safety assessment process or decision, in order to achieve a stated protection goal in the context of repeated-dose systemic toxicity. This 'conceptual framework' was used for organisation of the level 3 case studies at the application level. These reflect three typical safety assessment scenarios: (i) the objective of the first case study was to extend the concept of the Threshold of Toxicological Concern (TTC) to dermal exposure. Initially developed for oral exposure, methods were developed within the **SEURAT-1** Research Initiative to allow the application of the TTC concept in the context of dermal exposure and the goal of this case study was to confirm that the approach is practical and yet scientifically sound enough to be useful for both regulatory bodies and industries. (ii) The objective of the second case study was to use the **SEURAT-1** methods in the context of 'read-across', that is, to demonstrate that information using **SEURAT-1** methods can be used to improve the validity of a 'read-across' justification so that toxicological properties from tested source substance(s) can be 'read across' to 'target' substance(s) within a chemical category. (iii) The objective of the third case study was to arrive at a point of departure for a particular chemical that can be used as a basis for a safety decision by conducting an *ab initio* assessment using the new methods developed within the **SEURAT-1** Research Initiative. Piperonyl butoxide was chosen as the test compound with a hypothetical use scenario defined for a skin cream. The liver is the target organ for piperonyl butoxide, even though the mechanism of action is unknown. Furthermore, the two **SEURAT-1** standard reference compounds, methotrexate and valproic acid (VPA), were selected as positive controls for fibrosis and steatosis.

This final volume summarises the final reports from the case studies which operated at the application level, with special emphasis on the read-across case study as this application scenario has the highest potential for regulatory acceptance in the near term (see above). Overall, the intention was to demonstrate how **SEURAT-1** ultimately supported safety

assessment through results of the proof-of-concept case studies facilitated by the innovative toolbox provided by the **SEURAT-1** projects. The definition and execution of the case studies on all three proof-of-concept levels was highly inclusive, in that the partners, research projects, working groups, **SEURAT-1** Scientific Expert Panel, and industry advisers, were all involved and contributed to the process.

The detailed project descriptions and their achievements are given in Chapter 4. The **SEURAT-1** Research Initiative is designed as a coordinated cluster of five research projects, supported by a 'data handling and servicing' project and a 'coordination and support' project. The tasks and highlights of each of the projects presented in this Annual Report are:

Stem cell differentiation for providing human-based organ specific target cells to assay toxicity pathways in vitro.

The aim of the *SCR&Tox* Project was to provide the biological and technological resources needed to assay toxicity pathways *in vitro* and to demonstrate on industrial platforms that these resources could be reliably and robustly implemented at the required scale. Human embryonic stem cells and induced pluripotent stem cells were harnessed for this purpose. Cell lines were banked at the undifferentiated stage, and new technologies, including automation, were developed in order to obtain ES and iPS cell lines optimised for use in standardised assays. Protocols were then designed for differentiating pluripotent cells into derivatives for each of 5 toxicological relevant lineages (Liver, CNS, Heart, Skin, Muscle). The existence and reliability of toxicity pathways in the biological resources developed from pluripotent stem cells were demonstrated using the activation of the Nrf2 pathway as a model for the generation of reactive oxygen species. Drug toxicology assays were then designed and tested in academic-scale conditions. This involved the discovery of the protein components of the toxicity pathway and how the pathway is altered by test chemicals. Implementation of prototype assays was carried out. The transfer of the technologies of the assay developed on the bench was performed toward use on appropriate platforms for industrial-scale implementation through establishment of all standard operating procedures (SOP) and associated instructions for biological resources.

Development of a hepatic microfluidic bioreactor mimicking the complex structure and function of the human liver.

The aim of the *HeMiBio* Project was to create a bioreactor culture system of hepatocytes alone or in combination with the non-parenchymal fraction of

the liver (hepatic stellate cells (HSCs) and liver sinusoidal endothelial cells (LSECs)) to allow repeated dose toxicity testing of cosmetics and chemicals for up to 2-3 weeks *in vitro*. Primary HSCs and LSECs were thoroughly characterised at the functional and transcriptional level. Furthermore, gradually improving methods were developed to create hepatocytes and cells with HSC-like properties from PSCs, yielding cells that can be used to study toxicants, even though the PSC-derived cells remain less mature than primary cells. In addition, three different bioreactor designs were generated in the project: an antibody-based microfluidic system, a flow-over and a flow-through bioreactor. Sensors to be incorporated in cells (based on genome-edited sets of stem cells developed in the project) or in the bioreactor (sensors for oxygen consumption, pH, glucose and alanine aminotransferase (ALT) activity) were created and tested. The ultimate goal was to exploit the technologies in toxicity studies. For this, an *in vitro* model for liver fibrosis, using co-cultures of HSCs and hepatocytes, was developed, which can identify fibrosis-inducing drugs.

DETECTIVE
Detection of Endpoints and Biomarkers for Repeated Dose Toxicity Using In Vitro Systems

Identification and investigation of human biomarkers in cellular models for repeated dose in vitro testing.

The aim of the DETECTIVE Project was to identify robust, sensitive and specific, human-relevant *in vitro* biomarkers and surrogate endpoints that can be used for safety assessments of chronically acting compounds. The work undertaken in DETECTIVE covered liver toxicity, renal toxicity and cardiotoxicity, as well as cross-organ strategies. Multiple data streams derived from '-omics' readouts evaluated along with traditional toxicological and histopathological endpoints were analysed using integrative statistical analysis, systematic verification and correlation with *in vivo* data. Based on this, highly predictive biomarkers were identified in a pathway- and evidence-based approach. As one of the major achievements from the project, a 'Biomarker Repository' was developed, which is a database detailing biomarkers (functional and '-omics') together with the experimental details (*in vitro* models from which they have been retrieved, compounds and relevant concentrations). Other significant achievements of the DETECTIVE Project include a newly developed liver-based *in vitro* system for studying liver steatosis based on hepatic cells differentiated from human skin-derived precursors, and a GFP-BAC toxicity pathway reporter platform, which consists of approximately 50 reporter cell lines and is applicable to identify compounds that induce liver toxicity.

COSMOS *Delivery of an integrated suite of computational tools to predict the effects of long-term exposure to chemicals in humans based on* in silico *calculations.*

The COSMOS Project was initiated to support the computational modelling of toxicity and specifically to address the assessment of safety of cosmetics ingredients. The COSMOS Database (DB) is a freely available legacy from the COSMOS Project that incorporates an inventory of over 5,000 chemical structures which are known cosmetics ingredients; toxicity data for over 1,600 chemical substances, including the results of over 12,500 toxicity studies, were compiled in the database. The TTC approach was supported by a scheme that allows for route-to-route extrapolation (i.e. from oral to dermal exposure) of toxicological information. Models to predict the activity of chemicals solely from their structure were developed for effects such as binding to DNA and protein and nuclear receptors, as well as predicting effects associated with liver toxicity. The modelling approaches were developed in concert with ongoing research into relevant adverse outcome pathways (AOPs). Biokinetics and the distribution of compounds in humans following dermal and oral exposure were also modelled. The full suite of models for biokinetics and toxicity prediction is freely available as a series of KNIME workflows with accompanying web-tutorials.

NOTOX *Development of systems biology tools for organotypic human cell cultures suitable for long-term toxicity testing, and the identification and analysis of pathways of toxicological relevance.*

NOTOX developed *in vitro* cultivation systems amenable to systems biology '-omics' analyses, specifically serum-free long-term cultivation. In order to mimic the *in vivo* situation as close as possible, human organotypic 3D cultivation of various cell systems comprising HepaRG cells, primary human hepatocytes, co-cultures with non-parenchymal cells and human stem cell-derived cardiomyocytes were developed. Modern microscopic methods were used to derive structural and activity data at different levels of magnification and resolution. NOTOX established a unique approach of deriving diverse and dynamic '-omics' data from the same experiment with close links to computational modelling. The '-omics' endpoints provided rich insights into the mechanisms of action of the tested compounds. These data were used to build up large-scale computational models of varying complexities including metabolic network models, PBPK models, and tissue organisation and regeneration models.

ToxBank *Data management, cell and tissue banking, selection of 'reference compounds' and chemical repository.*

ToxBank developed infrastructure and service functions to create a sustainable predictive toxicology support resource going beyond the lifetime of the programme. ToxBank established (i) a dedicated web-based warehouse for toxicity data management and modelling; (ii) a 'gold-standard' compound database and repository of selected test compounds; and (iii) a reference resource for cells, cell lines and tissues of relevance for *in vitro* systemic toxicity research carried out across the **SEURAT-1** Research Initiative.

COACH *Cluster-level coordination and support action.*

The main roles of COACH were to (i) facilitate cluster-wide internal cooperation; (ii) to provide strategic guidance with the help of the Scientific Expert Panel (SEP); (iii) to prepare and distribute the **SEURAT-1** Annual Reports; (iv) to organise the **SEURAT-1** Annual Meetings, meetings of the SEP, and workshops supporting cross-cluster activities and collaborations with external partners, and (v) to coordinate cluster-level dissemination and outreach activities. COACH provided centralised scientific administration to the **SEURAT-1** Research Initiative (the 'COACH Office'), organised cluster-level interactions and activities, and was the main cluster-level entry point for all participants, including funding organisations such as the European Commission and Cosmetics Europe, as well as any external organisation looking to liaise with the **SEURAT-1** Research Initiative.

Chapter 4 also contains reports about the meetings of each of the specific projects. These meetings were conducted to disseminate the achievements of the projects. An overview of the **SEURAT-1** roadmap, highlighting the contributions of the individual projects to the achievement of cluster-level objectives, is presented together with an outline of the **SEURAT-1** Tools & Methods Catalogue in a section describing cross-cluster cooperation. Working groups played a vital role in the effort to make the whole greater than the sum of its parts, and final activity reports on activities and workshops conducted under the umbrella of these working groups are also included in this section, highlighting the fact that the cross-cluster working groups were the driving force behind cluster-level progress.

A particular highlight was the **SEURAT-1** Symposium, 'Painting the Future Animal-Free Safety Assessment of Chemical Substances', held in Brussels on 4 December 2015. The Symposium allowed participants to network with renowned experts and get acquainted with the activities of other ongoing and future initiatives. High-level presentations showcased **SEURAT-1**'s success stories in a practical, accessible manner, while an exhibition allowed

for deeper discussions. Furthermore, participants learned how the extensive research efforts over the last 5 years can translate into solutions for safety assessment ultimately replacing animal testing. Other related ongoing and future initiatives from the EU and the US also contributed, showcasing progress in the field and stimulating exchange and networking. There was also the opportunity to take part in a guided educational tour, which was developed to guide interested participants, who are not necessarily experts in the field, through a virtual 'case' in which non-animal testing tools and methods are combined to answer the question of whether a new compound is safe for consumers when used in a defined exposure context. Furthermore, a new film summarising the **SEURAT-1** strategy and achievements from the scientific projects in plain language was produced and projected in the auditorium. The 25-minute film, entitled 'SEURAT-1 – Testing Chemicals without Animals', can be watched on the **SEURAT-1** homepage *(http://www.seurat-1.eu)*, as well as on YouTube.[1]

Chapter 5 describes the related international activities of the **SEURAT-1** Research Initiative. The list of short project descriptions that was included in the previous Annual Reports has been further updated, with special emphasis on initiatives focusing on repeated dose toxicity and the replacement of animal testing in the field of human safety assessment. Collaborations between **SEURAT-1** partners and these various related international activities were highlighted, underlining the integration of **SEURAT-1** in the field. Indeed, for **SEURAT-1** to be successful, it was important to cooperate with the various complementary international research programmes on the way 'towards the replacement of *in vivo* repeated dose systemic toxicity testing' and various collaborations were set up from the start of the **SEURAT-1** Research Initiative. Although **SEURAT-1** has come to an end, SEURAT (i.e., Safety Evaluation Ultimately Replacing Animal Testing) as the long-term strategic target persists and was taken up by a new consortium that has been formed based on a call for proposals published under the umbrella of the European Commission's new Framework Programme, Horizon 2020. The project is called 'An Integrated EUropean 'Flagship' Program Driving Mechanism-based Toxicity Testing and Risk Assessment for the 21st Century' *(*EU-ToxRisk; *http://www.eu-toxrisk.eu)* and started on 1 January 2016. It follows the overall strategy developed in **SEURAT-1** with new case studies focussing on the implementation of mechanism-based integrated testing strategies in regulatory safety assessment scenarios. The planning of these new case studies was discussed at the workshop 'Exploring the regulatory use of alternative approaches in toxicology for the safety assessment of chemicals' on 9-10 November 2016 in Ispry / Italy, which was co-organised by EURL ECVAM (the European Union Reference Laboratory for alternative methods to animal testing), **SEURAT-1** and EU-ToxRisk. This workshop was one of the last activities of the **SEURAT-1** Research Initiative and the new case studies were discussed in the light of the experience gained in **SEURAT-1** together with the partners from the EURL ECVAM regulatory framework and its stakeholder forum in order to further explore the applicability of the regulatory use of the scientific progress. A short description of the design of the new six-year, EUR 30 million project EU-ToxRisk concludes this last **SEURAT-1** Annual Report, indicating that the end of **SEURAT-1** is the beginning of a new initiative.

1 https://www.youtube.com/watch?v=Ymzsh9p5pwM

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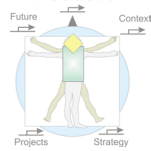


1 INTRODUCTION

Tilman Gocht, Michael Schwarz

'The most rewarding researches are those which, inasmuch as they are joy to the thinker, are at the same time of benefit to mankind.'

Christian Doppler

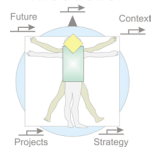
Background

On 11 March 2013, the full EU ban on animal testing for cosmetic products came into force. From this date, animal testing for the marketing of new cosmetic products in the European Union was prohibited. Data from animal testing carried out before the entry into force of the marketing ban can continue to be used in the safety assessment of cosmetic products. The implementation of the marketing and testing ban follows the seventh amendment to the Council Directive on the approximation of the laws of the Member States relating to cosmetic products (76/768/EEC, 'Cosmetics Directive'), which defined the step-by-step phasing out of animal testing for cosmetic products as well as for cosmetic ingredients over the last 10 years. Accordingly, animal testing for cosmetic products has been prohibited since 2004, but the deadline for the most complex fields of repeated dose toxicity, reproductive toxicity and toxicokinetics was extended to 11 March 2013. This deadline was not further extended, even though an expert panel of scientists came to the conclusion that they cannot estimate the required time for establishing alternative methods for full replacement of animal testing in the field of repeated dose systemic toxicity. This was due to unresolved questions related to the complex cellular mechanisms involved (*Adler et al., 2011*).

Triggered by this legislative pressure, Cosmetics Europe – The Personal Care Association (previously named COLIPA) proposed a contribution of EUR 25 million at the beginning of 2008 to support research work in the area of repeated dose systemic toxicity. 'Safety Evaluation Ultimately Replacing Animal Testing' (SEURAT) was presented by the HEALTH Theme of the Directorate General of Research and Innovation of the European Commission in 2008 as the long-term target in safety testing. Cosmetics Europe and the European Commission agreed to set up a Research Initiative for the development of a research strategy 'Towards the replacement of *in vivo* repeated dose systemic toxicity testing'. It was called '**SEURAT-1**', indicating that this is a first step in a specific area addressing the global long-term strategic target SEURAT.

In June 2009, the framework for the **SEURAT-1** Research Initiative was created through a Call for Proposals under the HEALTH Theme of the 7th European RTD Framework Programme: 'Alternative Testing Strategies: Towards the replacement of *in vivo* repeated dose systemic toxicity testing' with a total budget of EUR 50 million. Cosmetics Europe published its financial commitment to the Research Initiative at the same time. EUR 25 million funding was provided by the FP7 HEALTH theme and EUR 25 million by Cosmetics Europe.

The **SEURAT-1** Research Initiative started in January 2011 and ended in December 2015. Although **SEURAT-1** was initially motivated by the urgent needs of the cosmetic industry, it is undoubtedly relevant for other related fields, as systemic toxicity testing is needed for a variety of applications: in the context of the European Union Regulation REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals); in the development of pharmaceuticals, and in other industrial sectors. Moreover, the scientific knowledge delivered by the **SEURAT-1**



Research Initiative is expected to be highly relevant in personalised medicine, in systems medicine, in the development of innovative diagnostic tools, in regenerative medicine, and in other fields. Therefore, broad impact was expected and brought the consortium into a leading position internationally in this field of research.

Goals and Objectives

The goal of the five-year **SEURAT-1** Research Initiative was to develop a consistent research strategy ready for implementation in further research programmes. This involved establishing innovative scientific tools for a better understanding of repeated dose toxicity and identifying gaps in knowledge to be bridged by future research work. It was expected that the end results would be *in vitro* testing methods and *in silico* tools which, within the framework of safety assessment, have a higher predictive value, are faster and cheaper than those currently used, and significantly reduce the use of animal tests.

The cluster-level objectives, which could not be achieved by individual projects alone, were:

- To formulate and implement a research strategy based on generating and applying knowledge of modes-of-action;
- To develop highly innovative tools and methodology that can ultimately support regulatory safety assessment;
- To demonstrate proof-of-concept at multiple levels – theoretical, systems and application;
- To provide a blueprint for expanding the applicability domains – chemical, toxicological and regulatory.

The research work in the **SEURAT-1** projects involved the development of innovative testing strategies including: organ-simulating devices equipped with human-based target cells for toxicity testing, the identification of relevant endpoints and intermediate markers, the application of approaches from systems biology, computational modelling and estimation techniques, and integrated data analysis. Overall, the **SEURAT-1** Research Initiative contributed significantly to the establishment of a new paradigm in toxicology, which is summed up by the term ‘predictive toxicology’.

Structure of the **SEURAT-1** Research Initiative

The **SEURAT-1** Research Initiative was designed as a coordinated cluster of five research projects, supported by a ‘data handling and servicing project’ and a ‘coordination and support project’ at the cluster level.

The following six projects formed the backbone of **SEURAT-1**:

- 'Stem Cells for Relevant efficient extended and normalized TOXicology' (*SCR&Tox*)

  Scientific coordinator: Marc Peschanski, INSERM/UEVE 861, I-STEM/AFM, Evry/France;

- 'Hepatic Microfluidic Bioreactor' (*HeMiBio*)

  Scientific coordinator: Catherine Verfaillie, Interdepartmental Stem Cell Institute, Katholieke Universiteit Leuven/Belgium;

- 'Detection of endpoints and biomarkers for repeated dose toxicity using *in vitro* systems' (DETECTIVE)

  Scientific coordinator: Jürgen Hescheler, Institute for Neurophysiology, University Hospital Cologne/Germany;

- 'Integrated *in silico* models for the prediction of human repeated dose toxicity of COSMetics to Optimise Safety' (COSMOS)

  Scientific coordinator: Mark Cronin, School of Pharmacy and Chemistry, Liverpool John Moores University/United Kingdom;

- 'Predicting long term toxic effects using computer models based on systems characterization of organotypic cultures' (NOTOX)

  Scientific coordinator: Elmar Heinzle, Biochemical Engineering, Saarland University, Saarbrücken/Germany;

- 'Supporting integrated data analysis and servicing of alternative testing methods in toxicology' (ToxBank)

  Scientific coordinator: Barry Hardy, Douglas Connect, Zeiningen/Switzerland.

Furthermore, a coordination project was designed in order to facilitate cluster interaction and activities:

- 'Coordination of projects on new approaches to replace current repeated dose systemic toxicity testing of cosmetics and chemicals' (COACH)

  Coordinator: Bruno Cucinelli, ARTTIC, Paris/France.

The scientific management and coordination of the **SEURAT-1** Research Initiative was strongly supported by the Scientific Expert Panel (SEP), which played a key role in providing scientific advice regarding the research work and future orientation of **SEURAT-1**. COACH provided a

central administration for the **SEURAT-1** Research Initiative and to the SEP. Support to the cluster was provided either directly through COACH or through the SEP.

An example of the scientific management and coordination was the development of a roadmap for the cluster as a whole (*Figure 1.1*). Key contributions from the research projects, which were essential to meeting the above-mentioned cluster-level objectives, were identified as the starting point and introduced in the second volume of this book series. They were used to define cluster-level milestones, and cross-cluster working groups were established and made up of delegates from the various project consortia. The working groups and the **SEURAT-1** projects needed to interact in order to achieve the three proof-of-concept levels which formed the backbone of the **SEURAT-1** roadmap published in the third volume of this book series. Relevant case studies addressing these three proof-of-concept levels were established later and reported in the fourth volume. Updates and first results from these case studies were presented in the fifth and conclusions from the case studies focussing at the application level will be presented in this sixth volume. The overall approach for the implementation of this roadmap was developed by COACH in close cooperation with the project coordinators, and was subsequently endorsed by the SEP.

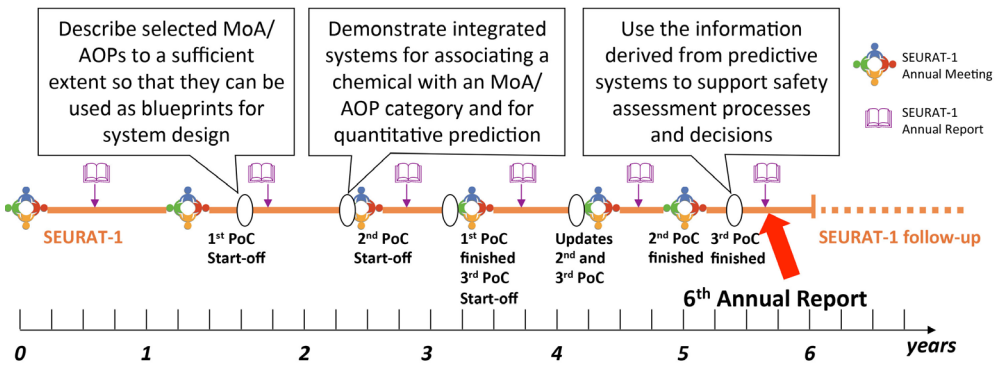


Figure 1.1 SEURAT-1 *roadmap illustrating the timing of the proof of concept (PoC) at three conceptual levels as the backbone for interactions between the* ***SEURAT-1*** *projects.*

The Annual Report: Something about 'Pathways'

This is the final volume of a series of six Annual Reports. The first volume presented a comprehensive overview of the planned work in the different projects of the **SEURAT-1** Research Initiative. The following volumes focused on highlights from the work periods in the research projects and steps towards reaching the final goal of the cluster. All six volumes together provide a complete overview of cutting-edge research 'towards the replacement of *in vivo* repeated dose systemic toxicity testing' and thus represent a 'pathway' regarding scientific progress.

This gives rise to the common theme running through the Annual Reports and the **SEURAT-1** Research Initiative, as introduced in the first volume. The structure of the Annual Reports, which was maintained over the six-year period, was inspired by one of the most important keywords of the field of research addressed: 'toxicity pathways' (*Figure 1.2*).

Chapter 2 briefly describes developments in the legislative, regulatory and scientific environment of the **SEURAT-1** Research Initiative. Chapter 3 outlines progress in the development of the long-term research strategy of the SEURAT initiative (i.e. **SEURAT-1** and beyond). In this sixth volume we report the results from the case studies and demonstrate how the data and information derived from the tools and methods developed within **SEURAT-1** can be used in safety assessment frameworks and scenarios, with a particular emphasis on read-across. This chapter is followed by detailed project descriptions in chapter 4 which provide an overview of achievements obtained over the entire funding period. Finally, chapter 5 focuses on related international activities and outlines collaborations which were established with the **SEURAT-1** Research Initiative highlighting the demand for collaborative future research and development work leading to pathway-based human safety assessments in the field of repeated dose systemic toxicity testing of chemicals.

Conceptual considerations related to biological pathways leading to toxicity consistently guide the report series. As a result, all six volumes together set out the pathway explaining how to perform the paradigm shift from describing phenomena to understanding processes in repeated dose toxicity.

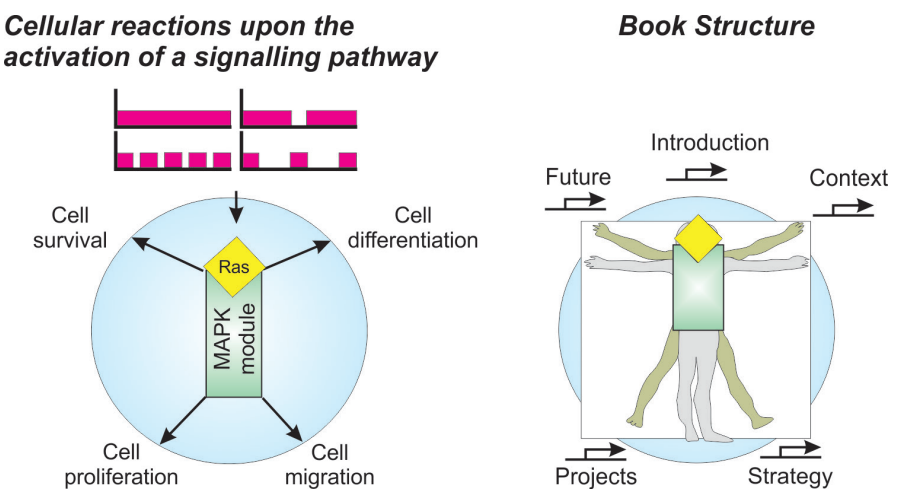
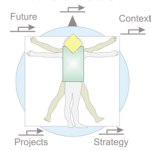


Figure 1.2 *The concept of 'Toxicity Pathways' (left panel) is mirrored by the book structure (right panel). Here we demonstrate the complexity of biology by means of the completely different cellular reactions depending on the length and sequence of the stimulation (input event(s), left panel).*



The Consortium and the Scientific Expert Panel (SEP)

The **SEURAT-1** Research Initiative combined the research efforts of over 70 European universities, public research institutes and companies. The composition was unique, with toxicologists, biologists from different disciplines, pharmacologists, chemists, bioinformaticians, material scientists and leading experts from other domains working closely together on common scientific objectives. The proportion of SMEs participating in **SEURAT-1** was high, at more than 30%.

As described above, the Scientific Expert Panel (SEP) advised the cluster on scientific matters related to specific topics within the area of repeated dose systemic toxicity. The SEP was made up of project coordinators and external experts and the membership is listed in *Table 1.1*.

Table 1.1 *Members of the* ***SEURAT-1*** *Scientific Expert Panel (co-chairs are highlighted in bold).*

Participant	Institution / Country	Project
Project Coordinators		
Marc Peschanski	INSERM/UEVE 861, I-STEM/AFM, Evry / France	*SCR&Tox*
Mark Cronin	School of Pharmacy and Chemistry, Liverpool John Moores University / UK	COSMOS
Catherine Verfaillie	Interdepartmental Stem Cell Institute, Katholieke Universiteit Leuven / Belgium	*HeMiBio*
Jürgen Hescheler	Institute for Neurophysiology, University Hospital Cologne / Germany	DETECTIVE
Elmar Heinzle	Biochemical Engineering, Saarland University, Saarbrücken / Germany	NOTOX
Barry Hardy	Douglas Connect, Zeiningen / Switzerland	ToxBank
External Experts		
Hans Juergen Ahr	Bayer Health Care AG, Wuppertal / Germany	
Ian Cotgreave	**Swedish Toxicology Sciences Research Center (Swetox), Södertälje/Sweden**	
George Daston	Procter & Gamble, Product Safety and Regulatory Affairs, Cincinnati / USA	
Derek Knight	**European Chemicals Agency, Helsinki / Finland**	
Catherine Mahony	Cosmetics Europe; (Procter & Gamble), London Innovation Centre / UK	
Russell S. Thomas	U.S. Environmental Protection Agency, National Center for Computational Toxicology, Research Triangle Park/USA	

Recent Developments: Human Safety Assessment and Horizon 2020

The continuation of the SEURAT programme is foreseen under the umbrella of the European Commission's new Framework Programme, Horizon 2020, which offers a total of EUR 80 billion in funding between 2014 and 2020. The theme 'Health, demographic changes and wellbeing' was identified as one of six societal challenges upon which funding will be focused. The Work Programme for the years 2014-2015 highlighted 'personalising health and care' as the particular area of interest, in which 34 topics in 7 focus areas will receive a total of EUR 1.21 billion in funding (*European Commission, 2014*). The most relevant call for **SEURAT-1** activities, entitled 'New approaches to improve predictive human safety testing' (call identifier PHC-33-2015), was published on 23 July 2014 in the area 'Improving health information, data exploitation and providing an evidence base for health policies and regulation' (*European Commission, 2014).* The overall aim is to improve the efficiency of predictive toxicological testing to address key areas of concern for human health and to meet regulatory requirements (e.g. EU legislation on REACH, cosmetics, biocides). The objectives are to develop and validate routine, non-animal approaches for toxicity testing of chemicals by means of mechanism-based understanding of complex biological pathways of toxicological relevance and identification of early markers predictive of toxicological effects in humans. The call highlighted the importance of international collaboration with similar initiatives in the USA and elsewhere. The deadline for proposals was 24 February 2015, and the successful consortium called 'An Integrated EUropean 'Flagship' Program Driving Mechanism-based Toxicity Testing and Risk Assessment for the 21st Century' *(EU-ToxRisk; http://www.eu-toxrisk.eu)* was selected in summer 2015. The project started on 1 January 2016 and follows the overall strategy developed in **SEURAT-1** with new case studies focussing on the implementation of mechanism-based integrated testing strategies into regulatory safety assessment scenarios. A short description of the design of this six-year, EUR 30 million project is given in the final section of this Annual Report, indicating that the end of **SEURAT-1** is the beginning of follow-up initiatives.

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2 CONTEXT

Science is wonderfully equipped to answer the question 'How?' but it gets terribly confused when you ask the question 'Why?'

Erwin Chargaff

2.1 Introduction

Tilman Gocht, Michael Schwarz

The Seventh Amendment to the Cosmetics Directive introduced a number of key requirements related to animal testing that were incorporated into the Cosmetics Regulation (Regulation (EC) No 1223/2009, 30 November 2009). In 2004, the testing of cosmetic products on animals was banned within the European Union. In 2009, an EU testing ban for cosmetic ingredients came into force with an extension to three specific areas: repeated dose toxicity (includes skin sensitisation, carcinogenicity and sub-acute/sub-chronic toxicity), reproductive toxicity (also includes teratogenicity) and toxicokinetics. On 11 March 2013, the full ban on animal testing for cosmetic products came into force and animal testing for the marketing of new cosmetic products in the EU was prohibited from this date.

This chapter outlines recent developments in the legal, regulatory and scientific context of the **SEURAT-1** Research Initiative. As the legal framework has been clearly defined since March 2013, the focus of this chapter is now on implementation in regulations and application by industry of the strategies and methods developed within **SEURAT-1**. The first contribution to this chapter deals with the latter aspect. The case study approach of **SEURAT-1** is discussed in the context of the research programme of the European cosmetic industry. This is taken as a starting point to highlight the next milestones on the strategic roadmap of the Cosmetics Europe Long Range Science Strategy in the effort to replace animal testing in toxicity testing of cosmetic compounds.

However, having new tools to hand is fine, but they need to be applied in safety assessment approaches. This requires acceptance by regulators and was the focus of a workshop organised by the European Chemicals Agency (ECHA) in Helsinki in April 2016. Read-across in the context of the REACH regulations seems to be the most obvious way to implement animal-free testing strategies, and **SEURAT-1** had the opportunity to discuss some of the case studies conducted within **SEURAT-1** (see also chapter 3) during the workshop. It also became clear that the barrier between method developers and regulators needs to become more permeable, and this was another purpose of the workshop (in fact, the meeting point between both communities is in the field of regulatory science). These topics are discussed in a summary report from the workshop, which is presented in the second section of this chapter.

The final section of this chapter compiles frameworks developed in recent years to provide structures and give orientation as to how to combine the available methods to answer a safety assessment question. The **SEURAT-1** Research Initiative developed such a framework, which is presented again with other approaches that were presented in previous volumes of this Annual Report. They may be redundant in parts, but they constitute the state-of-the-art in risk-based and animal-sparing approaches to evaluating chemicals for safety, beyond the

read-across framework. The purpose of this compilation is to stimulate discussion about how to eventually combine the various proposals in order to produce a consolidated version giving guidance for both the next generation of risk assessors and future research projects in the field of method developments for the replacement of animal testing.

2.2 SEURAT-1 - Continuing the Successful Journey

Horst Wenck

2.2.1 Introduction and Rationale

Almost 20 years after the establishment of its first formal research body in 1992, the European cosmetics industry is now looking back on its long-standing track record in the development of alternatives to traditional animal based safety testing (AAT). In fact, the Industry has been the leading contributor to driving AATs towards official regulatory acceptance, and has been key to the validation of about a dozen methods altogether.

Naturally, AAT research by the European cosmetics industry (Cosmetics Europe) historically focussed on replacement methods for local toxicity, as cosmetic products are largely about application and efficacy on the skin. If we consider the time around 2007, when the industry initiated the idea of **SEURAT-1**, we can see substantial progress in the Cosmetics Europe research programme, even in skin sensitisation, the most difficult local toxicity field, whereby comprehensive evaluation studies have begun to reveal which testing strategies may be suitable for hazard identification and what directions for potency evaluation as the basis safety assessment are promising. Furthermore, the eye irritation programme has been brought to successful validation by ECVAM and wide acceptance by the OECD (*OECD, 2015; Barroso et al., 2016*). The genotoxicity portfolio shows very promising directions for reducing the large number of false positive results which characterise the old testing strategy. This has received favourable recognition from regulatory bodies including the OECD. Substantial refinement has also been achieved in skin penetration assays as the basis for exposure determination.

However, when **SEURAT-1** was conceived, nearly a decade ago, there had been rather little tangible progress in most aspects of AAT for chronic systemic toxicity endpoints. Facing the pressure of the upcoming full replacement obligations imposed by the EU Cosmetic Directive bans, the cosmetic industry had to act, despite slow progress in the field. Basic research at that time was still in its early stages. The hallmark paper of the U.S. National Research Council, 'Toxicity Testing in the 21st Century: A Vision and a Strategy' (*NRC, 2007*), had only

just been published, constituting the theoretical strategic framework for the approach to a broad reform of traditional toxicology.

In light of this difficult starting point, limited evolution of basic science and limited own capabilities, the European cosmetics industry thus saw its main responsibility at that time in driving research into systemic toxicity AAT and proposed to engage in a public private partnership with the European Commission, combining the best of both fields of expertise and creating a larger financial offer by equally sharing an overall funding frame of EUR 50 million in a programme designed to spearhead the development of AAT in systemic toxicity. **SEURAT-1**, carefully called 'Safety Evaluation Ultimately Replacing Animal Testing', was hence conceived to constitute both the foundation and the catalyst of systemic toxicity AAT. Cosmetic industry scientists expected **SEURAT-1** to be mainly a basic research project, advancing knowledge and understanding but without any real output that would be immediately useful for routine safety assessment. Some decision makers in the cosmetic industry probably also agreed to shoulder the high investment in **SEURAT-1** because of political considerations. **SEURAT-1** would very prominently demonstrate commitment to AAT, thus possibly leading to fully fact-based political decision-making with respect to the final 2013 deadline of the European Cosmetics Directive bans.

2.2.2 SEURAT-1 from an Industry Practitioner's Perspective

We shall now review the outcomes of **SEURAT-1** with reference to the hopes and expectations at its conception.

First, any political hopes were clearly not fulfilled. The European Cosmetics Directive bans came into force despite and regardless of scientific facts and reality:

- Although scientifically valid replacement methods were not sufficiently available, the bans were brought in force as an act of political decision-making.
- The industry had heavily invested and made remarkable progress in replacement methods, but science simply could not implement full replacement for all animal tests within the 10-year deadline set by the European Cosmetics Directive in 2003.
- Cosmetics and their ingredients accounted for only a negligible fraction of all laboratory animals employed in testing in Europe (less than 0.1%), and so the bans had essentially no effect on animal welfare in or outside Europe.
- The Industry's replacement activities focussed on the area of greatest need in the local toxicity endpoints, which is where animal use was greatest although still a negligible fraction of all animals employed in public- or private-sector testing before 2009.

➠ Industry insiders are already feeling the predicted detrimental effects of the bans now, which weaken the industry's ability to operate in the marketplace. It is no longer able to adequately defend existing ingredients against the mounting pressure of public opinion questioning their safety. Being unable to provide publicly convincing evidence to counter wrong allegations of safety concerns, the industry has no option but to remove any ingredient with faint public concern from its portfolio. This mechanism is predicted to result in regular losses of valuable ingredients and to limit the choices of products for European consumers in the medium term. Equally importantly, the bans also impede innovation of one of Europe's few leading world champion industries. In one early warning sign, Chinese patent applications in cosmetics recently overtook those of Europe.

Second, however, and fortunately, **SEURAT-1** has somewhat overachieved initial expectations scientifically. The network project has indeed produced some remarkable results that have begun to find their use in daily practice.

The traditional approach of the cosmetics industry AAT programme had been to first understand the mechanism causing the toxicological response concerned and then to develop a portfolio of tests simulating and providing a read-out for the particular toxicological pathways (*Figure 2.1*).

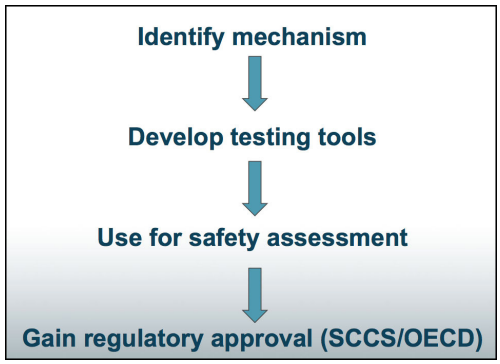

Figure 2.1 *Traditional strategic approach of the cosmetic industry's alternatives to animal testing (AAT) programme.*

However, during the **SEURAT-1** Research Initiative, it became increasingly clear that such a rigid systematic approach would not allow substantial progress in terms of tangible methods, tools and strategies for the more complex and challenging repeat dose endpoints, which would in turn facilitate uptake of new method-based safety assessment in routine industrial research and development. **SEURAT-1** would have run the risk of not passing the first phase of generating insight into the highly complex mechanistic network of biological processes

leading to the ultimate toxicological phenomenon. Such knowledge-building is the societal function of basic research pursued in academia. It is neither the core expertise nor the role of the industry, whose expertise resides in applied research. Hence, after intensive project discussions, the **SEURAT-1** strategy was adjusted to also comprise a distinctively different approach in advancing systemic toxicity. This is the concept of case studies with a dual role:

- They constitute a challenge test to identify whether the state of science already lends itself to being applied and used in practical safety assessment examples. This would allow for early spin-off results.
- Secondly, the findings of case studies help steer basic research and method development, as they pinpoint gaps and weaknesses in achieved and proposed conceptual systems (*Figure 2.2*).

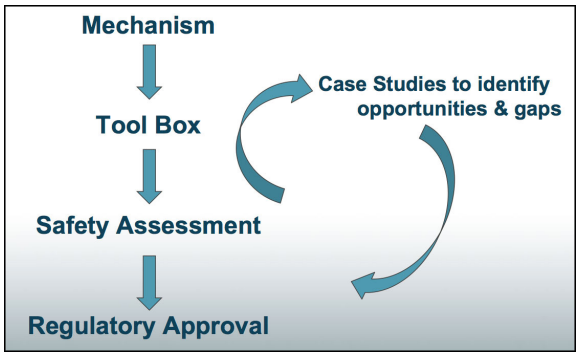

Figure 2.2 *R&D approach relying on case studies to drive AAT programs.*

From the viewpoint of applicability to industrial R&D, the most prominent results delivered by the **SEURAT-1** programme are:

- Broadening the applicability of the TTC (Threshold of Toxicological Concern) concept: TTC was previously regarded only as a principle allowing a proper dealing with chemical impurities. During the **SEURAT-1** project, it became clear that TTC would indeed lend itself to being an integral part of safety assessment for topically applied cosmetic substances. The highly effective skin barrier typically prevents any large-scale penetration of most chemicals. Systemic exposure from topically applied substances is thus often low, allowing use of the TTC concept. **SEURAT-1** built the COSMOS database *(http://www.cosmostox.eu/what/COSMOSdb/)*, which is essential for assessing the TTC of cosmetically relevant ingredients. Moreover, these results found a preliminary favourable reception of TTC by the European Scientific Committee on Consumer Safety (SCCS), which elaborated on the principle in its notes

of guidance (*SCCS, 2016*). This will allow the cosmetic safety assessor to increasingly employ TTC as a tool in daily practice.

➠ Read-Across Framework: **SEURAT-1** developed a strategy, workflow, templates, and case studies and thus gave guidance on how to use the Read-Across principle in safety assessment using new method data.

➠ Blueprint for Liver Toxicity; Workflow for *ab initio* chemical safety assessment: **SEURAT-1** focussed substantially on liver toxicity, where it ultimately developed a blueprint on how to deal with liver toxicity and yielded practical knowledge of how *in vitro* safety testing and assessment strategies could finally evolve based on a mechanistic understanding. Admittedly, this is just the first step of the ultimate scheme, yet it provides a clear roadmap for follow-up programs, and most prominently the new Horizon 2020 framework project EU-ToxRisk *(http://www.eu-toxrisk.eu)*. The workflow and case study developed by **SEURAT-1** on *ab initio* chemical safety assessment gave guidance on how to assess a chemical without relying on generating new animal data. Again, it is just a first step, but the cosmetic safety assessor is starting to see how to build up a hypothesis based on existing data, *in chemico* and *in silico* modelling and biokinetic considerations, and then by targeted new method testing.

2.2.3 Future Research in the Field of Alternatives to Animal Testing of the Cosmetics Industry / Cosmetics Europe

Cosmetics Europe has made ambitious plans for the 2016-2020 period to drive further replacement of animal toxicity data-based safety assessment. The future programme of the Cosmetics Europe Long Range Science Strategy (LRSS) will largely rest on three main pillars:

➠ Finalising achievements in full replacement of eye irritation tests and a markedly increased specificity of genotoxicity, respectively. Here, the focus of eye irritation and genotoxicity will be to finalise the proposed strategies and secure their comprehensive regulatory acceptance.

➠ Establishing assay systems and testing strategies for full replacement of skin sensitisation tests, and prepare for their regulatory approval. While hazard identification of sensitisers is well on its way, potency determination as a prerequisite for safety assessment ultimately accepted by regulatory authorities remains the major scientific challenge.

➠ Enhancing the scientific basis for dealing with systemic toxicity in the cosmetic industry.

The strategic roadmap of the industry's AAT programme for the 2016-2020 period is shown in *Figure 2.3*.

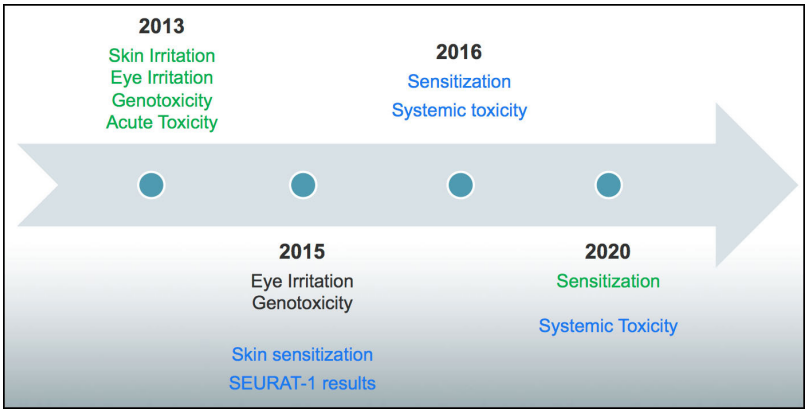

Figure 2.3 *Strategic roadmap of the Cosmetics Europe LRSS.*

The programme for systemic toxicity will follow the principles depicted in *Figure 2.4*. The concept resides on three main practical questions: first, calculating and assessing exposure at the relevant systemic target site; secondly, characterising the hazard of the investigated substance; and thirdly, combining the two in final safety assessment. The programme thus aims at fusing toxicokinetics with toxicodynamics in a safety assessment framework.

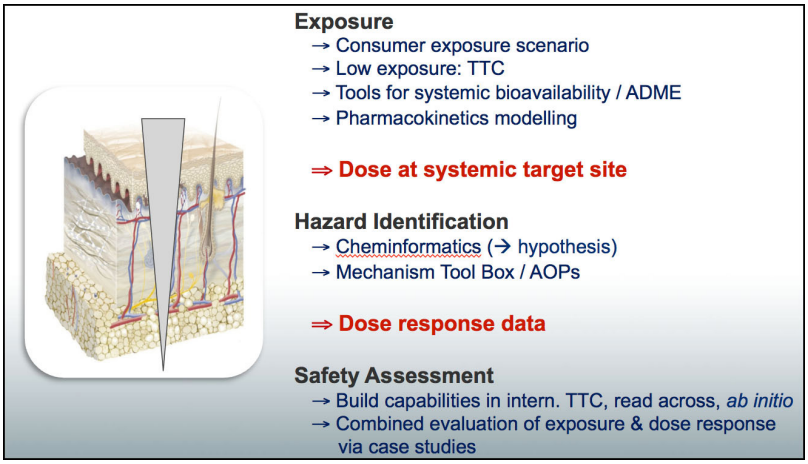

Figure 2.4 *Strategic principles of the Cosmetics Europe LRSS.*

In a nutshell, the safety assessment framework involves moving from dealing with low systemic exposure of known chemicals to increasing exposures of less-known chemicals, and developing testing arrays and safety assessment principles *en route*. As mentioned before, case studies will be the decisive element of such an applied science approach. As cosmetic

ingredients are quite unlikely to evoke specific toxicity in a given target organ, the safety assessor is mainly tasked to rule out any low level of unspecific toxicity. A more overarching general conceptual framework therefore seems more suitable than a primary focus on closing gaps in the complex, partially unknown network of adverse outcome pathways (AOPs), which is a very complex, long-term task requiring years of systems biology progress. This is why toxicokinetics, i.e. systemic ADME, will also play a very prominent role in the LRSS programme, and toxicodynamics will evolve to a great extent and be employed in an applied research context, in line with the generation of new knowledge and concepts.

2.2.4 Regulatory Acceptance and Validation

One of the key issues also to be solved by all those working on alternatives to animal testing is the late phase and essential goal of replacement programs: their regulatory acceptance. Already, the apparently 'well understood' endpoint of eye irritation clearly showed the difficulties associated with a traditional validation approach (*Barroso et al., 2016*). Validating a newly developed *in vitro* assay system has so far essentially relied on comparing its results against the data of the traditional, officially accepted animal tests. Such a strategy requires two main elements: first, the animal tests have to be true 'gold standards' that accurately predict human safety. Secondly, the developer of the alternative test strategy needs a sufficient number of chemicals with robust animal data, partly for the method development itself, and a set of 'innocent' chemicals for validation.

Both fundamental requirements will likely constitute major issues in future validation programmes, as illustrated by the eye irritation validation study (*Barroso et al., 2016; SCCS, 2016*), which was fully funded by Cosmetics Europe under the supervision and governance of ECVAM. As far as test substances are concerned, the eye irritation validation study faced the difficulty of properly populating the required set of more than hundred chemicals with a sufficient number of 'innocent' candidates that hadn't been used previously for method development (*Barroso et al., 2016*). Experts see no realistic chance of identifying sufficient numbers of test substances for traditional-type validation studies of test strategies for the upcoming repeated dose toxicity endpoints. Secondly, a traditional validation is also elusive for these more complex endpoints, as alternative testing strategies for these endpoints consist of systems of assays which exclude traditional 1:1 comparisons with the respective animal test. Lastly, it is increasingly accepted in the scientific community that traditional animal tests fail to fully predict human safety. Some of these traditionally grandfathered methods would not pass the rigid modern standards of at least 90% accuracy (*Hartung, 2009*).

There is, therefore, no alternative than for all involved parties, regulators, industry and the public research community, to jointly drive the AAT cause forward with intense collaboration, each sector contributing what it can do best and each daring to stretch the limits and step

beyond traditional dogmas and boundaries. This aspired collaboration is depicted in *Figure 2.5*. The European cosmetic industry stands ready to shoulder its part and to work closely with all parties involved.

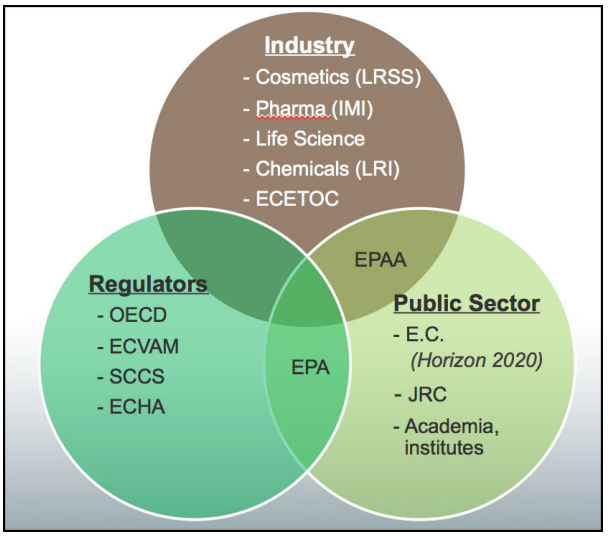

Figure 2.5 *Aspired cooperative development of alternatives to animal testing.*

2.2.5 Concluding Remarks

Fortunately, the increasing urgency of developing methods and strategies for non-animal based safety assessment is now mainstream thinking and has led to a substantial increase in research initiatives worldwide. All these and more are much-needed to revolutionise the discipline of toxicology, which has to move from being fully based on animal testing to a systems biology, chemo- / bioinformatics based safety assessment principle. This enormous task will take many more years to complete, but step by step work can deliver tangible results.

The European cosmetic industry can proudly claim a key institutional role in having changed the old dogma that a full-fledged replacement of traditional, often grandfathered animal testing is impossible. It has achieved regulatory acceptance of an impressive number of alternative methods with final or near-final regulatory acceptance. The industry also initiated and contributed its 50% share to the generally recognised outcome of **SEURAT-1**, which has given us hope and paved the way to future systemic toxicology. The cosmetic industry will continue its essential contributions, play its pivotal role in applied research by bridging the gap between findings of basic research and the dearly needed, practically possible safety assessment that is supported and officially approved by regulatory authorities. Until this Herculean task has been achieved, the European cosmetics industry will have to address and suffer from the

impact of marked competitive disadvantages with companies operating in other world regions where safety assessment regulations better reflect scientific reality than in Europe.

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2.3 New Approach Methodologies in the Regulatory Context: Impressions from the 2016 ECHA Scientific Workshop

Kaihsu Tai, Tomasz Sobański, Tatiana Netzeva, Derek Knight

2.3.1 Introduction

New Approach Methodologies and the 3Rs

ECHA held a scientific workshop on new approach methodologies on 19-20 April 2016.[1] In the spirit of the impressionist painter Georges Seurat, this is a subjective, selective perception from this workshop, as captured by a few ECHA staff members who were involved in preparing and organising it. For a complete rendering, please refer to the proceedings to be published on the ECHA website in autumn 2016.

The scene is set by two growing expectations in recent public discourse in the European Union. There is a higher demand for better chemical safety, but at the same time also for avoiding unnecessary animal testing. To transcend this, it is important to pay attention to all of the so-called '3Rs' principles for more ethical use of animals in testing: replacement, reduction and refinement. In other words, refinement and reduction are just as important as replacement, at least for now. In each of these 3Rs, 'new approach methodologies' (NAM) may help.

For the purposes of the workshop, we defined NAM to include a variety of new testing tools. They range from more conventional methods aiming at improved understanding of toxic effects, all the way through to so-called 'high-throughput screening' and 'high-content methods', such as genomics, proteomics and metabolomics. All these methods may help fill frequently-occurring knowledge gaps when assessing the hazards of chemicals, as in toxicodynamics and toxicokinetics.

Placing animal testing in the wider context, it should also be recalled that testing for toxicological and other safety evaluation of industrial or household chemicals is a small part of the total animal testing carried out in the EU (approximately 1 %; *European Commission, 2013*). Nevertheless, any efforts on the 3Rs – such as developments in NAM – in similar regulatory areas – such as pesticides, cosmetics and pharmaceuticals – will also have an impact on industrial chemicals within the scope of EU's REACH Regulation, which is ECHA's main operational field.

We are aware that the 3Rs do not become reality through a few large research projects, such as **SEURAT-1**, ToxCast and EU-ToxRisk, although these will be extremely helpful. This is a

1 http://echa.europa.eu/view-article/-/journal_content/title/topical-scientific-workshop-new-approach-methodologies-in-regulatory-science

project for a generation of new scientific developments and regulatory transformations. ECHA has been paving the way to the 3Rs in manifold ways: by developing advice on alternatives to animal testing, by promoting the use of the OECD QSAR Toolbox, by investing in and promoting read-across, and by contributing to relevant conferences in and beyond Europe. ECHA is also attentive to the regulatory relevance of alternative methods, building on the state of the art from the European Commission's Joint Research Centre (JRC).

Workshop Themes and Key Discussion Points

The unifying characteristic of the various NAMs is that they explore why something would happen, not just what happened. That is to say, they pursue mechanistic explanations to help assess the hazard of chemicals. They might be also useful for predicting the human relevance of toxicology studies, leading to new prospects for regulatory science.

In the workshop, we focussed on three themes:

- Definitive hazard assessment: improvement of read-across
- Screening and priority setting
- Prospects for future regulatory uses

During the workshop, it became clear that NAM could already be applied to each of these, to various extents. However, for regulatory acceptability, a number of elements still needed to come into place for every one of them. ECHA would need to be in a position to assess and take advantage of these.

The workshop saw a collaborative atmosphere, with open discussions and the right expertise present. This was a concrete meeting where many important issues were put on the table. The workshop thus helped achieve the strategic objective for ECHA to serve as a hub for building scientific and regulatory capacities in dealing with regulatory challenges.

Below, we will first describe the case studies discussed at the workshop under Theme 1. We will then offer our personal impressions on the key discussion points for Theme 1 before discussing the remaining themes. Finally, we will present our personal conclusions from our own observations of the workshop and at the meeting of the workshop's scientific advisory committee immediately afterwards.

2.3.2 Theme 1: Definitive Hazard Assessment - Improvement of Read-Across

For Theme 1 on definitive hazard assessment, particularly to improve read-across, the workshop discussed three case studies. The first two used the collaborative work between

Cosmetics Europe and **SEURAT-1**: read-across for selected perfluoroalkyl acids (PFAAs) and for β-olefinic alcohols (β-AO; see also section 3.2.2). The third case study was about phenoxy herbicides using metabolomics as a read-across tool, presented by BASF.

Case Study 1: Read-Across for 90-day Rat Oral Repeated-dose Toxicity of Selected Perfluoroalkyl Acids

The PFAA case extensively reviewed literature of the various studies available for different species, and with different durations. For the purposes of the study, however, it was presumed that the 90-day rat oral study NOAEL for perfluorooctanoic acid (PFOA) could serve as a conservative source for the carboxylic acids with different chain lengths, from C_7 to C_{10}. In addition, crucial evidence for the toxicodynamics of the substances was provided by ToxCast.

The initial hypothesis suggested that the substances were well absorbed orally, poorly eliminated, not metabolised, and would have similar mechanisms of distribution and elimination between the species. PFAAs were found giving a standard set of symptoms, including liver toxicity. While the molecular mechanisms of the PFAA-induced liver toxicity are not completely characterised, numerous studies suggested that PFAAs supress the immune system and induce fatty acid transport and metabolism due to PPARα/PPARγ interaction. Identifying the most likely initiating event (e.g. PPAR activity) was thus seen as key to establishing toxicodynamic similarity.

ToxCast screened up to 800 distinct *in vitro* assays. PFOA showed the strongest evidence for PPAR activity compared to all other tests in concentration ranges not associated with cytotoxicity. It was also found *in vitro* that PPAR activity increased with increasing carbon number from C_4 to C_{11}. A somewhat bell-shaped curve was observed *in vivo,* with maximum at C_8. Reading across from this most conservative result was, therefore, considered sufficient for the purposes of the example. However, it was noted that, in the case of these compounds, toxicokinetic similarity is key to establishing toxicological similarity, due to the fact that elimination half-life values vary significantly between species and sexes, and that excretion mechanisms may be compound-specific.

Case Study 2: Read-Across for 90-day Rat Oral Repeated-Dose Toxicity of Selected ß-Olefinic Alcohols

The β-AO case was based on the hypothesis that metabolites, acting by the same mode of action, can be considered as ultimate toxicants, if all other toxicokinetic and toxicodynamic factors are about the same. To illustrate this case, a series of short-chain β-AOs were selected, each containing 3 to 6 carbon atoms in the main chain. It was assumed that, in such a small range, there would not be not major differences in bioavailability. It was known that these substances are rapidly and extensively absorbed by the gut, and the metabolism goes mainly

via alcohol dehydrogenase (ADH) to corresponding aldehydes and ketones. The effects were driven by liver toxicity.

The NAM evidence in this case was generated by **SEURAT-1** (see section 3.2.2), and was based on knowledge of the reactivity of the parent compounds and their corresponding metabolites, which were shown to react in a Michael-addition type of mechanism with glutathione (GSH). It is interesting that additional data generated by **SEURAT-1** suggested removing the acetylenic alcohols from the initial group, due to different patterns of toxicological effects. Also, tertiary β-AOs were excluded because they do not undergo ADH activation.

The specific evidence in this case was generated by combining 2-hour isolated perfused liver assay with GSH as the final measure of produced activity. This activity was compared to the activity of the α,β-unsaturated metabolites, where possible, and the difference was attributed to the metabolic activity and capacity of the isolated liver. In addition, a human hepatic organoid model was used to study the effect patterns within the group.

There were many open questions associated with this case, mainly because the NAM data did not uniformly cover the members of the group due to financial and time constraints. There were five identified structural sub-groups and three main factors. At the end, read-across from 2-propen-1-ol was done on the assumption that this was conservative, but many uncertainties remained for this group of substances.

Case Study 3: Read-Across with Metabolomics for Phenoxy Herbicides

In this case, there were one target (MCPP) and two potential source substances (2,4-DP and MCPA). The question was which of the sources represented a closer analogue to the target, and how it could affect the read-across result. In terms of mechanism, the best match was found with peroxisome proliferator-activated receptor (PPAR) activity. Among the PPARα agonists, 2,4-DP and MCPA were found to be the best analogues to MCPP. In a principal component analysis with relevant mechanistic parameters, it was found that 2,4-DP is closer to MCPP than MCPA. This determined the source substance from the two candidates.

In this study, understanding the methodology was of huge importance. Unlike the previous study, where stable metabolites could be predicted and followed during the study, the metabolomics study used blood from *in vivo* 28-day study to determine the basic biomolecular components with which a substance may react. The result could be used as fingerprinting, or be translated with the help of historically compiled databases to biological pathways of activity/toxicity. These might, in turn, be used as biological descriptors for comparing toxicological similarity.

There were several advantages and disadvantages of the method discussed. An advantage is analysing blood at several time intervals in real repeated-dose study (if it has to be performed anyway). The authors (BASF) showed other examples where metabolomics was found to

provide important information on the mode of action, which is useful in read-across. However, questions of the time for blood sampling, the relatively high doses required, and the variability of the results were raised. The case was an interesting example of a potentially universal NAM (if the list of cellular reagents is exhaustive; what about receptor-mediated effects?), but it obviously requires a robust database for interpretation of results, and will need much more standardisation as well as transferability of the analyses and tools.

The Authors' Reflections on the Discussions from the Meeting of the Scientific Committee for Theme 1

Information from *in vitro* molecular screening, '-omics' assays and computational models can be useful to improve the robustness of read-across cases. This can be done by making intelligent use of the known toxicological profile of the source substance to choose assays pertinent for the relevant biological pathways. In all three case studies, NAM brought an added value to establishing the read-across hypothesis. In the long run, we could even envisage cases where the read-across reasoning could be based exclusively on NAM data.

ECHA's Read-Across Assessment Framework (RAAF; *ECHA, 2015*), developed for examining the scientific justifications for read-across cases, was affirmed by participants to be a useful means of examining read-across approaches, including the added-value of NAM in providing mechanistic evidence.

There remains a major knowledge gap regarding toxicokinetics, as we will see below. Toxicokinetics is the *sine qua non* for regulatory acceptance of NAM. No matter which approach is used, the chemical's toxicokinetics needs to be addressed.

On the other hand, metabolomic NAM data obtained from *in vivo* studies point to a possibility of 'biological read-across' without reliance on structural similarity. To build confidence in this approach, we still need to compile an open-access database from augmented *in vivo* studies for future toxicokinetic comparison.

2.3.3 Theme 2: Screening and Priority Setting

NAM evidence is already used for screening and prioritisation, mostly in the United States and in Canada. In the United States, de-prioritisation is done solely based on NAM data. However, a definitive conclusion on hazard cannot yet be based on NAM evidence.

In comparison with other jurisdictions' regulatory programmes, EU's REACH offers a radically different framework not just for hazard and risk assessment but also for screening and priority setting. In addition, REACH as a data-collection exercise offers a new basis for further 'big data'-style analysis.

There was support for the idea that arose during the workshop of a rolling research preference shortlist of 5-10 chemicals of highest priority to ECHA, as a 'challenge' to industry and the research community, e.g. EU-ToxRisk, to undertake testing on these particular substances.

2.3.4 Theme 3: New Prospects for Regulatory Science

It is evident that NAM methods are advancing rapidly. Regulators should therefore consider more extensive use of this type of evidence in various regulatory processes. In the short-term, combining NAM with standard methods under 'weight of evidence' is acceptable in regulatory application.

A philosophical point for consideration is how to ensure the current high level of protection for human health when using NAM evidence. In other words, is there a cost-effective yet trustworthy 'shortcut' to the same (or a higher) level of protection using NAM data, without resorting to strict coverage of apical endpoints? This may be a challenge for future research projects such as EU-ToxRisk.

A related point, for future public policy discussion, is whether we can explicitly state the level of protection using NAM (or even increase confidence), and without satisfying all the equivalent elements of the standard information requirements (e.g. annexes VII to X in the REACH Regulation) through stringent reasoning to adapt (e.g. REACH Annex XI). Currently, the desired level of protection is encoded only implicitly in law. Can the level of protection be expressed explicitly? Can or should the regulatory framework, with the knowledge offered by NAM, then be amended to calibrate or adjust the level? NAM may offer new prospects to define desired goals and levels of protection without direct reference to apical endpoints as observed in classical *in vivo* tests.

2.3.5 Provisional Summary: Barriers, Ideas, Priorities

As stated in the beginning, one of ECHA's strategic objectives is to 'address scientific challenges by serving as a hub for building the scientific and regulatory capacity of Member States, European institutions and other actors'. To achieve this objective, ECHA is looking forward towards the horizon for scientific developments and their potential regulatory impact. It is apparent that both the validity and regulatory acceptability of alternative approaches will continue to develop over the next few years.

The discussions at the workshop and in the scientific committee identified the following barriers to the wider use of NAM in regulatory contexts:

- Lack of understanding by industry and regulators in judging the relevance of NAM evidence, especially in relation to risk assessment and classification.

➠ Lack of quality standards on performance of NAM tests (to demonstrate robustness and reproducibility) and lack of common standards for reporting NAM evidence.

➠ NAM's focus on toxicodynamics (e.g. reactivity) while the toxicokinetics aspect (mainly metabolism) is often missing.

The authors of this article observed that the following ideas and priorities for the way forward of using NAM in a regulatory context emerged from the discussions at the workshop and in the meeting of the scientific committee:

➠ At a high level, a strategy is needed with defined pathways for future action; this should set clear goals and express the level of ambition.

➠ For application of NAM in the short-term and for maximum impact, the priority should be on read-across enhancement:

- For mechanistic support of read-across justifications, to address toxicodynamic mechanistic explanations.

- Improved grouping using NAM based on bio-profiling.

➠ Scientists and funders of research should receive clear input on regulatory needs from regulators, so that pure and applied research and method development can be targeted to best impact practical application. Within this context, there should be direct input into the ongoing EU-ToxRisk research programme.

➠ Toxicokinetics is a key knowledge gap; a clear priority is therefore research targeted to support read-across justifications.

➠ A high-level mapping of NAM techniques, and perhaps also a more detailed comprehensive NAM methods inventory, would be useful in facilitating research.

➠ A key means of communication from regulators could be drawing up a research preference shortlist of chemicals of regulatory interest. A 'challenge' can then be issued to researchers to explore use of NAM for the shortlisted chemicals.

➠ An effective means of working is using case studies. Read-across case studies should include more NAM data pertinent to the mechanistic aspect being addressed. An aspiration could be for some case studies to be submitted to regulators in a registration dossier. For *ab initio* case studies, the research preference shortlist could serve as a common pool of substances of regulatory interest.

➠ Some international consensus on the regulatory application of NAM is essential, probably under the auspices of the OECD, but perhaps also in the International Programme on Chemical Safety of the World Health Organization (WHO). Is there a need for further standardisation of reporting for weight of evidence and NAM?

➠ There is a clear need for regulators to better understand NAM and how it might be used. Exchange of experience between the various regulators and with the regulated community will facilitate this. In addition, training and education of all stakeholders on NAM, in particular on '-omics' interpretation, is an on-going need.

Regulators such as ECHA are in a good position to steer the development efforts for these ideas and priorities through international cooperation (e.g. bilaterally and via OECD, WHO) to develop more standardised approaches (e.g. Integrated Approaches to Testing and Assessment) and enhanced standard protocols. The ultimate aspiration, the 'grand vision', could be mutual acceptance of hazard predictions internationally.

The outcome from the workshop will be reported in its proceedings, to appear in autumn 2016. We hope promoting NAM in this way will allow *in silico* and *in vitro* NAM data to be more routinely incorporated into hazard assessments of chemicals, so that they will meet generally agreed standards, and that quality will be assured with regard to their methods, reporting and interpretation. Eventually, by exploring and promoting the use of NAM for regulatory purposes, these efforts will contribute to the reduction of animal testing.

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Disclaimer

The views expressed in this paper are solely those of the authors and the content of the paper does not represent the views or position of the European Chemicals Agency.

2.4 Frameworks for Using New Approach Methods in Human Health Risk Assessment

Tilman Gocht, Michelle R. Embry, Russell S. Thomas, Derek J. Knight

2.4.1 Introduction

Increasing demand for animal-free safety assessment procedures has stimulated the development of frameworks providing structures and giving guidance as to how to combine available methods to answer a safety assessment question. In previous volumes of this Annual Report, we highlighted some of these frameworks that we will now recapitulate to provide an overview of these concepts. All of them are flexible in the sense that progress in the related fields is expected and can be considered: new methods and technologies can be incorporated into these frameworks. The frameworks can, therefore, combine the greater understanding of modes of action, adverse outcome pathways, toxicokinetics, high throughput assays, exposure models, databases, *in silico* algorithms and other advances which are being made by many groups. As these develop, a framework is needed that allows them to work together. The framework will be constant, but the components within it will evolve as the science develops.

We start with the RISK21 roadmap that highlights the common principles of combining the formulation of the problem with tiered approaches to assess exposure and hazard. The most important point to understand is that we cannot simply follow an overall pathway in safety assessment; rather, the problem defines the route we must take. In other words, the formulation of the problem defines the boundary conditions for exposure and hazard assessments. This is also the starting point for the conceptual framework developed within the **SEURAT-1** Research Initiative. As the application of the developed tools and methods in the safety assessment context was part of the focus of the **SEURAT-1** Research Initiative from the outset, the consortium developed its own framework incorporating knowledge about mode-of-action and adverse outcome pathways in an effort to link early key events with adverse effects at the organ level. It also takes into account the important distinction between general and organ-specific toxicities, as highlighted in the third, data-driven framework discussed below. This data-driven framework suggests following different assessment strategies for 'non-selective' chemicals (chemicals with many biological targets) as compared with 'selective' chemicals (chemicals with a limited number of biological targets).

2.4.2 RISK21 - A Roadmap for 21st Century Human Health Risk Assessment

The Health and Environmental Sciences Institute (HESI) created the RISK21 Project to address

and catalyse improvements in human health risk assessment by developing a framework to enable integration of information obtained by both conventional and emerging methods. RISK21 is coordinated by HESI, a global branch of the International Life Sciences Institute (ILSI, see also a short characterisation in section 5.2.2), and is a multi-sector programme with participants from government agencies, academia, industry, and others (*www.hesiglobal.org*). The project has involved over 120 participants from 12 countries, 15 government institutions, 20 universities, and 2 non-governmental organisations since it was formed in 2010.

RISK21 Principles

RISK21 (*Pastoor et al., 2014*) derived a flexible framework for bringing together knowledge to enable effective, efficient decision-making. It is based on a set of principles:

- Focussing on problem formulation;
- Utilising existing information to the greatest extent possible;
- Starting with exposure rather than hazard;
- Using a tiered approach to data development and decision-making.

Focussing on problem formulation: Problem formulation is built around the fundamental question: 'What decision do you need to make?' For example, for priority setting, the formulation of the problem would limit the scope of the assessment to determining which chemicals are of greatest concern. For a full human health risk assessment, the formulation of the problem would focus on identifying sufficient information on use, exposure and toxicity to establish a margin of exposure (MoE) and a decision as to whether or not that MoE provides reasonable certainty of no harm. Risk assessments should begin with the end in mind by considering physical/chemical properties, usage characteristics, existing exposure and toxicological data, and the risk management context. By starting with these disciplined and transparent steps, there is greater likelihood that appropriate and necessary data are developed without unnecessary commitment of resources.

Utilising existing information to the greatest extent possible: There are few chemicals that are so isolated in their properties, effects, or exposure characteristics that some indication of their potential toxicity, mode of action or human exposure cannot be estimated from similar chemicals or uses. By collating and mining the extensive knowledge that now exists on chemistry, fate, usage characteristics and toxicity, useful predictions about the behaviour of a chemical can be obtained.

Decades of data generation, much of which is now available in online databases, can be utilised to provide estimates of exposure and toxicity that may be sufficient to make a decision or, if not, to guide focussed data generation.

Starting with exposure rather than hazard: Human safety depends on both exposure and hazard. An early estimate of potential human exposure in relevant scenarios and populations, including susceptible populations, will characterise the degree of specific toxicological data needs. For example, chemicals with exceedingly low potential exposure should lead to less allocation of toxicological resources than those with higher exposures, which could call for a more extensive toxicological database to inform risk assessment.

Using a tiered approach to data development and decision-making: RISK21 uses a tiered approach for both exposure and hazard assessment. This leads to optimal use of available resources and establishes a value of information approach for decision-making. Guided by problem formulation and taking advantage of existing information, both exposure and toxicity estimates can be generated, initially at a basic level, that may be sufficient for a decision on human safety.

Data acquisition can stop when there is enough precision to make a decision. 'Precision' is used here to represent the degree of accuracy in the data, which is usually an estimate bounded by a confidence interval or range, the size of which is typically proportional to the quality and quantity of knowledge used to generate the estimate.

Usually, greater investment of resources is rewarded with narrower ranges of estimates, and hence greater precision. In some cases, a broad estimate of exposure or toxicity may be sufficient, while in other cases, more precision will be necessary.

The RISK21 Roadmap

RISK21 organised the above principles into a transparent, tiered framework called the RISK21 roadmap (*Embry et al., 2014*). Estimates of exposure and toxicity are plotted on the RISK21 matrix, with an estimate of their respective bounds of uncertainty, providing a highly visual representation of the margin of exposure. It can easily be ascertained whether there is likely to be a health concern and, if so, whether it would be more productive to refine the estimate of exposure or of toxicity (*Figure 2.6*). Use of this matrix allows dynamic illustration of the impact of moving through the respective assessment tiers (exposure and toxicity) and the consequences of reducing uncertainty in estimates. The axes can be any suitable dose metric, from external exposure to target tissue concentration, so long as it is the same on both. In this way, the matrix can readily accommodate data generated *in vivo*, *in vitro* or using a combination of approaches.

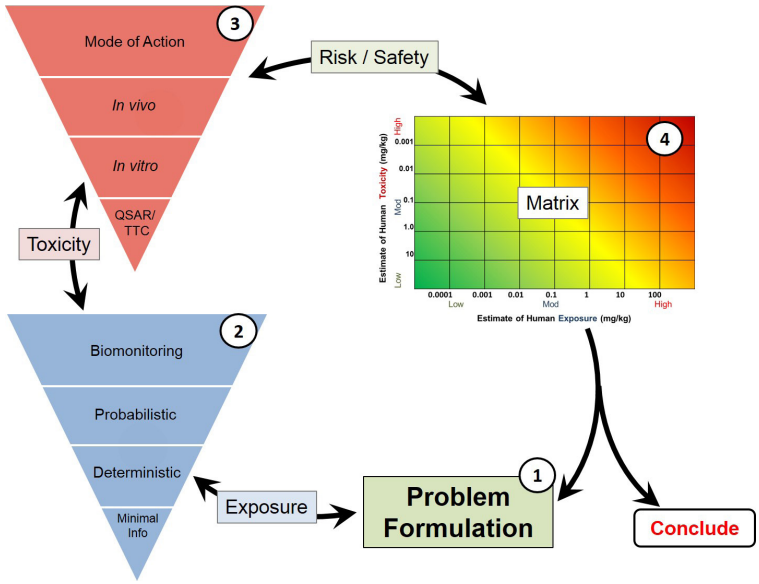

Figure 2.6 *The RISK21 roadmap. This diagram is a schematic representation of a multifunctional tool that provides a transparent process for obtaining rational risk-related decision points. The inverted triangles for exposure and toxicity represent the proportional investment of resources needed for each tier. The following steps describe the use of the roadmap and are described in additional detail in Embry et al. (2014): 1) Problem formulation; 2) Exposure estimate; 3) Toxicity; 4) Matrix (Source: Embry et al., 2014).*

The key is to understand the precision of the methodology and the degree of exactness of the data in predicting effects on human health. Once this is known, the RISK21 road map allows a method to be incorporated into the tiered approach. The approach is non-judgmental with regard to the methodological origin of the data, so long as they can be expressed in a common metric. The greater the precision of the methodology, the higher the tier at which it can be used. However, many decisions about the safety of chemicals in use can be made using lower tier assessments. It is also important to emphasise that the RISK21 approach does not mandate a rigid hierarchical application of the tiers. These can be applied as appropriate, with different tiers for exposure and hazard, if necessary, and moving to whichever higher tier is most suitable.

2.4.3 The SEURAT-1 Conceptual Framework

The **SEURAT-1** Conceptual Framework is inspired by the initiative's overall research strategy, which is to adopt a toxicological mode-of-action approach to describe how any substance

may adversely affect human health, and to use this knowledge to develop complementary theoretical, computational and experimental (*in vitro*) models that predict quantitative points of departure needed for safety assessment. The aim is to develop a flexible approach to deliver fit-for-purpose assessment of the toxicological properties of a substance, taking into account its properties and the (regulatory) purpose of the prediction.

A flexible 'conceptual framework' has emerged from **SEURAT-1** that can be used as a basis for rational combination of information derived from predictive tools to support a safety assessment process or decision to achieve a stated protection goal in the context of repeated-dose systemic toxicity. This framework is intended to set out a structure to guide assessors in devising a fit-for-purpose (or 'bespoke') Integrated Assessment and Testing Approach (IATA) for the particular circumstances and case. The overall outcome is anticipated to be robust, as it is not based on single pieces of evidence, but rather a weight of evidence combined in a biologically rational manner.

An indicative diagrammatic representation of the latest version of this conceptual framework is shown in *Figure 2.7*. Before beginning the assessment, the degree of confidence needed for the prediction is decided, for example to replace a standard toxicological study in a regulatory submission or an industry risk assessment of the substance in a cosmetic product. Within a particular exposure context, the assessor may be able to accept a moderate or low degree of confidence in the prediction if human exposure from the use is well controlled and low. The idea is then to begin by examining existing knowledge from various lines of evidence. In particular, it is important to consider whether this is a 'general chemical' expected to be unselective in interacting with biological targets, or a drug/pesticide designed to be selectively biologically active. Other evidence could include toxicological studies on the substance; read-across from chemical or biological analogues; QSARs and structural alerts; and expert judgement. There are then two parallel lines of consideration:

- 'General' adverse effects not associated with a particular organ but associated with targets present in many cells, tissues and organs or resulting from general chemical activity (e.g. alkylation);
- 'Organ-based' adverse effects.

Both lines of consideration require a review of toxicokinetics/toxicodynamics. The target organs for the parent substance and metabolites would be the focus of more rigorous assessment, with non-target organs also examined using a simplified assessment. Effects on organs can be assessed by (several) AOPs; with the Molecular Initiating Event (MIE) and Intermediate Events (IEs) within an AOP; incorporating existing knowledge and with new data as a combination of *in vitro* assays ('-omics' data, high-throughput data, etc.); and *in silico* predictions, forming a battery of assessment tools.

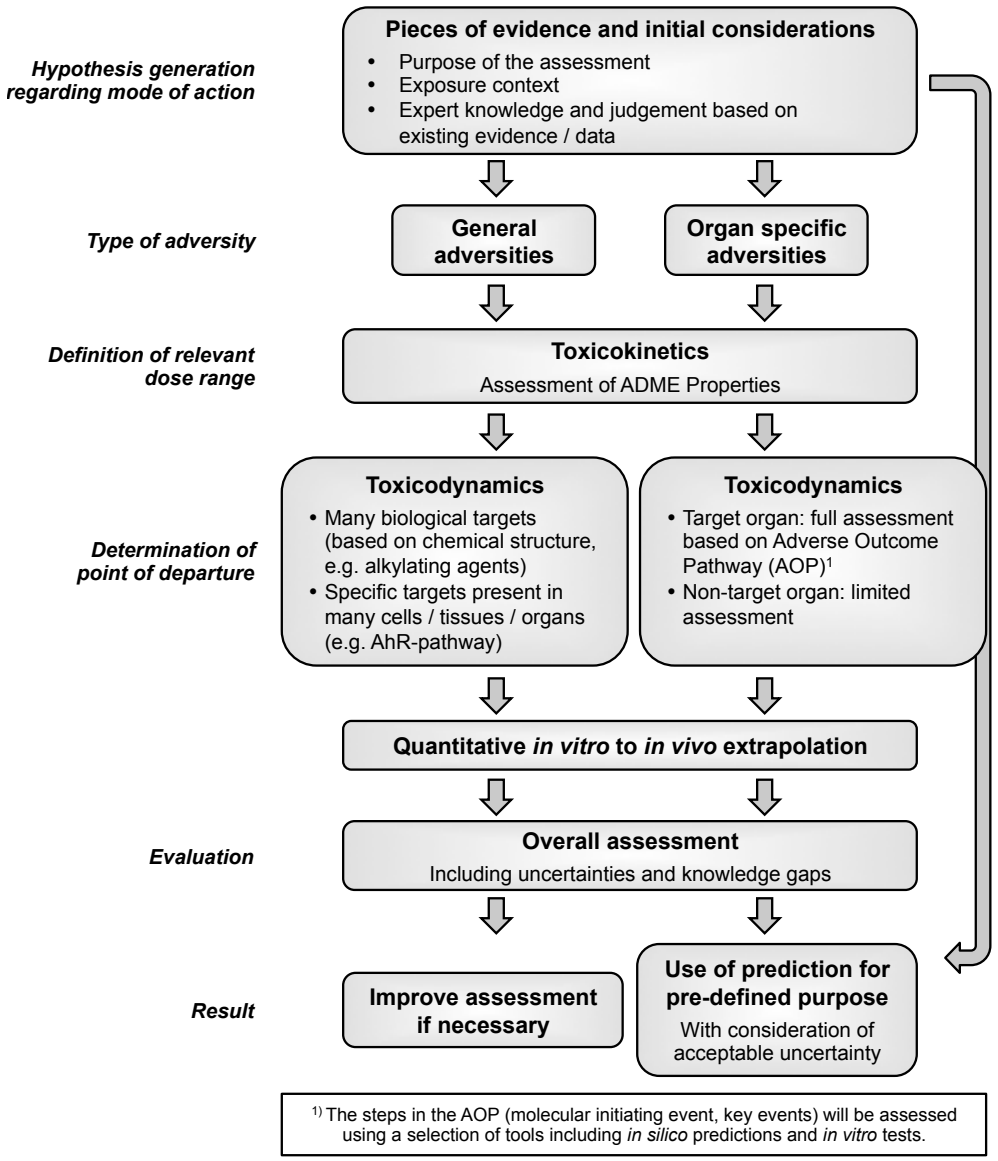

Figure 2.7 *'Conceptual framework' as a structure for assessors in devising a fit-for-purpose 'bespoke' Integrated Assessment Strategy for a particular case (Source: Gocht et al., 2015).*

The overall assessment is achieved through the evaluation of this information and evidence, including the assessment of the uncertainty associated with the prediction. It may be necessary to improve the assessment if the result is not fit for purpose.

2.4.4 A Data-Driven Framework for Incorporating New Technologies into Chemical Safety Assessment

Introductory remark: This is an extended abstract of a manuscript that was published in Toxicological Sciences (Thomas et al., 2013).

Selective Versus Non-Selective Chemicals

The data-driven framework presented below is inspired by the efforts of the U.S. ToxCast programme. The aim of this programme is to develop a cost-effective approach for prioritising the vast number of chemicals that still need toxicity testing, and to predict the potential toxicity of chemicals. A series of studies has been conducted that together may begin to formulate a new data-driven framework to toxicity testing and risk assessment (*Figure 2.8*). The first study is a comprehensive cross-validation model comparison to evaluate the predictive performance of the more than 600 *in vitro* assays from the ToxCast Phase I screening effort across 60 *in vivo* endpoints using 84 different statistical classification methods. The predictive performance of the *in vitro* assays was compared to that of chemical structure descriptors. The results showed that the current suite of ToxCast high-throughput toxicity assays have limited applicability for predicting *in vivo* chemical hazards using standard statistical classification methods (*Thomas et al., 2012a*). Despite these limitations, the *in vitro* assays do provide a broad survey of the potential proximal biochemical and cellular targets for a chemical, and can be used to separate chemicals into either those that cause toxicity primarily through non-selective interactions with cells and cellular macromolecules or those that act through more selective interactions (e.g., receptor-mediated chemicals). When analysed from this perspective, the data suggest that the majority of chemicals represented in the ToxCast Phase I library likely act via non-selective interactions with cellular macromolecules.

The internal dose of a chemical is an important determinant of toxicity. In previous studies, data from *in vitro* hepatic metabolic clearance and plasma protein binding assays were used to parameterise an *in vitro*-to-*in vivo* extrapolation (IVIVE) model to estimate the daily human oral dose, called the oral equivalent dose, necessary to produce steady-state *in vivo* blood concentrations equivalent to the *in vitro* AC_{50} value for each of the high-throughput *in vitro* assays (*Rotroff et al., 2010; Wetmore et al., 2012; 2013*).

For the selective chemicals, the oral equivalent dose values could be used to identify potential key events in a mode-of-action (MoA). The MoA for a chemical consists of a series of key events triggered by a chemical at a molecular or cellular level that causally link with an *in vivo* adverse effect. The key event must, therefore, be triggered at doses lower than or equal to doses at which the adverse effect is observed. Among a subset of ToxCast Phase I chemicals, eight were active in the *in vitro* peroxisome proliferator-activated receptor alpha (PPARa) assays and caused rat proliferative lesions, while four also caused rat liver tumours. For each

chemical, the lowest observed effect level (LOEL) and no observed effect level (NOEL) for rat liver proliferative lesions and tumours were overlaid with the oral equivalent dose values for the three high-throughput *in vitro* assays measuring PPARa-related activation (*Wetmore et al., 2013*). In all cases, activation of the PPARa *in vitro* assays occurred at or below the dose for the *in vivo* effects, thereby demonstrating consistency as potential key events on a dose level. From these selectively activated or inhibited assays, probable key event(s) could be determined and the oral equivalent dose value for the key event could be used as the point-of-departure (PoD) in the dose-response assessment.

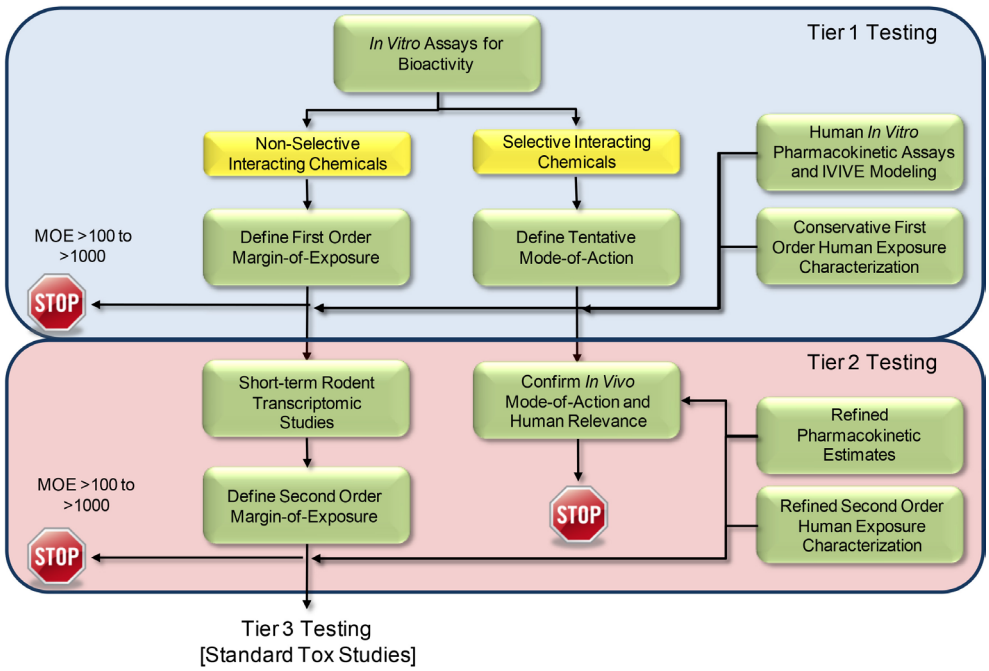

Figure 2.8 *Flow-chart outlining a proposal for a data-driven framework for toxicity testing and risk assessment.*

For the non-selective chemicals, identifying the MoA is neither efficient nor economical since, by definition, the chemical will interact with and perturb multiple cellular processes within a narrow dose range. In a previous study, rat-specific oral equivalent doses were calculated for 59 ToxCast Phase I chemicals and compared with LOEL values for a variety of *in vivo* apical responses in the rat (*Wetmore et al., 2013*). Although not significantly correlated, the oral equivalent dose for the most sensitive *in vitro* assay was less than the LOEL for the most sensitive rat *in vivo* endpoint for approximately 95% of the chemicals. On average, the most sensitive *in vitro* assay was 66-fold lower than the most sensitive LOEL value. These results

demonstrate that the oral equivalent dose for the most sensitive *in vitro* assay can be used as a conservative estimate of the PoD for the non-selective chemicals in the dose-response assessment.

Estimates of human exposure would be combined with the PoD from the dose-response assessment to calculate a margin-of-exposure (MoE). For the selective chemicals, the MoE could be calculated relative to the oral equivalent dose from the *in vitro* assay(s) associated with the proposed key event. For the non-selective chemicals, the MoE could be calculated relative to the oral equivalent dose for the most sensitive *in vitro* assay. For those chemicals with an MoE greater than a defined cut-off, no further testing would be required while chemicals with an MoE below the cut-off would progress on to the next tier (*Figure 2.8*). In a previous study on the ToxCast Phase I chemicals, an MOE cut-off of >100 would eliminate additional testing on ~40% of chemicals, while an MOE cut-off of >1,000 would eliminate additional testing on ~25% of chemicals (*Wetmore et al., 2013*).

For chemicals progressing on to the next tier, data requirements would be different for the selective and non-selective chemicals. For the selective chemicals, focussed *in vivo* MoA studies would be performed to confirm the tentative MoA defined in the first tier and would be defined by the type of MoA proposed. Assuming the MoA is relevant to humans, the PoD would be determined based on the associated endpoints in the *in vivo* studies.

For non-selective chemicals, a series of previous studies have examined the relationship between transcriptional and apical responses (*Thomas et al., 2007; 2011; 2012b; 2013*). Both non-cancer and cancer-related responses were analysed using benchmark dose (BMD) methods to identify PoDs. The dose-response changes in gene expression were also analysed using BMD methods and the responses grouped based on signalling pathways. A comparison of transcriptional BMD values for the most sensitive pathway with BMD values for the non-cancer and cancer apical endpoints showed a high degree of correlation (*Thomas et al., 2012b*). The correlation was also robust across multiple time points (*Thomas et al., 2013*). Dose-response studies could be performed on the non-selective chemicals at any single time point between 5 days and 13 weeks. The studies would be performed in mice and rats of both sexes. A battery of eight tissues that include those most frequently positive in rodent cancer bioassays (liver, lung, mammary gland, stomach, vascular system, kidney, hematopoietic system and urinary bladder) would be collected. These eight tissues cover 92% and 82% of targets for all mouse and rat carcinogens, respectively (*Gold et al., 2001*). Gene expression microarray analysis on these tissues would allow the estimation of pathway transcriptional BMD and BMDL values. The signalling pathway with the lowest transcriptional BMD value across all analysed tissues would be used to derive the PoD.

Similar to the initial tier, refined estimates of human exposure would be combined with the PoD from the dose-response assessment to calculate an MoE. For the selective chemicals, the MoE could be calculated relative to the PoD of the associated endpoints in the *in vivo* studies.

For the non-selective chemicals, the MoE could be calculated relative to the PoD derived from the signalling pathway with the lowest transcriptional BMD value across all analysed tissues. For those chemicals with an MoE greater than a defined cut-off, no further testing would be required while chemicals with an MoE below the cut-off would progress on to the final tier (*Figure 2.8*). Using data from ToxRefDB on the *in vivo* LOEL values for the ToxCast Phase I chemicals and the corresponding human exposure estimates, MoE values were calculated using the minimum *in vivo* LOEL value (i.e., the most sensitive *in vivo* endpoint) and the exposure estimate for the most highly exposed subpopulation. Assuming that this ratio is representative of the MoE values for a larger set of chemicals, an MoE cut-off of >100 would eliminate additional testing on ~97% of chemicals, while an MoE cut-off of >1,000 would eliminate testing on ~85% of chemicals.

The studies proposed for 3-15% of chemicals passing into the final tier are not explicitly defined, but they would be conceptually equivalent to the current guideline *in vivo* studies performed on high-value chemicals with significant potential for human exposure. These studies could include rodent cancer bioassays, developmental toxicity studies and two-generation reproductive toxicity studies. Depending on the MoE cut-off values imposed, the vast majority of chemicals would be screened out in the preceding tiers.

Conclusions

The proposed tiered testing framework is an attempt to integrate data from new technologies into toxicity testing using the best science available at this point in time. The framework is a significant departure from the current way we evaluate and test chemicals for toxicity and will require a shift in thinking about chemical safety. The first shift revolves around the separation of chemicals into selective and non-selective mechanisms and the acceptance that the majority of non-pharmaceutical chemicals act via non-selective means. The prevailing thought is that the MoA approach can be efficiently applied to all chemicals; however, imposing an MoA approach on non-selective chemicals would waste valuable resources and unnecessarily delay decision-making since each MoA requires agreement on the underlying key events followed by extensive peer review. These efforts should be focussed on the selective chemicals where a series of standardised MoAs could be developed that are associated with specific cellular targets.

A second shift in thinking will be required for the non-selective chemicals. The current hazard-based labelling approach that relies on apical responses will need to be transitioned to a 'region of safety' approach where the most sensitive adverse apical effect is not known, but the PoD and subsequent decisions are based on the lack of biological perturbation. This shift in thinking may be more challenging to achieve due to (i) the entrenched reliance on apical responses and concerns relating to the broad applicability of these associations; (ii) the potentially conservative nature of relying on molecular perturbations as PoDs; and (iii) the difficulty of associating molecular perturbations with actual risk.

2.4.5 Final Remarks

The various frameworks presented above may be redundant in parts, but this is the state of the art and highlights the fact that there is no commonly accepted approach on the market. They provide risk-based and animal-sparing means to evaluate chemicals for safety, but represent only initial steps along the way to a fully knowledge-based approach to evaluating chemical safety. Progress towards a better future in chemical safety assessment will require implementation of pragmatic approaches by national regulatory agencies, cooperation of international organisations, and stakeholder education and involvement. This compilation may provide a useful starting point for discussions about how to combine the various proposals in order to come up with a consolidated version that gives guidance for both the next generation of risk assessors and future research projects in the field of method developments for the replacement of animal testing.

Disclaimer

The views expressed in this paper are solely those of the authors and the content of the paper does not represent an official position of the U.S. Environmental Protection Agency or the European Chemicals Agency.

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3 PROVING THE SEURAT-1 RESEARCH STRATEGY

'No experiment can prove me right. Only one proves me wrong'
Albert Einstein

3.1 Introduction

Elisabet Berggren, Tilman Gocht, MIchael Schwarz, Maurice Whelan

Chapter three of each **SEURAT-1** Annual Report has been dedicated to the development of the SEURAT strategy. In Volume 1, the **SEURAT-1** vision and strategy was outlined as a backbone to all **SEURAT-1** activities (*Whelan & Schwarz, 2011*).

The SEURAT vision is to fundamentally change the way we assess the safety of chemicals, by superseding traditional animal experiments with a predictive toxicology that is based on a comprehensive understanding of how chemicals can cause adverse effects in humans.

The SEURAT strategy is to adopt a toxicological mode-of-action framework to describe how any substance may adversely affect human health, and to use this knowledge to develop complementary theoretical, computational and experimental (in vitro) models that predict quantitative points of departure needed for safety assessment.

This strategy was then further developed into the **SEURAT-1** objectives, including the idea of proving the **SEURAT-1** concept in Volume 2 (*Whelan & Schwarz, 2012*). In Volume 3, the proof-of-concept was expanded to theoretical, systems and regulatory application levels (*Whelan & Schwarz, 2013*) and the fourth volume provided detailed descriptions of the **SEURAT-1** proof-of-concept case studies (*Berggren et al., 2014*). The fifth volume discussed progress in the various case studies with particular emphasis on the methods developed within the **SEURAT-1** projects contributing to them (*Berggren et al., 2015a*). In this final volume, we build on these reports and focus on the application level, demonstrating how new *in vitro* and *in silico* tools can be applied in the context of chemical safety assessment, thus supporting the development of animal-free assessment strategies.

These activities were not originally foreseen when setting up the individual projects but, by developing trust and fruitful collaboration between **SEURAT-1** partners, the vision become a common basis for further commitment, and all the projects contributed to proving **SEURAT-1** concepts.

3.2 The Read-Across Case Study for Safety Assessment

Elisabet Berggren in collaboration with the SEURAT-1 Safety Assessment Working Group

3.2.1 Background

The read-across case study was initiated in the **SEURAT-1** Safety Assessment Working Group and further developed based on the conclusions of an expert workshop hosted by the JRC in Ispra on 29-30 April 2014 (*Berggren et al., 2015b*). At that meeting, participants agreed to investigate four different read-across scenarios, covered in the sections below, which were considered a classification of possible read-across situations, to better manage their assessment and estimate the related uncertainties. The scope in read-across, incorporating *in vitro* and *in silico* data, was defined as the replacement of the animal study for repeated-dose toxicity. Additional biological evidence for the chemical structure-based similarity argument needs to be provided using relevant alternative method(s), and the uncertainty in a read-across argument should be reduced using 'new methods' *in vitro/in silico* data to confirm the similarity in the mechanism of action and perhaps assess relative potency between analogues.

As a follow-up to the workshop, a read-across strategy was drawn up (*Schultz et al, 2015*) including templates to assist in organising the data for the read-across assessment and evaluating uncertainties, reaching a conclusion of the overall final confidence in the read-across assessment. Four chemical categories corresponding to four different read-across scenarios were defined: (1) perfluoroalkyl acids (read-across scenario: toxicity caused by parent compound, i.e. not necessary to consider metabolism), (2) β-unsaturated alcohols (read-across scenario: toxicity caused by metabolites), (3) saturated alcohols (read-across scenario: low or no toxicity) and (4) alkyl phenols (read-across scenario: structural similar compounds, possible to subdivide by different modes of action).

On 6-7 October 2015, the experts met again, at the invitation of the JRC, to review the progress of the read-across case study. The categories corresponding to three of the four scenarios were almost finalised, and could be discussed in detail, while the last one was only initiated. In addition, the group of p-glycol ethers was defined as another chemical category for the scenario of low toxicants, and the assessment was carried out independently of the **SEURAT-1** read-across strategy.

The chemical category assessments were supported by external experts from the US EPA ToxCast program, who provided guidance and expert advice on the use of ToxCast™ *(http://www.epa.gov/ncct/toxcast/data.html) in vitro* data as supporting evidence. In addition, the COSMOS project provided data for the case studies from *in silico* methods developed within

the **SEURAT-1** Research Initiative, and the projects DETECTIVE and *HeMiBio* contributed with *in vitro* data from **SEURAT-1** methods, as supportive evidence for the β-unsaturated alcohols and the short-chained carboxylic acids.

3.2.2 The Read-Across Strategy

The read-across strategy had been developed prior to moving forward on the read-across exercise for the various categories, providing a tool to describe similarity and logically guide the read-across prediction. The strategy also provides templates to structure the information used.

The **SEURAT-1** read-across case study was carried out in two steps. First, traditional read-across based on structural similarities and available existing data was performed. Then, as a second step, the added value of using data from 'new' *in vitro* or *in silico* methods, such as developed in the **SEURAT-1** Research Initiative or from ToxCast, was evaluated. The hypothesis was that the additional data would improve confidence in the traditional read-across. The new data could also assist in better framing sub-categories and a more refined selection of analogues, which might be more appropriate, or suggest what additional studies may need to be performed to strengthen read-across or reduce uncertainty. Additional biologically relevant data could also discourage reliance on chemical structure similarity-based read-across between analogues.

The outcome of the case study is basically an evaluation of how much the read-across was improved by adding data from 'new *in vitro* and *in silico* methods' and, consequently, identification of data or information that could lead to further improvement. However, the templates were developed as a basis for any read-across case study, summarising data and confidence in the data of the source substance(s) and the additional new data generated in order to strengthen the read-across. The main issue in this case study is thus the reduction of uncertainty by means of data from new alternative methods. Overall, confidence in the mode of action was considered to be crucial to providing enhanced evidence through data from new methods.

Scenario I: Chemical similarity of compounds that do not require (or do not undergo) metabolism to exert a potential adverse human health effect) – Perfluoroalkyl Acids

The read-across for the perfluoroalkyl acids (PFAAs) was originally supposed to include molecules with chain lengths from six to twelve carbons. The most data-rich substance, 'the source', is perfluorooctanoic acid (PFOA). The endpoint to be read across is 90-day rat oral repeated-dose toxicity. PFAAs are direct-acting toxicants and the common mode of

action is most likely a combination of PPARα and PPARγ interactions, leading, after repeated exposure, to perturbations of fatty acid metabolisms related to liver toxicity. However, the molecular mechanism of PFAA-induced liver toxicity is not confirmed. The structural similarity between category members is based on identical active groups and the carbon chain length is the only difference. The mechanism of action is therefore assumed to be the same, which was also confirmed by ToxCast™ data. The remaining problem is the toxicokinetics; the PFAAs are known to have large variations in ADME properties in different species. PFOA has a half-life in humans of 2-3 years, while the half-life in the monkey and rodent is 10-20 days (*Han et al., 2012*).

The kidney reabsorption and extremely long excretion time in human compared to animal data caused concern among the experts at the October 2015 workshop. It was considered that small changes in chain length might have a large impact and it was suggested that concentrations in blood should be looked at. It was also observed that the water solubility varies significantly between different molecules in the category. However, the experts considered that the bell-shaped potency curves across the category (PFOA, C-8, being most active) were related more to binding effects than to the physico-chemical properties of the compounds. It was considered that greater understanding was needed of the ADME properties of the category. Further evidence could be gained by making a quantitative comparison with existing biokinetic data. Transport information could be of added value.

Furthermore, it was hypothesised that uncertainty could be reduced by limiting the category from C-6 to C-10, and thereby having the source substance (most active according to ToxCast data) in the middle and also avoiding inclusion of C-11 and C-12, as first suggested, as no clearance data was available for these two analogues. The category members that were finally considered in the case study are shown in *Figure 3.1*.

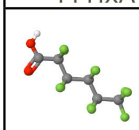
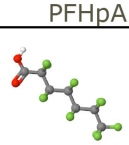
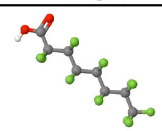
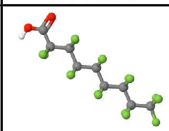
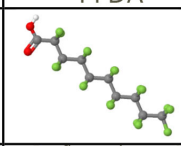
PFHxA	PFHpA	PFOA	PFNA	PFDA
				
Perfluorohexanoic acid	Perfluoroheptanoic acid	Perfluorooctanoic acid	Perfluorononanoic acid	Perfluorodecanoic acid

Figure 3.1 *Category members of the PFAA case study with PFOA as source substance.*

The overall assessment of the improvement of the read-across arguments through the inclusion of the data from new *in vitro* and *in silico* methods is summarised in *Figure 3.2*. Based on the results, the authors of the PFAA case study suggested applying an uncertainty factor of 1. However, the experts that reviewed the study at the October workshop did not

agree with this, as it was not exactly clear how to derive the uncertainty factor. Read across for the purpose of replacing a 90-day rat study is clearly different from its application in the context of human risk assessment and with the broader goal in mind, and whilst confidence had been gained in toxicodynamic aspects, the conclusion was that more investigations are needed to take account of toxicokinetic differences.

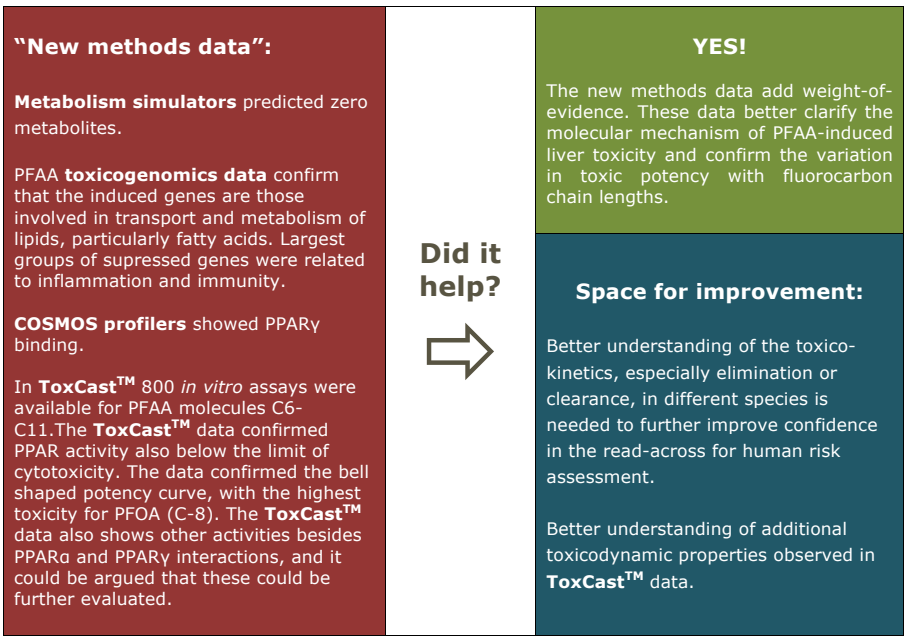
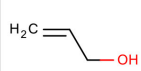
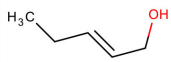
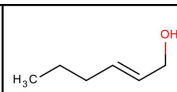
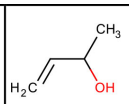
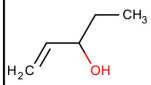
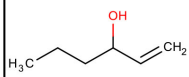
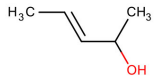
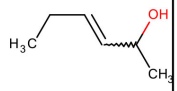
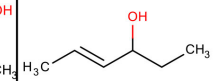


Figure 3.2 *Data and methods used in the PFAA read-across case study and qualitative evaluation of the approach.*

Scenario II: Chemical similarity involving chemical metabolism (resulting in exposure to the same/similar proximal toxicant) – ß-unsaturated alcohols

This category originally included small primary and secondary β-unsaturated alcohols (chain lengths of 3-6 carbons with β-positioned vinylic or acetylenic moiety, *Figure 3.3*) that are readily metabolised by alcohol dehydrogenase (ADH) to polarised α, β -unsaturated aldehydes and ketones. These metabolites react via Michael addition interaction with thiols in proteins resulting in cellular apoptosis or necrosis, which may lead to liver fibrosis. The electrophilic activity of the metabolites also indicates that they would contribute to toxicity. Rodent 90-day oral repeated-dose toxicity study is to be read across from the well-studied allyl alcohol to the other substances in the category.

				
1. Allyl alcohol	2. 2-buten-1-ol	3. 2-penten-1-ol	4. 2-hexen-1-ol	5. 1-buten-3-ol
				
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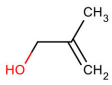	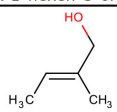	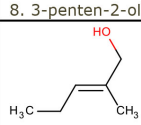	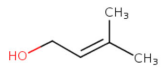	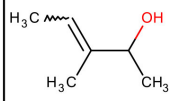
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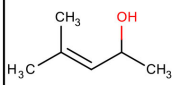	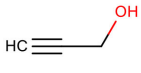	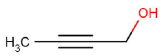	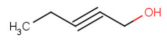	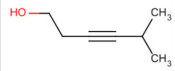
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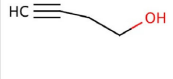
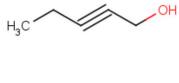
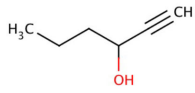
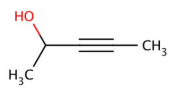
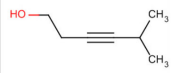
				
21. 1-Butyn-3-ol	22. 2-Pentyn-1-ol	23. 1-Hexyn-3-ol	24. 3-Pentyn-2-ol	25. 3-Hexyn-2-ol
				
26. 4-Hexyn-3-ol				

Figure 3.3 *Category members of the case study focussing on β-unsaturated alcohols with allyl alcohol as source substance.*

The assessment for this scenario started with an emphasis on predicting whether the various category members would be parent compounds to identical or slightly different metabolites but with the same toxic effect. Relevant data was collected for a large category including both allylic and propargylic compounds – all 26 compounds depicted in *Figure 3.3*. Allyl alcohol

was the only source substance in the category, but the data collection revealed that 2-propyn-1-ol (propargyl alcohol) is also a well-studied chemical with *in vivo* and *in vitro* data and was, therefore, included as supporting evidence for the toxicity of β-unsaturated alcohols rather than a category member. The data collection was further complemented by additional *in vitro* data from the *HeMiBio* and the DETECTIVE projects. A 3D liver fibrosis model suitable for studying collagen formation from the *HeMiBio* project (*Figure 3.4*), and reporter cell lines developed in the DETECTIVE for studying stress responses (*Figure 3.5*), were applied for this purpose.

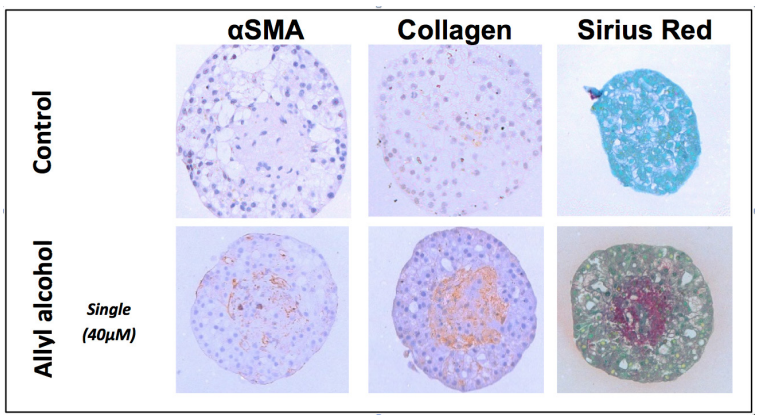


Figure 3.4 *Collagen formation after repeated exposure to allyl alcohol in the HeMiBio co-cultured liver fibrosis model.*

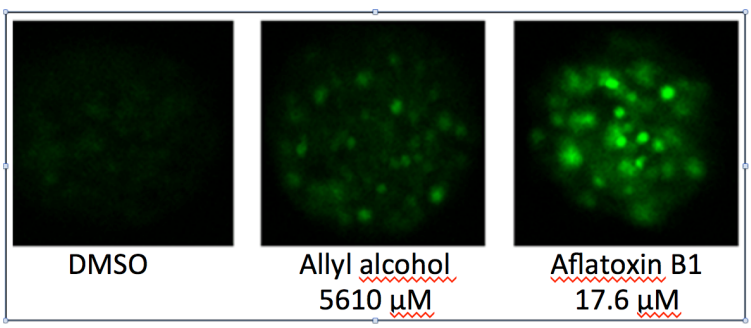


Figure 3.5 *Analysis of P21 activity by high content imaging using reporter cell lines developed in the DETECTIVE project. P21 activation is analysed after exposure to DMSO (negative control) and aflatoxin (positive control), and compared with allyl alcohol after 48 h exposure.*

As an outcome of the expert workshop in October 2015 in Ispra, substances 18-26 in *Figure 3.3* were excluded from the study. The toxicokinetics within the category were assumed to be similar enough. However, there were observed differences in short-term effects between the category members, and it was questioned whether these would also be relevant for long-term effects. The overall summary including the evaluation of the improvement of the read-across arguments through the inclusion of data from new *in vitro* and *in silico* methods is summarised in *Figure 3.6*.

Figure 3.6 *Non-animal data and methods used in the read-across case study focussing on β-unsaturated alcohols and qualitative evaluation of the approach.*

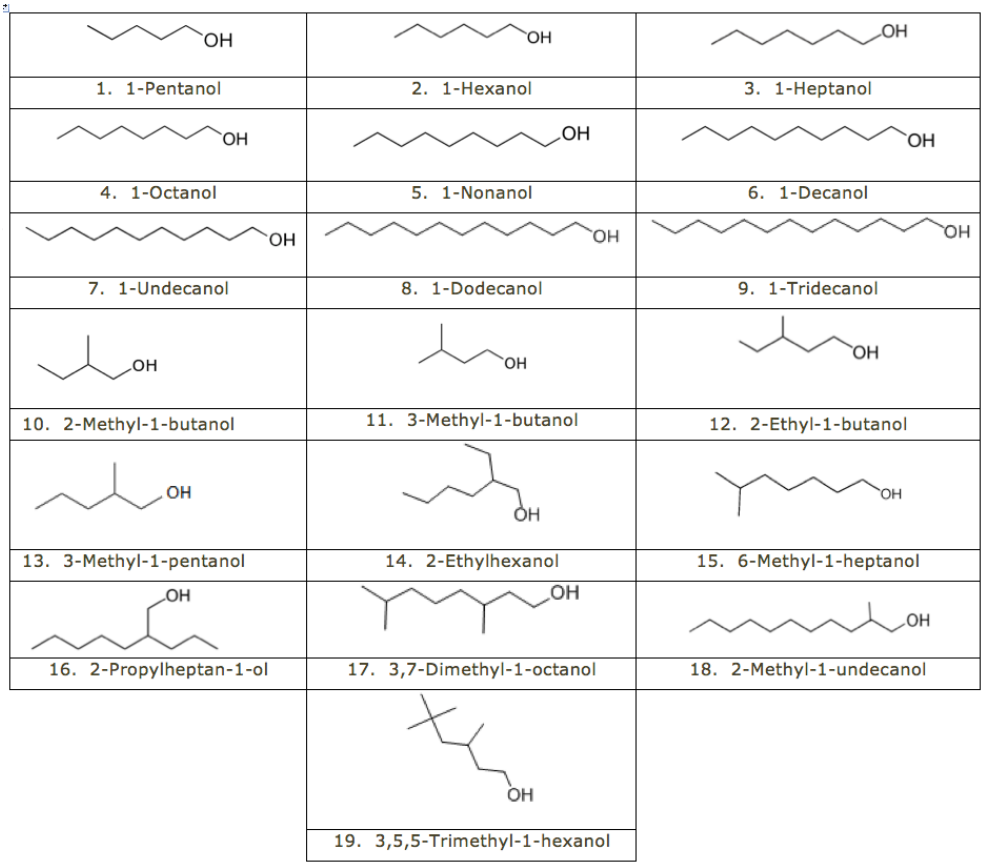


Figure 3.7 *Category members of the case study focussing on primary alcohols.*

Besides the investigation of literature data, *in silico* tools including profilers developed in the COSMOS project, as well as *in vitro* data from the ToxCast™ programme, were used to support the read-across arguments. The argument to combine linear and branched compounds was that there were more studies available for the branched ones. The compounds with linear chains undergo complete degradation, while the branched compounds become ketones and are those that would be both more active and persistent. The initial thought was, therefore, that one large group would actually strengthen the arguments for low toxicity as the branched members did not show higher toxicity, which would have been expected. However, due to the differences in the metabolic pathways, the branched secondary alcohols were excluded from the category (compounds 12-19 in *Figure 3.7*), and only two of them were maintained to better identify the borders of the category (compounds 9 and 11 in *Figure 3.7*). In addition, 1-pentanol (compound 1 in *Figure 3.7*) was excluded to avoid coming into the range of the more toxic short-chained alcohols.

The weak points in this read-across scenario are related to the uncertainty in the understanding of narcotic effects and the possibility of metabolism to reactive aldehydes. There might also be a concern for kidney toxicity for the branched compounds. The overall summary, including the evaluation of the improvement of the read-across arguments through the inclusion of the data from new *in vitro* and *in silico* methods, is summarised in *Figure 3.8*.

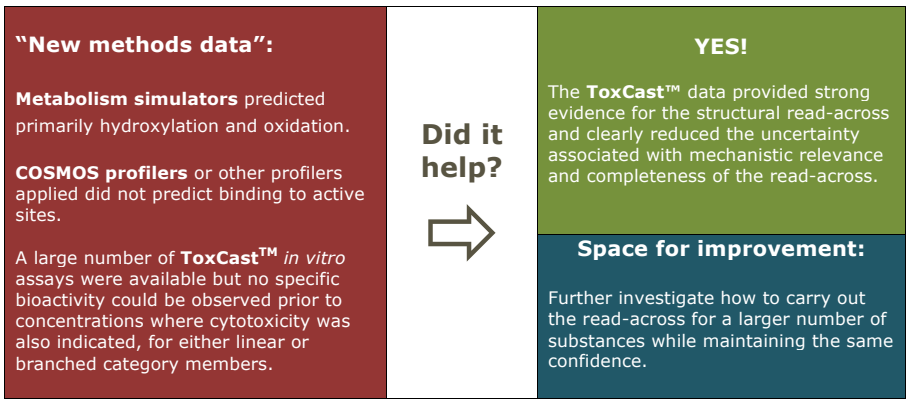


Figure 3.8 *Non-animal data and methods used in the read-across case study focussing on primary alcohols and qualitative evaluation of the approach.*

Scenario IIIb: Chemicals with general low or no toxicity – p-glycol ethers

P-glycol ethers (*Figure 3.9*) were investigated as a second group of chemicals addressing the scenario of low or no toxicity.

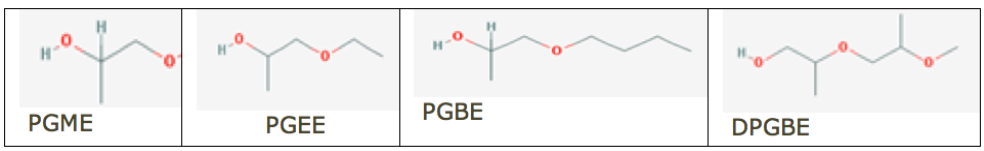


Figure 3.9 *Chemical structures of some p-glycol ethers as a category for the scenario of chemicals exhibiting low or no toxicity.*

The general problem with the low toxicity scenario is the lack of a specific toxicity or effect. It is therefore necessary to strengthen confidence by proving the similarity in phenotype in addition to structural similarity. An integrated approach was developed based on utilization and interpretation of traditional toxicity, chemical structure and also *in vitro* data, to offer a workflow for transparent and effective communication. ToxPi *(http://comptox.unc.edu/toxpi.php)* and Chemical-Biological Read-Across (CBRA) software (*Low et al., 2013*) was used to

demonstrate low/no concern for toxicity in the category comparing data to the more toxic e-glycol ethers (*Figure 3.10*). Graphical presentation was suggested to be key in the low toxicity scenario to evidence observed effects for the assumed low toxicity compounds and compare them to the activities of known toxicants, as in the comparison between e- and p-glycol ethers.

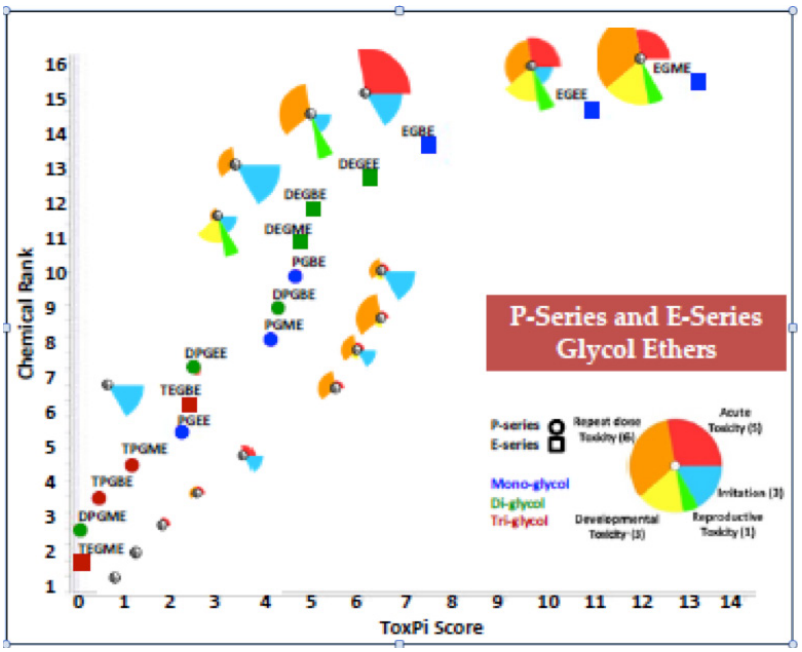


Figure 3.10 *ToxPI-based groupings for both the p-series (circles) and e-series (squares) of glycol ethers. The ToxPI images corresponding to individual chemicals illustrate similarity based on structure even across p- and e-series, indicating that the structural similarity is not sufficient evidence for reading across.*

The read-across would be reinforced by the *in vitro* biological profiles including all available endpoints to provide an overall view of possible biological activity and toxicity trends, and showing that *in vitro* trends would reproduce trends based on existing animal data. Chemical descriptors were also used to support the similarity between the components of the category. The experts at the October 2015 workshop stressed that besides biological profiles and trends, it would still be important at low toxicity to identify Molecular Initial Events and then to demonstrate through *in vitro* data whether effects were observed related to the expected initiated pathways, and at relevant concentrations. The overall summary including the evaluation of the improvement of the read-across arguments through the consideration of data from new *in vitro* and *in silico* methods is summarised in *Figure 3.11*.

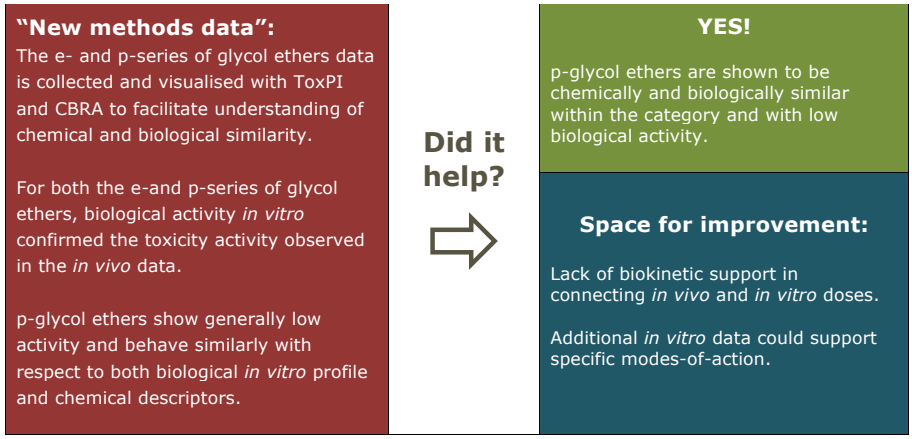
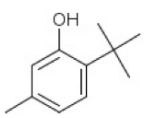
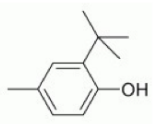
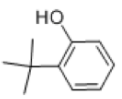
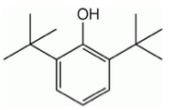
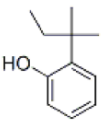


Figure 3.11 *Non-animal data and methods used in the read-across case study focussing on p-glycol ethers and qualitative evaluation of the approach.*

Scenario IVa: Distinguishing chemicals in a structurally similar category with variable toxicities based on Mode of Action hypothesis – alkyl phenols

This chemical category includes short-chained alkyl-substituted simple phenols, structurally similar with one or two alkyl groups with chain lengths of 1-5 carbons in different positions on the benzene ring in relation to the hydroxyl group (*Figure 3.12*).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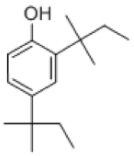	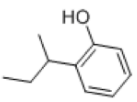	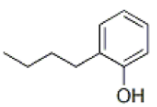	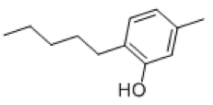	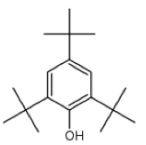
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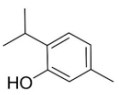	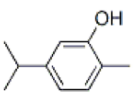	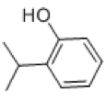	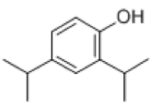	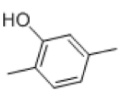
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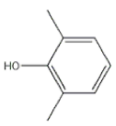
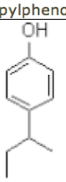
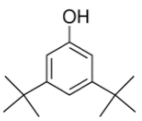
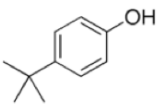
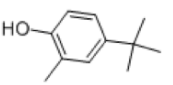
				
2,6-Dimethylphenol	4-*sec*-Butylphenol	3-*tert*-Butylphenol	4-*tert*-Butylphenol	4-*tert*-Butyl-2-methylphenol

Figure 3.12 *Category members of the case study focussing on alkyl phenols.*

In spite of structural similarity, the phenotypic expressions of repeated-dose toxicity weaken the read-across evidence between category members, as no structural trends are observed, but basically fall into two groups of different effect levels, one with NOEL around 12 mg/kg bw/d and the other with 100 mg/kg bw/d. Investigation is therefore suggested as to whether further sub-categorisation based on the position of the alkyl group in the phenolic ring would lead to two sub-categories where read-across could be strengthened by alternative data confirming predicted different mechanisms of action of the two sub-categories. The hypothesis is that altered metabolism of the hydroxyl group is the mechanistic rationale for sub-categorisation.

The results of the alkyl phenols are still in a phase of elaboration, and were not discussed at the October 2015 workshop.

Scenario IVb: Distinguishing chemicals in a structurally similar category with variable toxicities based on Mode of Action hypothesis – short-chained carboxylic acids

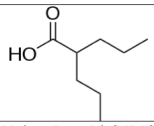
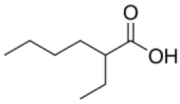
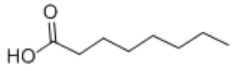
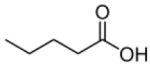
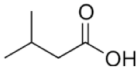
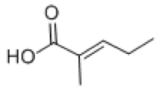
		
Valproic acid (VPA)	2-Ethylhexanoic acid	Octanoic acid
		
Valeric acid	2-Methyl butyric acid	trans-2-Pentenoic acid

Figure 3.13 *Category members of the case study focussing on short-chain carboxylic acids.*

Valproic acid (VPA) is a branched carboxylic acid which is known to induce liver steatosis in both humans and rodents. 10 biomarkers covering relevant toxic mechanisms related to liver steatosis, e.g. lipid and energy metabolism, metabolism of xenobiotics, endoplasmatic reticulum and general stress responses, were identified using transcriptomics data from the TG-Gates database *(http://toxico.nibiohn.go.jp/english/)*. The 10 biomarkers were strongly upregulated after VPA treatment. To test whether the biomarkers could be used to predict VPA-like hepatotoxicity, 11 analogues structurally similar to VPA, consisting of branched and unbranched carboxylic acids, were identified. For the six category members shown in *Figure 3.13*, historical *in vivo* data showed hepatotoxic activity. The other five did not cause any adverse effects in rat liver up to the highest tested doses. The 11 substances were then tested in the BAC-GFP toxicity pathway reporter platform developed within the DETECTIVE project, using human HepG2 cell lines. Although this HepG2 is not metabolic competent, three of the candidate biomarkers were identified as being predictive for the category members that had been identified as positive.

3.2.3 Concluding Remarks

A read-across assessment strengthened with alternative data must be based on a hypothesis of the mode-of-action of the chemical analogues or category. We need to define questions to verify the hypothesis and then produce targeted testing and modelling to answer the questions. ADME (Absorption, Distribution, Metabolism, and Excretion) properties for both source and target chemicals are essential for establishing a hypothesis, predicting relevant internal concentrations and interpreting data. Targeted *in vitro* testing should provide robust data using well-defined test systems (known specificity, variability and metabolic competence), relevant concentrations (predicted through biokinetic modelling), relevant exposure scenarios, and a

number of replicates statistically guaranteeing reproducibility. The test system should be fit for purpose, i.e. a simpler model might provide time- and cost-efficient results, while certain effects can only be observed in more complex multidimensional and co-cultured cell systems (e.g. to predict liver fibrosis). A chemical library with well-defined modes-of-action and known adverse outcomes would enable benchmarking of test systems for both positive and negative read-outs. The actual concentration available in a test system must be determined and related to the *in vivo* dose through *in vitro-in vivo* extrapolation, to enable comparison of data from different methods and to relate the data to a realistic external exposure. In the case of low toxicity, when specific target organ effects are difficult to detect, all endpoints should be evaluated and compared to identify trends and possible biological activity. Uncertainty must be evaluated in the existing animal data for the source substance, in each alternative method performed, and in the biokinetic assumptions.

Both the Scientific Committee on Consumer Safety (SCCS) *(http://ec.europa.eu/health/scientific_committees/consumer_safety/)* and the OECD Hazard Assessment Task Force indicated interest in the strategy and templates developed for read-across assessment (*Schultz et al., 2015*). In addition, ECHA, in collaboration with Cosmetics Europe and the JRC, applied their Read-Across Assessment Framework (RAAF; *ECHA, 2015*) to two of the **SEURAT-1** read-across case studies, the perfluoralkyl acids and the β-unsaturated alcohols, at the ECHA Scientific Topical Workshop in April 2016 (see section 2.3).

The EU-ToxRisk project *(http://www.eu-toxrisk.eu/)* is financed under the EU Horizon 2020 Framework Programme for Research and Innovation by DG RTD and started in January 2016. It offers case studies to assess repeated dose target organ toxicity and developmental and reproductive toxicity, with the opportunity to build further on experiences gained in the context of the **SEURAT-1** case studies (see section 5.4).

3.3 The *Ab Initio* Case Study for Safety Assessment

Elisabet Berggren, Gladys Ouedraogo, Alicia Paini, Andrea Richarz, Andrew White, Catherine Mahony

The **SEURAT-1** *ab initio* case study is an attempt to structure knowledge and data in a logical workflow that would be the basis for full risk assessment and a first integrated assessment relying only on alternative methods. This will demonstrate that there is a feasible way forward while also pointing out weaknesses and knowledge gaps, thus helping shape a more focussed strategy to advance alternative assessment approaches. The approach was also outlined as

a so-called 'Guided Educational Tour', developed to show how existing knowledge can be integrated and complemented with non-animal data to answer a safety assessment question. This tour was shown during the final **SEURAT-1** Symposium in Brussels on 4 December (see section 4.10.4).

Based on the **SEURAT-1** conceptual framework (see section 2.4.3), we have developed a general workflow to assist in risk assessment. We assume that the workflow starts from the same considerations regardless of the type of safety assessment, and only once it has been concluded that it is impossible to apply a Threshold of Toxicological Concern (TTC) or a read-across assessment; we continue to construct the logic on how to predict whether the intended exposure could be considered safe based on data solely from alternative methods. We also include uncertainty estimates for each step in the workflow.

The substance selected to illustrate the case study is piperonyl butoxide (PBO) in an imagined exposure scenario of it being a new ingredient introduced in a body lotion applied daily to the skin (overall body surface). The supportive alternative data (*in vitro* and *in silico* results) were obtained from ToxCast *(http://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data)* or generated using methods developed within **SEURAT-1** projects. PBO is a known hepatotoxin, even though the mechanism of action is unknown, and was therefore considered suitable as most methods developed within **SEURAT-1** were focussed on hepatotoxicity. It could thus provide relevant data for the assessment.

The complete case study will be described in a paper currently in preparation.

3.4 Threshold of Toxicological Concern Case Study for Safety Assessment

Chihae Yang, Mark Cronin, Elena Fioravanzo, Judith Madden, Andrew Worth, Stéphane Vidry, Andrea Richarz

The Threshold of Toxicological Concern (TTC) approach is a non-testing method that allows a preliminary risk assessment of chemicals based on the availability of reliable exposure information. It is an exposure threshold for chemicals, below which there is a low probability of an appreciable risk to humans. The concept was originally based on the efforts of the Center for Food Safety and Applied Nutrition (CFSAN), at the US Food and Drug Administration (FDA), to address the challenges in the safety assessment of food contact substances. When there are no data available for a target compound for use at low exposure, TTC can be a

pragmatic assessment tool. It is currently used for evaluation of flavouring agents by the FAO/WHO Joint Expert Committee on Food Additives, as well as for the evaluation of genotoxic impurities in pharmaceuticals and natural health products. There is increased interest in broadening the use of the TTC concept within the regulatory context. However, the fact that the original TTC approach is based on data from oral toxicity testing studies is a critical issue for applications with different exposure routes, as in the case of cosmetics. This concerns both the chemical space of the TTC concept developed from oral exposure studies as well as the extrapolation of exposure routes and related bioavailability differences.

The COSMOS project within the **SEURAT-1** Research Initiative has supported the evaluation and extension of the TTC approach to cosmetics ingredients and chemicals in formulations. In collaboration with ILSI Europe, COSMOS has set up two expert groups to deliver opinions and recommendations to guide COSMOS with these efforts. The two expert groups addressed two major tasks. The first Expert Group contributed to the creation of a new non-cancer TTC dataset of No Observable Adverse Effect Levels (NOAELs) with the aim to enrich the existing TTC dataset with cosmetics ingredients and chemicals used in cosmetics formulations. The majority of the NOAEL data in the COSMOS TTC dataset has been compiled from regulatory sources and stored in the COSMOS oral repeated dose toxicity database (oRepeatTox DB; see section 4.5.3.2). This work included the planning and execution of three different rounds of quality control through numerous study reviews by the external experts. The second Expert Group evaluated oral-to-dermal extrapolation and addressed the difference in exposure scenarios and bioavailability issues, such as dermal absorption and skin metabolism. Research into skin permeability has been supported by collation of skin permeability data, which were implemented in COSMOS DB, as well as the redevelopment of models for the calculation of permeability coefficients. A decision-making approach has been devised that comprises various steps to evaluate whether the TTC methodology can be applied to dermal exposure. A number of examples for use case scenarios for cosmetic-relevant materials were developed to illustrate the applicability of the approach (*Williams et al., 2016*).

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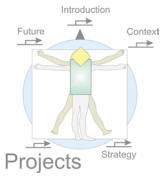

4 THE PROJECTS

"The characteristic of scientific progress is our knowing that we did not know."

Gaston Bachelard

4.1 Introduction

Tilman Gocht

This chapter provides a comprehensive overview of the achievements of the **SEURAT-1** Research Initiative projects, thus generating the backbone of the Annual Report. Although the focus from a cluster-level perspective shifted over time towards the case studies reported in chapter 3, one should keep in mind that the work programmes of the **SEURAT-1** projects were formulated independently of them. There was therefore much progress in the various projects outside of the **SEURAT-1** case studies, and this is reported in the subsequent sections.

Overall, the **SEURAT-1** Research Initiative was designed as a coordinated cluster of five research projects supported by a 'data handling and servicing project' and a 'coordination and support project' at the cluster level.

The following integrated projects formed the core of **SEURAT-1**:

- 'Stem Cells for Relevant efficient extended and normalized TOXicology' (*SCR&Tox*): Stem cell differentiation for providing human-based organ-specific target cells to assay toxicity pathways *in vitro;*
- 'Hepatic Microfluidic Bioreactor' (*HeMiBio*): Development of a hepatic microfluidic bioreactor mimicking the complex structure and function of the human liver;
- 'Detection of endpoints and biomarkers for repeated dose toxicity using *in vitro* systems' (DETECTIVE): Identification and investigation of human biomarkers in cellular models for repeated dose *in vitro* testing;
- 'Integrated *In Silico* Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimise Safety' (COSMOS): Delivery of an integrated suite of computational tools to predict the effects of long-term exposure to chemicals in humans, based on *in silico* calculations;
- 'Predicting long-term toxic effects using computer models based on systems characterization of organotypic cultures' (NOTOX): Development of systems biology tools for organotypic human cell cultures suitable for long-term toxicity testing, and the identification and analysis of pathways of toxicological relevance;
- 'Supporting Integrated Data Analysis and Servicing of Alternative Testing Methods in Toxicology' (ToxBank): Data management, cell and tissue banking,

- selection of ‘reference compounds’ and chemical repository.

Furthermore, a coordination action project was designed in order to facilitate cluster interaction and activities:

- ‘Coordination of projects on new approaches to replace current repeated dose systemic toxicity testing of cosmetics and chemicals’ (COACH): Cluster-level coordination and support action.

All of the projects started on 1 January 2011. The first Annual Report volume focussed on the plans and challenges of the various projects and the second and third contained first results from the research conducted within the **SEURAT-1** Research Initiative. This was continued in the fourth and fifth volumes and supplemented with the contribution of the projects to the **SEURAT-1** proof-of-concept case studies, which were drawn up in the meantime. In this final volume, we summarise achievements and provide an overview of the potential impacts of these achievements at the scientific and technological levels.

A section summarising meeting reports at the project and cluster levels follows the detailed project descriptions. Cross-cluster activities emerged increasingly over the lifespan of the **SEURAT-1** Research Initiative. The second volume of this Annual Report series described the *modus operandi* of cross-cluster Working Groups as the central elements for facilitating cooperation between projects and people. The third and fourth volumes focussed on the development of a **SEURAT-1** roadmap as a tool to monitor progress towards the achievement of the cluster-level objectives. This was extensively reported and is briefly summarised and updated in this final volume, and further supplemented with an outline of the ‘**SEURAT-1** Tools and Methods Catalogue’, which should, together with the research data stored within ToxBank, become a sustainable resource for future research programmes operating in the field of predictive toxicology. The Working Groups played a major role in efforts to provide a blueprint for future implementation of mechanism-based integrated toxicity testing strategies in modern safety assessment approaches based on case studies, demonstrating how far we can move away from the existing *in vivo* toxicity testing paradigm, and short activity reports by the Working Groups supplement this section. In total, the following six Working Groups were active during the lifetime of the cluster: (i) the Gold Compounds Working Group, (ii) the Data Analysis Working Group (these two have been active since the beginning of the **SEURAT-1** Research Initiative), (iii) the Mode-of-Action Working Group, (iv) the Biokinetics Working Group, (v) the Stem Cells Working Group and (vi) the Safety Assessment Working Group. All Working Groups were made up of members from different projects, enabling targeted discussions on the needs and contributions of the **SEURAT-1** research projects to meet the cluster-level objectives.

The most important event in the last year was the **SEURAT-1** Final Symposium, which took place in Brussels on 4 December 2015. In total, 120 researchers, regulators, EU officials and industry representatives in the alternative methods field came together and focussed on

the presentation of the main results in various formats. The highlights from the projects were communicated in oral presentations as well as in an exhibition. Furthermore, a guided tour showcased how the new tools and methods can be applied to answer a safety assessment question. Additionally, a film summarising the **SEURAT-1** strategy and achievements from the scientific projects in plain language was screened to the audience. These activities are reported in a separate section, which is followed by the final section describing other outreach activities during the final year of the **SEURAT-1** Research Initiative. The central aspects here are: organisation of and participation in workshops (with a strong focus on the implementation of the scientific tools developed into regulation); dissemination activities at conferences; the **SEURAT-1** public website; and the adoption of a dissemination strategy aimed at increasing coverage of **SEURAT-1** activities in mass media, which includes the involvement of professional journalists and the establishment of an internal Editorial Review Board. Besides the Annual Report, these activities were the most important cluster-level tools to promote the dissemination of knowledge.

4.2 SCR&Tox: Stem Cells for Relevant efficient extended and normalised TOXicology

Marc Peschanski on behalf of the SCR&Tox consortium

4.2.1 Executive Summary

The need for a profound shift in the way toxicology testing is carried out for chemicals in the pharmaceutical and cosmetic industry is clearly acknowledged by all, in the industry and academia as well as in institutional bodies. The extrapolations – across species, from high test doses to low exposures, and from descriptive endpoints in animals to their possible human correlates – are handicapped by the lack of underlying mechanistic information. A number of expert reports and publications have called for re-orienting testing to the molecular level, highlighting the concept of 'toxicity pathways' within human cells that would be triggered by a toxicant exposure at a low dose that by itself does not provoke major cell toxicity but induces changes in cell homeostasis to cope with the phenomenon.

The ***SCR&Tox*** consortium has been formed in order to evaluate the value of human pluripotent stem cell lines for elaborating assays of toxicity pathways that meet all challenges of the new strategy. The aim of the ***SCR&Tox*** programme was to provide biological and technological resources needed to assay toxicity pathways *in vitro* and to demonstrate on industrial platforms that these resources could be reliably and robustly implemented at the required scale.

The first part of the ***SCR&Tox*** Project was dedicated to the provision of biological resources required for assaying toxicity pathways. Human embryonic stem cells and induced pluripotent stem cells have been harnessed. Their use requested mastering and orienting their major attributes – self-renewal and pluripotency. Cell lines were banked at the undifferentiated stage, and new technologies, including automation, developed in order to obtain ES and iPS cell lines optimised for use in standardised assays. Protocols were then designed for differentiating pluripotent cells into derivatives for each of 5 toxicological relevant lineages (Liver, CNS, Heart, Skin, Muscle).

The second part of the ***SCR&Tox*** Project provided all technologies and methodologies required for developing efficient cell-based assays seeking quantitative assessment of

biomarkers associated to toxicity pathways. This involved validating the applicability on pluripotent stem cells derivatives of a multi-parametric array not only of available techniques analysing molecular components but also of newly designed methods for exploring functionally cells responses to potential toxicants, with special mention to bioelectronics. Optimisation of the biological resources to their use for industrial-scale assays additionally required genetic engineering.

The third part of the project explored toxicity pathways 'on the bench', making use of the obtained biological and technological resources. The existence and reliability of toxicity pathways in the biological resources developed from pluripotent stem cells were demonstrated. Then, drug toxicity assays were designed and tested in academic-scale conditions. This involved the discovery of the protein components of the toxicity pathway and how the pathway is altered by test chemicals. Implementation of prototype assays was carried out and demonstrated the value of the resources developed by the consortium. Unfortunately, due to delays introduced by the inappropriate withdrawal of the key partner in the task, the last objective of that part, i.e. prevalidation of the assay and documenting test methods according to ECVAM criteria, fell beyond the life time of the network.

The fourth and last part of the ***SCR&Tox*** Project aimed at operating the promotion of the selected cell-based assay up to the industrial scale. The transfer of the technologies of the assay developed on the bench was performed toward use on appropriate platforms for industrial-scale implementation through establishment of all standard operating procedures (SOP) and associated instructions for biological resources. Protocols were then adapted to the industrial platforms, with particular emphasis on miniaturisation and standardisation. Unfortunately, the demonstration of the robustness, specificity and sensitivity of the prototype assay for testing toxicology could not be fully performed within the lifetime of the ***SCR&Tox*** programme.

4.2.2 Project Context and Objectives

The need for a profound shift in the way toxicology testing is carried out for chemicals in the pharmaceutical and cosmetic industry is clearly acknowledged by all, in the industry and academia as well as in institutional bodies. Change is inevitable because the current system is not based on fundamentally sound science, but rather on descriptive data from high dose animal tests. The extrapolations – across species, from high test doses to low exposures, and from descriptive endpoints in animals to their possible human correlates –are handicapped by the lack of underlying mechanistic information. Although this has been often instrumental in the past, it has also shown sometimes clearly unreliable. In addition, our current approach is too expensive and too slow, capable of only limited throughput.

A number of expert reports and publications have called for re-orienting testing to the

molecular level, highlighting the concept of 'toxicity pathways' within human cells that would be triggered by a toxicant exposure at a low dose that by itself does not provoke major cell toxicity but induces changes in cell homeostasis to cope with the phenomenon. Evaluation of toxicants calls, therefore, for new models to be created that will allow assessing toxicity pathway responses *in vitro*, that will deliver a more accurate profile of acute toxicity in humans and possibly also reveal more subtle chronic toxic contraindications. Implementation of this new strategy based upon *in vitro* tests requires the most relevant and reliable model systems, which should also be robust and scalable in order to be instrumental at an industrial scale.

The ***SCR&Tox*** consortium has been formed in order to evaluate the value of human pluripotent stem cell lines for elaborating assays of toxicity pathways that meet all challenges of the new strategy, from the most basic issues on mechanisms of differentiation up to the demonstration of normalised assays on industrial-scale platforms and validation. It has used fully the diversity and versatility offered by those cell lines, for analysis of multiple cell phenotypes (in 5 different organs of interest for toxicology), multiple conditions of exposure (single *vs.* repeated – low *vs.* high doses) and multiple approaches (both in terms of analyses, in particular with 'functional '-omics', and engineering to optimise and standardise). Overall, the aim of the ***SCR&Tox*** programme was to provide biological and technological resources needed to assay toxicity pathways *in vitro* and to demonstrate on industrial platforms that these resources could be reliably and robustly implemented at the required scale.

The first part of the ***SCR&Tox*** Project was dedicated to the provision of biological resources required for assaying toxicity pathways. Human embryonic stem cells and induced pluripotent stem cells have been harnessed. Their use requested mastering and orienting their major attributes – self renewal and pluripotency – toward their specific use in testing toxicity of chemicals, namely their diversity of origins, scalability at the undifferentiated stage and pluripotency in order to create the conditions for production in needed quantity and quality.

The first objective was to provide cell lines at the undifferentiated stage, with emphasis on a diversity of donors relevant to analysis of the impact of genetic polymorphism on toxicity assays. Diversity is a major advantage of pluripotent stem cell lines, as it allows exploring impact of genetic polymorphisms on the responses to chemical in human. The first task of the programme was to master that capacity by managing banking of the many cell lines deemed necessary for the project. Procedures for amplification and quality control of hES cell lines were well established and banking of cells at the undifferentiated stage were straightforward. This was not fully the same for iPS cell lines. First, a small set of induced pluripotent stem cell lines was available at the start of the programme, in order to explore iPS capacities in parallel to hES. Then, newly developed technologies were used to obtain iPS cell lines optimised for use in standardised assays.

A second objective was to establish conditions for scaled-up production of large amounts of pluripotent cells at the undifferentiated stage. Scalability is a major advantage of pluripotent

stem cell lines since they can be amplified without ever entering into a senescence process. The consortium aimed at developing culture systems that would allow growing cells in flasks in a scalable way and at implementing conditioned media or more defined culture media for feeder cell replacement.

A third objective was to identify protocols for differentiating pluripotent cells into derivatives – both at a 'full' terminal stage and in applicable cases at an intermediate, amplifiable stage – for each of 5 toxicological relevant lineages (Liver, CNS, Heart, Skin, Muscle). Pluripotency is a main attribute of ES and iPS cell lines: cells are theoretically capable of providing any cell phenotype at any stage of differentiation, with the only qualification that they spontaneously do so in a stochastic non selective way, i.e. they differentiate into multiple cell phenotypes at the same time if left undirected. Specific protocols have been designed and implemented in order to obtain specifically the cell phenotypes of interest.

The fourth and last objective in the first part of the programme was to make use of the technologies and methodologies described above in order to provide cells ready, quantitatively and qualitatively for toxicology pathway assays in dedicated formats for direct use in different assays.

The second part of the ***SCR&Tox*** Project aimed at providing all technologies and methodologies required for developing efficient cell-based assays seeking quantitative assessment of biomarkers associated to toxicity pathways. This involved validating the applicability on pluripotent stem cells derivatives of a multi-parametric array not only of available techniques analysing molecular components but also of newly designed methods for exploring functionally of cells responses to potential toxicants. Optimisation of the biological resources to their use for industrial-scale assays additionally required genetic engineering and new developments compatible with the overall objective of standardisation.

The first objective of this second part was to implement classical '-omics' for gene and protein expression analysis on ES and iPS cells-derivatives in order to demonstrate the value of the biological resources for exploring signalling pathways. Large-scale evaluations of the status of gene expression and protein concentrations in cells has allowed us to understand the integrated biologic activities and to catalogue changes after *in vitro* treatment with toxicants. Gene and protein profiling have been adapted to pluripotent stem cells derivatives in order to define capacities and limitations of those technological resources applied to the test cells.

A second objective was a search for scalable techniques applicable to pluripotent stem cells derivatives that would allow dynamic identification of genes involved in signalling pathways triggered by toxicants, including (i) functional genomics based upon large-scale screens for siRNA or cDNA, (ii) functional proteomics with particular emphasis on protein-protein interaction, and (iii) bioelectronics. Functional approaches are especially useful in addition to classical '-omics' for seeking and characterising toxicity pathways, and the consortium has set-up and characterised value and limitations of such approaches. Functional analysis used high-

throughput methods that permitted automation of cell-based assays with libraries of cDNAs and siRNAs. Functional proteomics was explored for complementary data. Last, an important effort was put on bioelectronics that allowed us to evaluate electrogenic changes associated to exposure to toxicants in cells that are physiologically electro-active like cardiomyocytes, muscle cells or neurons in microarrays and microcavity arrays.

A third objective in this second part was to develop homologous recombination and protein transfer for optimising biological resources to be implemented in large-scale screens, focusing on newly developed tools, endonucleases. This aimed at engineering cells to make them most adequate for use on industrial HTS platforms. Engineering of the cells to be tested for toxicant effects using specifically designed tools would allow optimising the assays by providing cells with purposely chosen properties that make them more fitted with test requirements.

A fourth objective of this second part of the project was to ensure 'clean' reprogramming of somatic cells in order to create iPS cell lines devoid of any remnant of the transgenes originally used for triggering stemness. Clean protocols for the reprogramming of iPS cells were deemed an absolute requirement for the implementation of those cell lines in standardised assays for industrial purposes.

Similar to the last objective of the first part of the project, the last one in the second part was the production of ready-to-screen cells for further use in the programme after differentiation, thorough quality control and documentation. This prepared for direct use of these biological resources in subsequent steps toward industrial-scale testing. Besides the actual production of the most relevant cells in terms of quantity and quality, particular emphasis was placed in the control of the relevance of these ready-to-screen cells with regards to the requirements of high throughput screening, i.e. demonstrate responses to tests that are robust, sensitive and specific.

The third part of the project explored toxicity pathways 'on the bench'. Starting at half-term of the programme, this part aimed at making use of the obtained biological and technological resources for developing 'at bench scale' at least one cell-based assay of a toxicity pathway. After checking for relevance, i.e. observing the test signalling pathway in pluripotent stem cells derivatives, a test assay was to be designed and implemented at low scale, up to results allowing prevalidation.

The first objective was the demonstration of the existence and reliability of toxicity pathways in the biological resources developed from pluripotent stem cells. Relevance of the prepared resources for analysing toxicity pathways was planned through the identification of the signalling pathways of interest in cells to be tested, and their triggering by a known toxicant in conditions that are compatible – particularly in terms of dose and duration of exposure – with those of an efficient assay.

A second objective was to design at least one assay to be tested further 'on the bench' (in

academic-scale conditions), based upon one identified toxicity pathway and associated biomarkers in pluripotent stem cells derivatives. Design of a prototype assay was planned upon the computed model defining the toxicity pathway, its biomarkers and endpoints as observed in the available derivatives of pluripotent stem cells. This involved the discovery of the protein components of the toxicity pathway and how the pathway is altered by test chemicals. On that basis, the project implied engineering specific gene constructs that would allow visualising and measuring in quantitative manners any perturbation elicited by toxicants in the toxicity pathway of interest.

A third objective was the implementation 'on the bench' of the designed assay for exploring the targeted toxicity pathway. Implementation of the prototype assay at academic scale was seen as a most determinant step to prepare for transfer of the technology to industrial HTS platforms.

The last objective aimed at ensuring pre-validation of the assay and documenting test methods as implemented 'on the bench' according to ECVAM criteria for validation under good laboratory practice (GLP) conditions. Pre-validation and normalisation of the assay "on the bench" by the specialist partner (ECVAM) was envisioned as the final step before implementation on industrial-scale platforms. There was clearly no time for a full validation of one prototype assay within the framework of the 5 year long programme, but we thought that there could be enough time for pre-validation and prospective normalisation on the basis of the results of the 'assay on the bench'.

The fourth and last part of the ***SCR&Tox*** Project aimed at operating the promotion of the selected cell-based assay up to the industrial scale. This required technology transfer as well as methodological adaptation and refinement in order to reach the proof-of-concept that pluripotent stem cells derivatives can show instrumental for testing a toxicology pathway in a relevant, efficient, extended and normalised assay on an industrial platform.

The first objective was the transfer of the technologies of the assay developed 'on the bench' toward use on appropriate platforms for industrial-scale implementation through establishment of all standard operating procedures (SOP) and associated instructions for biological resources. Technology transfer to industry platforms was a major endeavour of the consortium as a whole, since it required combining all expertise and using biological and technological resources obtained during the Project. This in particular implied establishment of standard operating procedures and instructions requested for provision of the biological resources. All characteristics of the biologic material in industry conditions were determined using basic parameters allowing for formal 'cell batch releases'. Quality Controls were assessed in parallel, providing for formal 'quality control reports'.

A second objective was to refine protocols designed 'on the bench' in order to adapt them to the industrial platforms, with particular emphasis on miniaturisation and standardisation. Adaptation and refinement were required for all analysis methodologies in order to make

them suitable for a large-scale application. Flexibility cannot exist in industrial platforms that are entirely based upon robotic systems which will strictly deploy exact protocols. Transfer of the assay successfully developed in an 'academic' set-up to the industrial robotic platform, therefore, required specific adaptation and refinement.

The third and last objective of this fourth part was the demonstration of the robustness, specificity and sensitivity of the prototype assay for testing toxicology, with particular emphasis on reproducibility of results in assays exploring repeated dose applications of potential toxicants. Demonstration of the reliability and effectiveness on industrial platforms of at least one assay was the last objective of the ***SCR&Tox*** Project. In order to be successfully reached, it would have required the full development of the prototype assay based upon an engineered cell line that could be transferred to the industrial platforms.

4.2.3 Main Achievements

4.2.3.1 Biological Resources

Banking of Undifferentiated hPSCs

Human pluripotent stem cells offer a unique opportunity to establish *in vitro* human cell culture models of *in vivo* tissue cells of any type. The availability of quality controlled hESC and iPSC lines to meet the needs of ***SCR&Tox*** partners and more widely for **SEURAT-1** partners is valuable to ensure that partners can work from the same reference seed stocks of research cell lines which have met agreed quality control (QC) and characterisation criteria.

Whilst an individual hPSC lines can provide useful tools for toxicology studies, variation has been observed between pluripotent cell lines, both within and between groups of hESC and iPSC lines. In addition, it is also highly valuable to have multiple lines generated by similar technologies available to enable selection of lines with the best capabilities for development of representative human cell-based systems for toxicology research. The use of techniques which do not involve integration into the genome, in theory avoiding genetic disruption, are considered important to provide cell systems that will provide responses most close to responses seen in native tissue cells.

This task comprised banking of caucasian and three non-caucasian hESC lines, all of which passed standard QC testing and banking of hiPSC lines ('classical' and 'clean'). All banking was completed according to partner needs following circulation of a questionnaire to partners. Generation of a reference hiPSC line for comparison of protocols was also performed.

Low passage stocks of 'clean' iPSC lines were established using 3 different state of the art non-integrating reprogramming techniques (episomal vectors, modified mRNA transfection and co-transfection with miRNA and modified mRNA). These lines were banked and stocks

subjected to quality control and characterisation testing including expression of surface markers (TRA-160, TRA 1-80, SSEA-4&1), expression of self-renewal genes, a pluripotency assay, karyology, mycoplasma and sterility testing. Safety testing for serious human blood born pathogenic viruses was also completed.

A subset of these lines was subject to extended characterisation in order to explore requirements of reference cells. This included additional analysis of pluripotent capacity by expression of lineage commitment genes and preliminary studies of response of the lines to a generic toxic challenge (hydrogen peroxide). Extended pluripotency assays showed a subtle range of lineage commitment profiles amongst the lines although all were characterised as 'pluripotent' by routine methods applied to QC of cell banks. One line NIBSC 7 produced by the mi/mRNA method used, was closer in profile to the 'reference' hESC line (H9) than the others. Further studies of this group of lines sought their response to oxygen radical challenge by analysing expression of genes associated with apoptosis, cell cycle state and nrf-2 expression. Some inter-line variability was observed.

Amplification of Quality-Controlled Undifferentiated Stem Cells

One of the main objectives of the ***SCR&Tox*** Project was to build the necessary biological resources needed for supporting the second part of the work programme. This included the mass cell production (automation) of undifferentiated pluripotent stem cells, as an important challenge for the final transfer of an assay on industrial platforms that may eventually require billions of cells to be produced from each cell line.

Optimal conditions for mass cell production of human pluripotent cells have been established and working cell banks of undifferentiated were produced. This has included the development of the necessary conditions for cell cultures of PSCs (either hESCs or iPSCs), automated at all the stages: culture, amplification and freezing. Cellartis DEF-CSTM feeder-free culture system has been successfully applied for the proof of principle production of feeder-free cell banks of hES cell lines. The feeder-free culture system has furthermore been tested for the expansion of hiPS cell lines.

A protocol has been established to amplify IPS cells in single cells and feeder free culture conditions on the CompactSelect automated cell culture platform. The amplification process allowed 130,000-fold amplification in 22 days. Those IPS cells were used to implement automated differentiation into mesodermal progenitor cells, also on the CompactSelecT platform. After 16 days (4 automated passages) and 260 fold amplification, Mesodermal progenitor cells conserved their characteristics and could be amplified 600-fold during 12 days, giving rise to a theoretical master cell bank of 2,000 cryotubes, at 10^6 cells/mL.

A fully automated system has been developed for cryopreservation and banking of large batches of pluripotent stem cells, while controlling several cryobiological parameters. This

technique allows the generation of consistent and standardised cryopreserved batches of cells, as checked by post-thawing quality control. The Cryomed system allows freezing by injecting liquid nitrogen in controlled conditions. The automation and distribution of cell cultures is possible by using the Fill-It robot, this module is capable to distribute a suspension of cells mixed to a cryoprotector into 96 vials, simultaneously and in less than 30 seconds.

QCs have been especially studied in order to obtain a set of tests that both comprehensively explore all relevant parameters and are in parallel workable within the framework of an industrial use. The following *Figure 4.1* and *Table 4.1* summarise the results.

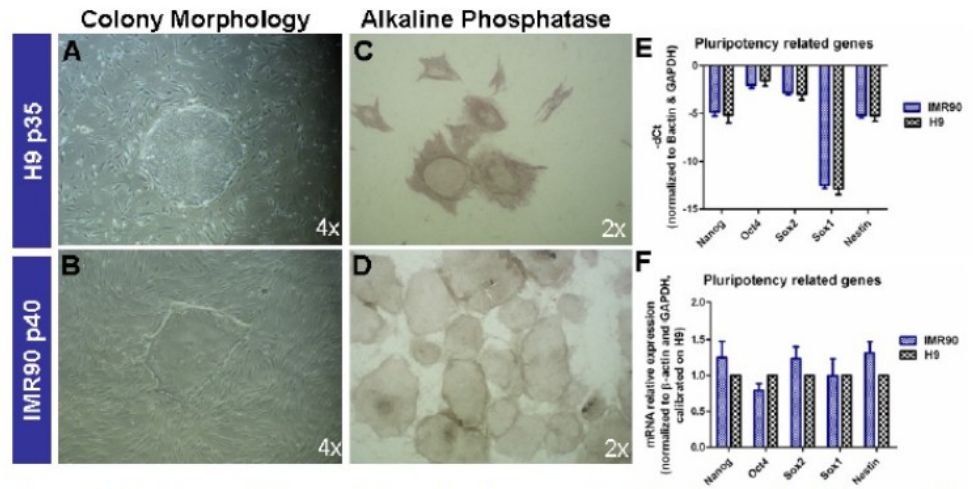

Figure 4.1 *Colonies morphology, alkaline phosphatase and qPCR analyses.* ***(A, B)*** *Representative phase-bright images of undifferentiated colonies (H9 p35 and IMR90 p40) (4x magnification;* ***(C, D)*** *representative images of AP stained colonies (same samples (2x magnification);* ***(E, F)*** *Bar graphs reporting qPCR analyses of Nanog, Oct4, Sox2, Sox1 and Nestin, normalised to B-actin and GAPDH (-DCt, in* ***E****) and of the same genes normalised to B-actin and GAPDH and then calibrated to undifferentiated H9 cells (DDCt method, in* ***F****). Mean of 3 independent analyses ±S.E.M.*

Table 4.1 *Quality control analyses, expected results and proposed preliminary thresholds for undifferentiated PSCs (hiPSCs compared to hESCs, H9) characterisation.*

Type of Analysis	Expected Results			Preliminary Threshold Values
Analysis of Colonies Morphology by cell microscopy and digital photography	Colonies should be round shape, large nucleolus and not abundant cytoplasm; flat, tightly-packed colonies			≥ 80% NB: when passaging, discard of morphologically differentiated colonies has to be performed
Alkaline Phophatase (AP) (with BCIP/NBT)	Undifferentiated colonies should be positive for AP activity			≥ 80%
IC analyses (qualitative analysis and HC-imaging platform, Cellomics)	Undifferentiated colonies should be positive for		Oct4 SSEA3 Tra1-60 Sox2	≥ 80% ≥ 80% ≥ 80% ≥ 80%
Flow Cytometric analyses	Undifferentiated cells should be positive for:		SSEA4-Alexa647 Tra-1-81-PE	≥ 80% ≥ 80%
	Undifferentiated cells should be negative for:		SSEA1(CD15)-Pacific Blue	≤ 5%
RQ-PCR analyses, using the ΔΔCt method. Housekeeping genes: GAPDH and Bactin	Significantly high expression levels of pluripotency related genes, as compared to undifferentiated H9:		Nanog POU5F1 (OCT4) Sox2	ΔCt ≤ 6 ΔCt ≤ 3 ΔCt ≤ 3.5
	Significantly low expression levels of ectoderm related genes, as compared to undifferentiated H9:		Nestin Sox1	ΔCt ≥ 6 ΔCt ≥ 13
TaqMan Human STem Cell Pluripotency Array (Applied Biosystem) using the ΔΔCt method. To be run every 10 passages	Undifferentiation/pluripotency related genes should result significantly expressed Significance is calculated using a 1-tailed t-test paired t-test comparing undifferentiated H9 cells at different passages and IMR90 cells at different passages vs. the H9-lowest available passage number. Germ layer-specific genes should be undetectable or not significant. Significance is calculated by using a 1-tailed paired t-test comparing undifferentiated H9 cells at different passages and IMR90 cells at different passages vs. the H9-lowest available passage number			
EBs formation (analysis at day 0/2/4/6/10/14): RQ-PCR analysis of 3-germ layers related genes, using the ΔΔCt method ($2^{-\Delta\Delta Ct}$).	Comparing EBs at day6 to undifferentiated call (day0), germ layer specific genes should result significantly expressed.	Ectoderm related genes	Sox1 Pax6 Nestin	$2^{-\Delta\Delta Ct}$ ≥ 3 (at day 6) $2^{-\Delta\Delta Ct}$ ≥ 10 (at day 6) $2^{-\Delta\Delta Ct}$ ≥ 2 (at day 6)
		Endoderm related genes	α-fetoprotein (AFP) Cytokeratin 18 (KRT18)	$2^{-\Delta\Delta Ct}$ ≥ 400 (at day 6) $2^{-\Delta\Delta Ct}$ ≥ 4 (at day 6)
		Mesoderm related genes	Brachyury (T) Atrial natriuretic factor gene (NPPA)	$2^{-\Delta\Delta Ct}$ ≥ 40 (at day 6) $2^{-\Delta\Delta Ct}$ ≥ 15 (at day 6)

Differentiation Protocols

SCR&Tox aimed to develop protocols for the differentiation of hPSCs to provide models of five different cell types (hepatocytes, neuronal cells, cardiomyocytes, keratinocytes and muscle precursor cells) that have significance for screening in drug safety testing and a variety of other applications. Differentiation protocols were successfully developed for models of these five different cell types.

In the case of protocols to establish models of hepatic cells, protocols were developed for hESCs grown on feeder cells and feeder-free and were optimised to provide robust differentiation of a range of hiPSC lines into high purity (>90%) functional hepatocyte like cells. Representative examples of the four successive phases of differentiation observed *in vitro* along the hepatocytic lineage are shown in *Figure 4.2*.

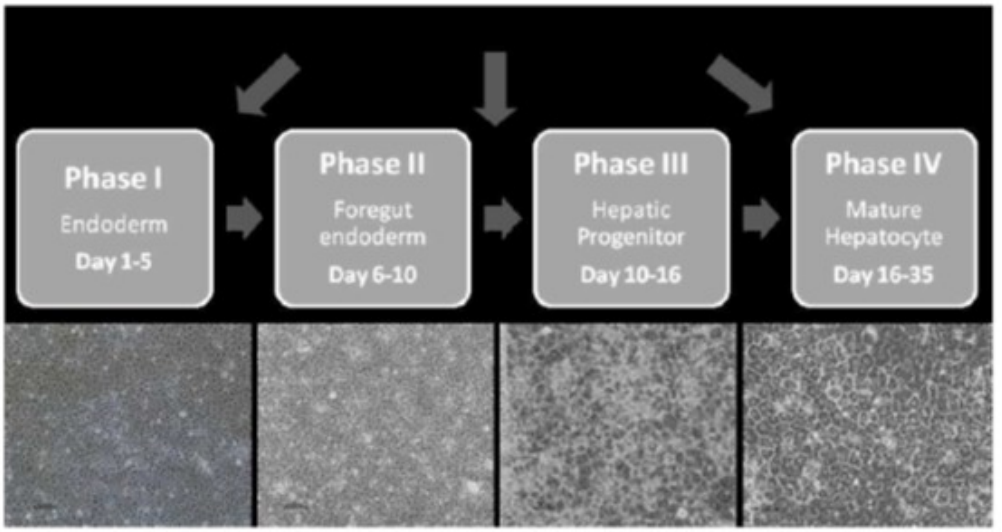

Figure 4.2 *Differentiation of hiPSC into functional hepatocyte-like cells.*

Neuronal differentiation protocols were developed for pre- and post-mitotic neurones (*Figure 4.3*) and also specialised neuronal cell types. These appear scalable for industrial application in drug screening. The generation of pure cultures of postmitotic neurons remains challenging. Nevertheless, it is also important to note that in the human brain there is a heterogeneous population of different types of neurons and supporting glial cells, and for this reason mixed populations of cells better represent the *in vivo* situation and may provide valuable complex culture systems which can be exploited in the future.

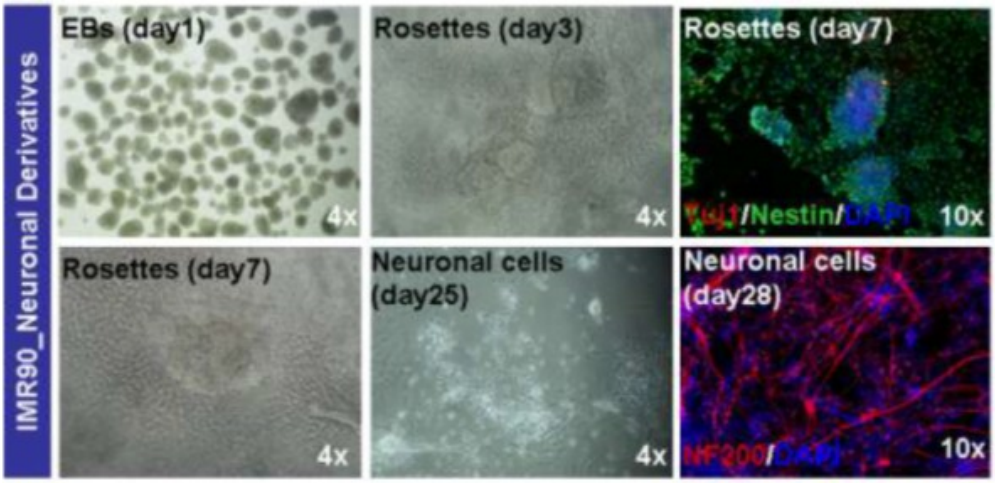

Figure 4.3 *Representative phase bright images showing the differentiation protocol for iPSC-derived post-mitotic neurons.*

The progress reported in the development of purified ventricular cardiomyocytes could provide a means of delivering significant improvements in these cell types, and also provides a potentially valuable approach for a range of other cell types. The illustrations in *Figure 4.4* demonstrate the successful differentiation of iPS cells into ventricular cardiomyocytes, using a 3D cluster technique.

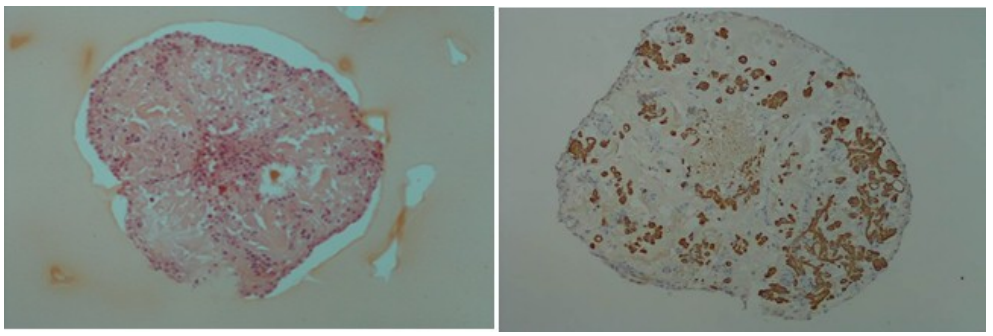

Figure 4.4 *Differentiation of iPS cells into ventricular cardiomyocytes (left: H&E staining; right: immunohistochemistry staining for myosin).*

The feeder-free protocol for generation of basal keratinocytes reported within the framework of the programme will facilitate the industrial application of this cell type in drug screening. A representative example of an epidermis reconstructed *in vitro* is shown in *Figure 4.5*.

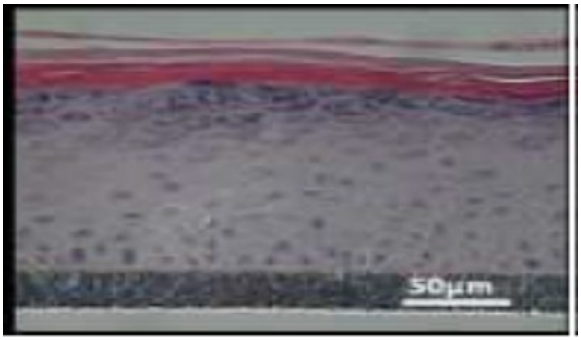

Figure 4.5 *Epidermis reconstructed in vitro following seeding of basal keratinocytes differentiated out of hES cells on a synthetic matrix.*

Homogenous populations of mesodermal precursor cells of the myogenic lineage can also now be produced at scale from both hESC and iPSC lines. This protocol enables the differentiation of hESCs into a homogeneous population of mesodermal progenitors after 3 weeks of culture on a gelatin coating dishes and in an appropriate medium. In order to improve the yield of this process, we proceeded in several optimisations of the culture media. Notably, the addition of two cytokines (FGF2 and ascorbic acid 2-phosphate) significantly improved the rate proliferation of the progeny. In the optimised conditions, the obtained population presents a doubling time of approximately 30 hours and can be maintained for more than 35 cumulated divisions. This protocol was successfully transposed to iPSCs.

Production of Ready to Use Cells for Toxicity Testing

Automated production of early stage mesodermal and neural stage precursor cells from cryopreservable hPSC suspension cultures has been established. For early mesodermal precursor cell expansion a 1000-fold expansion was achieved over 16 days whilst retaining stable phenotypic marker expression levels. The automation of early neural precursor expansion could be initiated from cryopreserved suspension cultures of hPSCs.

The scalable automated production of late neural precursors from hESCs was also established, and furthermore, automated differentiation into motor neuron progenitor phenotypes was shown to be at least as efficient as standard manual methods. In this case the capability to monitor the differentiated cell state was also established using an hESC line transfected with a reporter gene activated in the motor neuron lineage.

Scalable production of fully differentiated hPSCs was established for neural and hepatic cell types. The hepatic system was reported to have achieved T225 flask (225cm3 culture surface) scale.

Automation to set up 96 and 384 multi-well micro-plates of single genotype early stage cells was achieved for mesodermal precursor cells using the Bravo (liquid handling)/BenchCel (plate manipulation) system. Process optimisation for cell seeding achieved significant improvement in inter-plate consistency. This system also provided culture stability for 2 weeks. A system for automated manufacturing single genotype 96 well plates of late stage neural cells from hESCs was also developed in the CellHOST system yielding cells with evidence of neuronal functional maturation.

Automated preparation of multiple genotype plates of early stage cells were also successfully established for mesodermal precursor cells and neural precursor cells in the Bravo/BenchCel and CellHOST systems respectively. Both systems were demonstrated with multiple hESC and iPSC lines processed simultaneously. The mesodermal system showed stability of the final cell types for two weeks indicating capability for repeat dose toxicity testing.

Altogether, this part of the work programme has provided scalable and automated manufacturing systems for industry scale studies of toxicity in mesodermal, neural and hepatic models. Both single and multiple cell lines can be handled automatically and in the case of the neural cell system this is achievable from thawing of a source vial of cells to harvesting or testing fully differentiated cells. In addition, it has provided comparative experience in use of a number of automation systems.

Another important outcome from this work is that it provided potential new technical procedures which could accelerate progress towards industry ready protocols. In particular, it has been demonstrated that undifferentiated hPSC lines can be expanded and cryopreserved as spherical aggregates in suspension and in addition that neural precursors can be preserved successfully and used in an automated system. These methods could provide important opportunities for more flexible planning of the manufacturing process and enable shorter assay timelines for industry users. In addition, stability demonstrated in one system and qualified over 2 weeks, paves the way for systems that will enable evaluation of repeat dose toxicity testing.

4.2.3.2 Technological Resources

Profiling and Functional Characterisation of Test Cells

Besides the biological resources described above, the ***SCR&Tox*** consortium aimed also at providing all technologies and methodologies required for developing efficient cell-based assays seeking quantitative assessment of biomarkers associated to toxicity pathways.

The first task aimed specifically at providing the ***SCR&Tox*** consortium with technologies for profiling cell and gene expression, as well as to explore the cell functions. The task was further divided into three groups, namely the

➠ characterisation of gene and protein profiles and functional analysis of stem cell derivatives;

➠ design and implementation of functional genomic and proteomic methodologies to analyse dynamically stem cell derivatives;

➠ characterisation of electrogenic properties of neural and cardiac stem cells derivatives.

Implementation of Transcriptomics

Regarding the task to design and implement technologies for defining cell profiles of gene expression, corresponding to the differentiation of definite endoderm cells and hepatic progenitors in three-dimensional cultures, the findings in this study suggested that directed differentiation of DECs and HPs in dynamic 3D perfusion culture provides a promising approach to effectively derive functional hepatic cells. In contrast to conventional 2D culture, the 3D perfusion culture systems induced more functional maturation to hESC-derived hepatocytes (*Figure 4.6*).

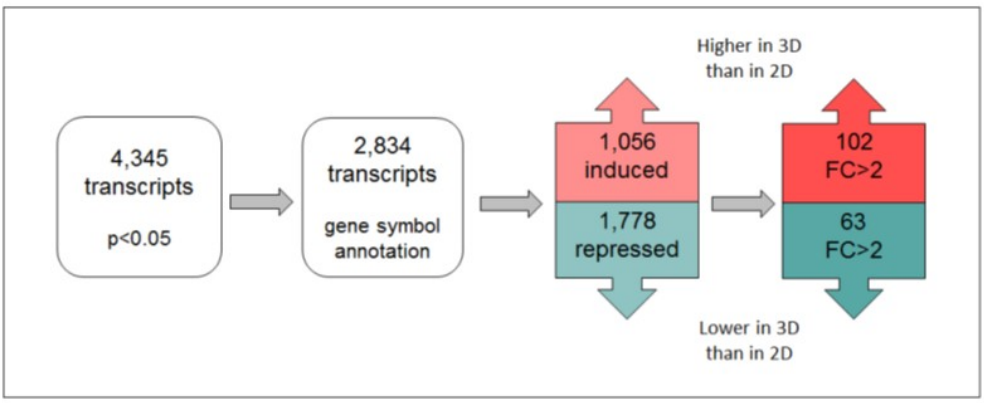

Figure 4.6 *Induced and repressed genes in 3D versus 2D.*

Regarding hepatotoxicity, we have also investigated the viability, steatosis, phospholipidosis, and changes in gene expression of spheroid cultures of primary human hepatocytes following chronic dosing (7 and 14 days) with aflatoxin B1, amiodarone, acetaminophen (APAP), chlorpromazine, troglitazone and ximelagatran (*Figure 4.7*). Further it has been compared with HepaRG and hIPS-Heps.

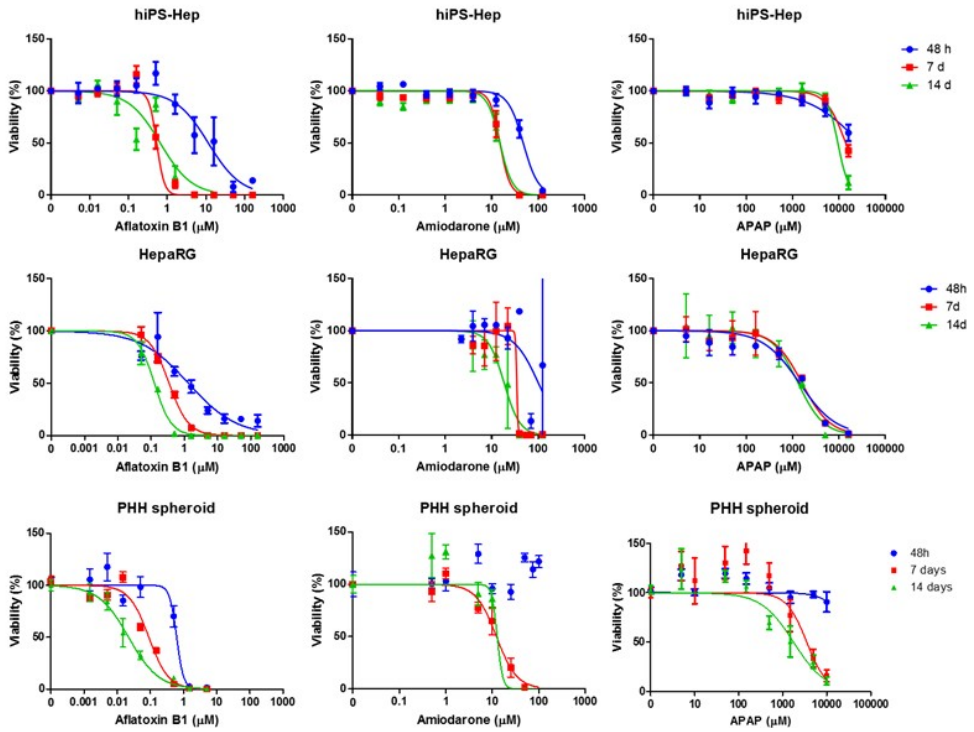

Figure 4.7 *Chronic toxicity of aflatoxin B1, amiodarone and APAP. Each cell type was dosed with the indicated compound every 2-3 days and viability was measured at 48h, 7 days and 14 days.*

Regarding the task to design and implement technologies for optimising the use of Mesodermal Progenitor Cells for their use in the Simvastatin subacute chronic muscle toxicity evaluation, we found that the differences in gene expression of the mesodermal progenitor cells treated with simvastatin 1 mM for 2 days taken together with the decrease of cell viability from the same time period, suggests that such dose of simvastatin may exert toxicity by alterations at the transcriptional level. Both the identification of the key players in hepatocyte differentiation, and the validation of toxicity models based on stem cells-derived systems, provide an essential mean for the *in vitro* evaluation of drugs and hence the substitution of animal based-systems.

Implementation of Interferomics

Development of optimal conditions for siRNA transfection in one hPSC progeny has allowed us to implement an RNAi modifier screen in order to explore repeated dose toxicity mechanisms and the possibility to apply this genome-wide technology to hPSC progenies. The mesodermal progenitor cells derived from hESCs or hiPSC can be efficiently transfected with siRNA, in 96 and 384 well plate, manually and with a liquid handling automat. Mesodermal progenitor cells

placed into a repeated dose toxicity state upon simvastatin pretreatment can be transfected as efficiently with siRNA as populations that did not receive any pretreatment and without excessive toxicity.

Implementation of Interactomics

By using new human protein-protein interaction network, we aimed to identify functional and topological signatures of simvastatin-induced toxicity patterns. We have built a new human protein-protein interaction network (*i.e.* interactome) from public sources. This interactome is considered as up-to-date and of good quality. It is composed of 73,212 protein-protein interactions linking 11,636 distinct human proteins. Then transcriptomic analysis of cells exposed 2 to 17 days to high and low doses of simvastatin was performed. The results reveal clear difference between acute and chronic treatment at the level of genes and proteins (*Table 4.2*). They can also be linked to cell phenotype (morphology and proliferation).

Table 4.2 *The betweenness distribution of the proteins from D2 (acute toxicity) SIM1 down genes is significantly higher than the betweenness distribution in the human interactome. This is not the case for repeated dose toxicity (D17) for the same concentration of Simvastatin (1 mM)*

Study	Differential expression	Mean betweenness	When relevant, p-value (U-test) and conclusion
D2 SIM1	up	1.08 10-4	
	down	1.169 10-4	Significantly > human interactome (4.47 10-4)
	all	1.095 10-4	
D17 SIM1	up	8.36 10-5	
	down	3.38 10-5	
	all	6.57 10-5	
D17 SIM0.4	up	1.75 10-4	
	down	/	
	all	1.75 10-4	

Implementation of Bioelectronics

Advanced and novel bioelectronic-based stem cell monitoring system, functional bioelectronic monitoring system for pluripotent stem cells, thereof derived cell lineages and differentiated cell types, the read-out amplifiers and multiplexers as well as the planar multielectrode array (MEA) has been developed, adapted and optimised based on the specifications needed for cardiomyocytes and neural cells.

Microarrays: We were able to adapt and optimise our bioelectronic measurement platform for the monitoring of pluripotent stem cell characteristics. Therefore, we evaluated the optimum electrode size, configuration, geometry and MEA substrates and conductive / semi-conductive electrode materials etc.. Finally, we could demonstrate the discrete detection of non-directed differentiation processes in hiPS cultures. Moreover, we could demonstrate the convincing suitability of our planar MEA measurement system and technology platform for real-time online recording for the functional analysis of pluripotent stem cell derived neuronal networks in the context of maturation (*Figure 4.8*). Based on the established system we initiated and intensified several collaborations where we already provided our self-developed and produced MEAs to the consortium partners.

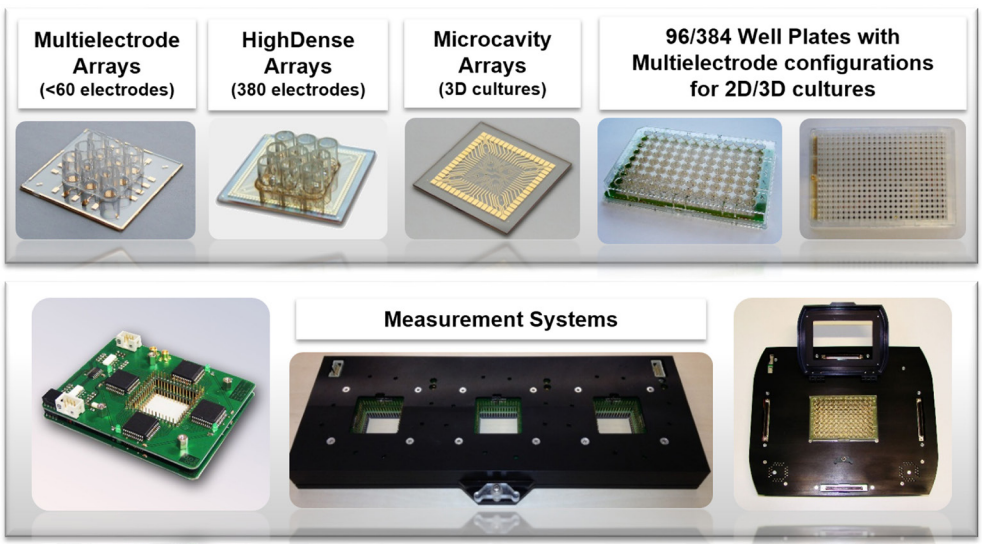

Figure 4.8 *Optimised multielectrode arrays for cell type-specific and assay-dependent sensitive monitoring of cell function and cellular alterations (top). For reliable and automated data acquisition, appropriate electronic boards were developed (bottom). Data source: University of Leipzig.*

Microcavity arrays: For the establishment of a microcavity array based bioelectronic monitoring system, the MCA as well as the amplifier and multiplexer devices had to be characterised by applying reference compound analysis using stem cell derived human cardiomyocytes, and had to be optimised for an optimum signal quality. The multimodal recording system had also to be tested and optimised for long-term measurements, especially to fulfill the demands on repeated dose toxicity testing. The established MCA measurement system could be adapted and optimised for the bioelectronic analysis of human cardiomyocytes (*Figure 4.9*). The use of the MCA technology showed superior advantages over commonly used planar MEA based analysis, especially in the context of long-term monitoring with improved signal stability that is a prerequisite for repeated dose toxicty testing. Exclusive data records can be made regarding the efficacy and (bio)availability of active pharmaceutical ingredients including cytotoxic side effects.

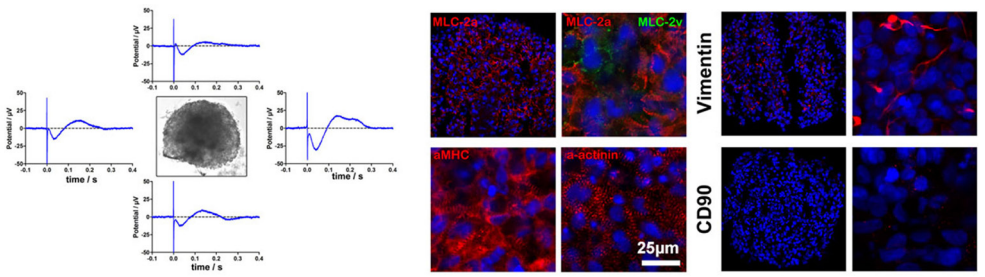

Figure 4.9 *The microcavity-based functional monitoring of hES-derived cardiomyocyte clusters allows the determination of beating frequency, excitation velocity and action potential duration (left). The validity of the obtained results depends on the quality of the cardiomyocyte clusters. The immunocytochemical characterisation of the developed hES-derived cardiomyocyte clusters revealed a highly enriched cardiomyocyte population with a high level of cardiac markers (MLC-2, aMHC, a-actinin), no fibroblasts (CD90) and only a small number of other mesodermal-derived cell types (vimentin) (right).*

Implementation: In order to obtain a bioelectronic measurement system that can be used in combination with stem cell derived cells e.g. neuronal and cardiomyocyte 2D and 3D-cultures, the analogue and digital measurement pathways have to be tested and adapted/optimised. Especially for electrophysiological recording of cardiomyocyte clusters but also for neuronal network cultures signal sensitivity as well as signal-to-noise ratios had to be investigated for a feasible data analysis. Therefore, also recording and analysis software had to be adapted and optimised with regard to a feasible and automatable data acquisition and processing system that can be easily up-scaled (*Figure 4.10*).

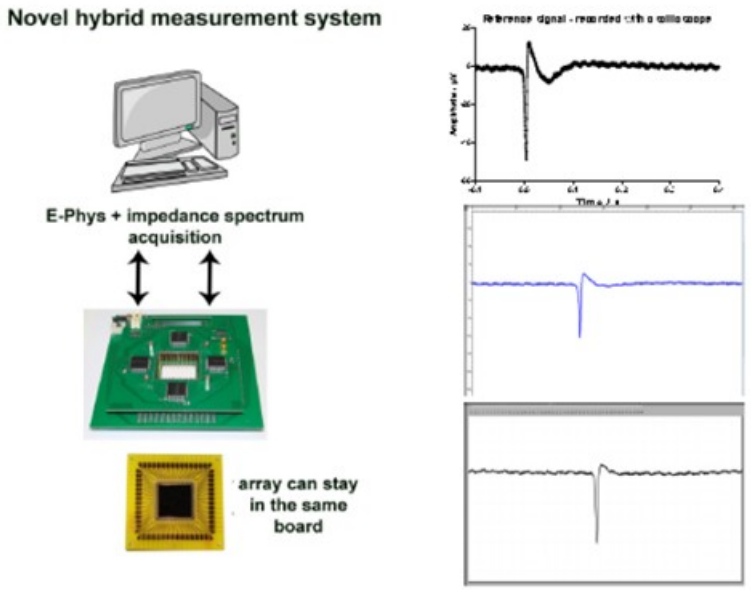

Figure 4.10 *Testing of hES-derived cardiomyocyte clusters a self-developed hybrid measurement system. In comparison to a human cardiomyocytes-derived reference signal (top), the recorded stream with a conventional amplifier (middle) as well as the stream recorded with our novel hybrid measurement system (bottom) are shown.*

Another focus was the integration of different bioelectronic detection methods to obtain a high content monitoring platform. Our self-developed and in the project provided hybrid measurement system including impedance spectroscopy and electrophysiological recording had to be evaluated and optimised for the use of stem cell derived cell cultures, especially in the context of repeated dose toxicity testing (*Figure 4.11*).

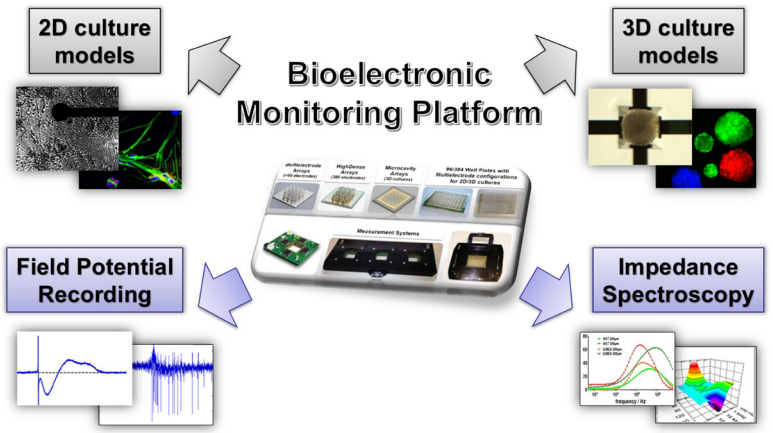

Figure 4.11 *Bioelectronic monitoring platform for the analysis of 2D- and 3D-culture models using field potential recording and impedance spectroscopy. This allows the functional monitoring of electrogenic cells (neurons and cardiomyocytes) including the detection of cellular effects due to toxic compounds. Data source: University of Leipzig.*

Based on our well-established measurement systems for electrophysiological recording and impedance spectroscopy we adapted and optimised both systems with regard to the novel microelectrode-based planar arrays and microcavity arrays within the ***SCR&Tox*** Project. To use and operate the developed bioelectronic measurement systems in a feasible and end-user friendly way the software packages had to be adapted and optimised for the specific needs and characteristics of stem cell and stem cell derived cell cultures. With the data recording and analysis software packages for electrophysiological recording and impedance spectroscopy on microelectrode-based planar arrays and microcavity arrays with up to 384 channels, our established systems can be used to monitor and analyse the stem cell derived cultures like cardiomyocytes and neurons but also muscle cells and hepatocytes/hepatocyte like cells. Since our bioelectronic measurement systems are label-free and non-invasive they perfectly match the needs for long-term monitoring in the context of chronic toxicity and repeated dose toxicity testing.

Genetic Engineering of Cells: Reprogramming of somatic into iPS Cells

Two different methods to reprogram somatic into induced pluripotent stem cells have been established. The reprogramming methods are non-integrative (foot print free). iPS lines have been generated with these technologies.

TALENs Homologous Recombination

Nucleases (meganuclease and TALEN) have been successfully used to integrate repair matrices into iPS genome, in several different loci (*Figure 4.12*). We have established a process, from the early design of nucleases and matrices to the harsh selection procedure where we on a routine basis can achieve homologous recombination in human iPS and ES cells. Efficiencies were, however, low.

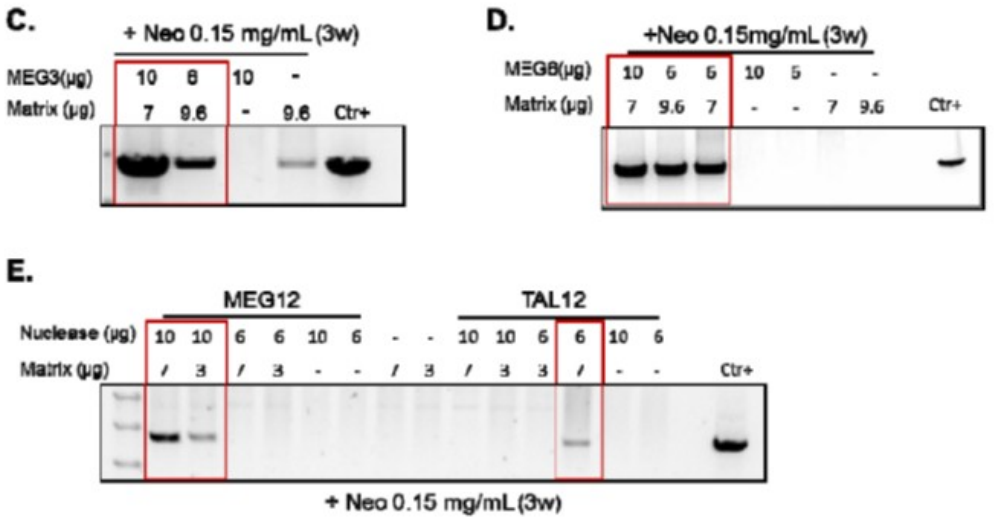

Figure 4.12 *Targeted integration with the meganucleases MEG3 (C), MEG6 (D.), MEG12 and TAL12 (E.) in iPS cells.*

Gesicles as a Tool to Deliver Gene Constructs to Stem Cells

Gesicle production relies on the coexpression of VSV-G, a viral glycoprotein, and a plasmid coding a protein of interest (POI) in a producer cells (HEK cells). Gesicles are next release from the supernatant of producer cells and can be collected/concentrated/stored. The objective was to create a robust gesicle-producing system for the transfer of transcription factors (*Figure 4.13*). These factors being nuclear and carrying nuclear localisation signal, their incorporation in gesicles budding from the membrane of producers cells was challenging due to a poor incorporation in budding gesicles.

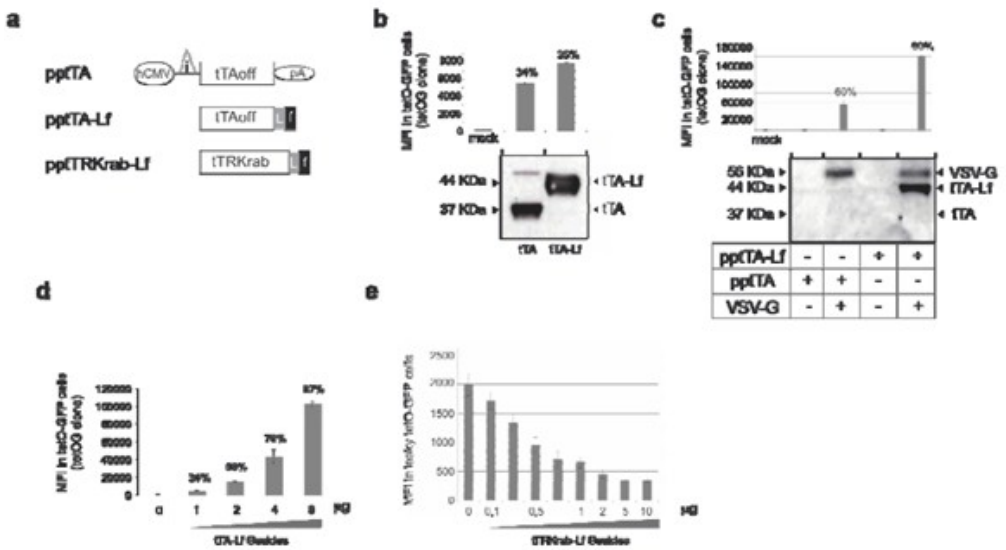

Figure 4.13 *Targeted integration with the meganucleases MEG3 (C), MEG6 (D.), MEG12 and TAL12 (E.) in iPS cells.*

iPS with Reprogramming Genes

The objective was to create 'clean' iPS cells, primarily by demonstrating the feasibility of the nuclease-based targeted integration into human fibroblast cells and to develop a robust and reproducible process of reprogramming. As a back-up of the nuclease-based reprogramming option and taking into account the recent advances in this area, it was decided to asses an enhanced version of the episomes reprogramming process as published by Yamanaka's group in 2011. Adult fibroblasts were therefore transfected and cultured as described previously. Fibroblasts from five different healthy donors, and with appropriate donor consents, were reprogrammed by employing the episome-based technology. The episomes were delivered via electrical based transfection, employing either an AMAXA or a microporator (Neon). Both type of machines gave rise to similar results. The cells were reprogrammed under feeder free conditions employing DEF-CS. When the emerging colonies were big enough they were picked and expanded individually under the same culture conditions. The depletion of the episomes was monitored via a qPCR approach. At the stage of cell banking, episomes could no longer be detected within the reprogrammed cells (*Figure 4.14*).

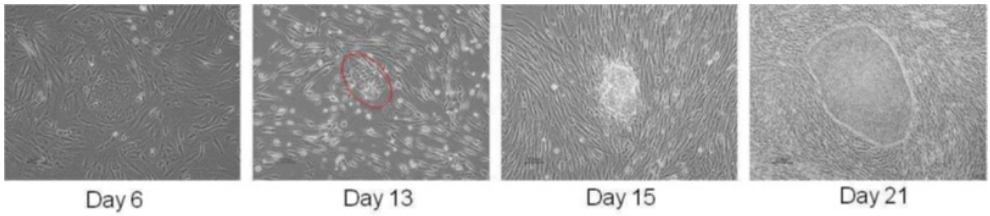

Figure 4.14 *Morphology of the iPS-like colonies observed with the episome-based reprogramming methodology.*

Demonstration of Gesicle-vectorisation of a meganuclease

I-Sce-1 gesicles were produced and analysed by western Blot to validate the protein incorporation (*Figure 4.15*). Monitoring different independent batches of gesicles, we estimated that a given batch of ISCE-1 gesicles contains 1-2 units per 10ul of product.

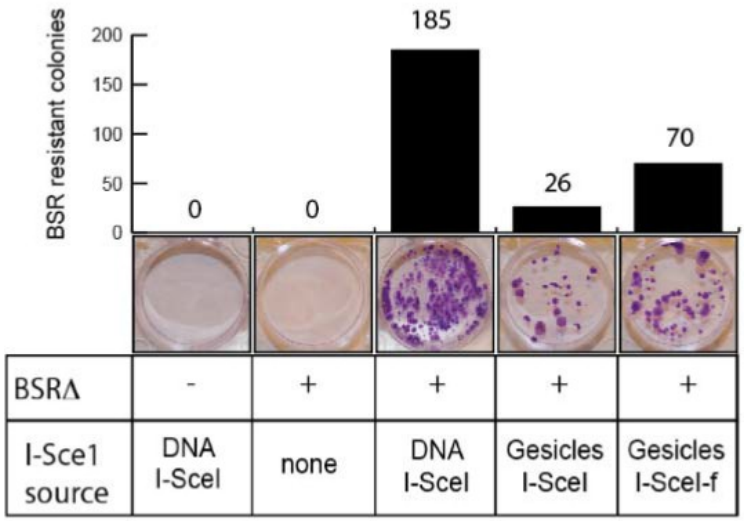

Figure 4.15 *Western Blot to validate the protein incorporation.*

iPS Lentivector for Temporary Engineering

Molecular feasibility of the meganuclease-based reprogramming strategy was successfully demonstrated into the 293H cell line. In order to transfer this process into primary fibroblast cells, twelve different meganucleases targeting twelve distinct loci into the human genome were assessed. Their expression and functionality (targeted mutagenesis) were demonstrated.

A reporter matrix was also correctly integrated into the MEG6 locus in the two fetal fibroblasts cell lines used within the study. However neither an integration of the reprogramming matrix, nor reprogramming events were observed so far with this methodology.

The episome-based back-up strategy was, therefore, selected rather than the endonuclease-mediated reprogramming, on the basis of the positive results presented above.

Supplementary Work

In light of ongoing discussion on challenges of identifying diploid iPSC lines without genetic mutation due to the reprogramming process and also on standardisation of stem cell based assays within ***SCR&Tox*** and at the **SEURAT-1** Stem Cell Working Group (see section 4.9.9), further work has been completed by one partner. iPSC line NIBSC8 was generated using Stemgent's mRNA/miRNA reprogramming kit from the cell line MRC9 on Vitronectin. In the ***SCR&Tox*** programme it had been difficult to identify iPSC lines which were diploid and free of genetic mutations which might affect the performance of these cells differentiated for systemic toxicity assays. The additional line generated using mRNA technology appears to have a stable diploid genetic makeup in the majority of cells. It thus offers a further potential candidate of unaltered wild-type biology for use in assay development.

Production of Optimised Ready-to Use Cells for Toxicity Testing

The aim was to design and implement technologies for introducing gene constructs into human pluripotent stem cells that can facilitate cell selection at a particular differentiation stage or promote differentiation into a specific cell phenotype. The focus of the work has been on two different cell types, stem cell derived hepatocytes and stem cell derived cardiomyocytes. For keratinocytes and neural cells the consortium already had very robust differentiation protocol or valuable reporter cell lines available so no efforts were spent on engineering work for these two cell types.

By using TALEN based genome engineering technology we first derived human pluripotent stem cells, which (after differentiation into hepatocytes) should overexpress the important upstream genes PXR-RXR and CAR-RXR, respectively. To further enhance the functionality of maturing hepatocytes derived from hiPS cells, three additional plasmid constructs were engineered. Two different approaches were considered, either to overexpress certain transcription factors that are known from literature to play a key role during differentiation (i.e. HNF4alpha and HNF6), or to overexpress a transporter protein involved in the CYP-metabolism (i.e. OAT1B1). However, analysis of the performance of the gene modified cells did not prove that more functional hPSC-derived hepatocytes were achieved.

Regarding the gene engineering of cadiomyocytes we initially made a reporter line generated using hESC-line SA002 and a NCX1-GFP construct. During evaluation of this transduced line,

the GFP signal was detected in differentiated CMs. However, the GFP expression appeared quite weak. In order to improve the outcome we therefore, as an alternative, generated another reporter line using a cTnT-GFP construct. In that case, the GFP signal was strong and readily detected in differentiated CMs originating from the transduced cell line.

4.2.3.3 Assay Development

This part of the programme was modified because the withdrawal of the partner in charge of the production of a genetically-engineered cell line for the selected toxicity pathway precluded full completion of the programme as scheduled. The iPS clones with ARE-Luc construction were produced and distributed to the relevant partners but with a significant delay. In the following we describe two types of activities related to the development of assays for exploring toxicity pathways. First, the progress made despite the absence of the engineered cell line is presented for the selected pathway, which concerned the NRF2 system. Second, other assays successfully developed using non-engineered cell lines are discussed.

Profiling and Functional Characterisation of Cells with Reference to the Selected Toxicity Pathway

The aim was to establish an assay using human pluripotent stem cells derivatives for one chosen toxicity pathway, relevant to repeated dose toxicity evaluation. The complexity of repeated dose toxicity involves a number of different target organs and a whole range of different pathways. These pathways are often referred to as toxicity pathways since their activation is triggered when cellular adaptive and defense mechanisms are being challenged by toxic insult. Obviously, pathways of toxicity that lead to adversities *in vivo* vary as they might be specific for different cell types. However, there is also strong evidence that the different manifestations can be triggered by the perturbation of the same pathways. It is widely accepted that oxidative stress contributes to a variety of target organ toxicities induced by different classes of chemicals and generation of reactive oxygen species (ROS) is one of the measured endpoints, widely used for oxidative stress evaluation. Therefore in this project Nrf2 pathway activation has been selected as a key event highlighting the oxidative stress as well as cellular defense mechanisms for assessing the hazard of chemicals across different cell types.

The NRF2 Model

Nrf2 target genes encoding phase II detoxification enzymes and antioxidant proteins such as NAD(P)H quinone oxidoreductase-1 (NQO1), glutathione *S*-transferases (GSTs), glutamate-cysteine ligase and heme oxygenase-1 (HMOX1) were studied as readouts for Nrf2 pathway activation. These target genes expression has been evaluated using neuronal, cardiomyocytes

and keratinocytes models derived from hiPSCs in the control cultures and after the exposure to the chemicals that activate Nrf2 signalling pathway.

The hiPSCs IMR-90-derived neuronal cultures responded to rotenone treatment by up-regulation of the antioxidant enzymes NQO1 and SRXN1 at the protein level in a concentration- and time-dependent manner. Dose-response curves were determined in mature neuronal culture derived from hESCs treated for up to 14 days with two reference compounds; rotenone, a well-known inhibitor of the mitochondrial complex I and AI-1 (ARE-Inducer-1), a synthetic molecule, a specific activator of the Nrf2 pathway and established IC_{50} values based on Alamar Blue reduction. Regarding the gene expression analysis after exposure to test compounds, low level of exposure to rotenone at 20 nM and 100 nM changed the expression of the neural genes, in particular of BetaTubulin, synapsin GABA and glutamate transporters as well as Nrf-associated genes (KEAP1, SRXN1, NQO1 and SRXN1) differently at different time points. At day 14 all the neural markers were down-regulated but the expression of NQO1 and SRXN1 was increased. HMOX1 instead returned near the control level in the two highest concentrations while it was up-regulated at 1 nM indicating not only a dose-response effect but also a time-dependent effect. After 14 days of AI-1 exposure the neural genes expression was down-regulated only at the highest concentration. At the not cytotoxic concentrations the neural genes maintained levels similar to the untreated control but the NQO1, SRXN1 and HMOX expression was significantly increased. The data indicate that the system can discriminate specific neurotoxic effects from unspecific cytoxic effects and the Nrf2 pathway can be specifically activated in this test system.

To assess the toxicological relevance of the Nrf2 pathway we first evaluated its basal and inducible activity in lt-NES and lt-NES-derived neurons of different maturation stages. The protocols established yield stable, proliferative, and tripotent RGL-NSC and lt-NES populations. They enable the generation of terminally differentiated subtype-specific cells of the glial and neuronal lineage, respectively, at high purities via distinct self-renewing intermediate populations, providing scalable and standardised hPSC-derived cellular resources for a variety of neurotoxicological studies. The generated lt-NES-based Nrf2 luciferase reporter cell line provided evidence that this system is amenable to dose-specific rotenone and AI-1-mediated modulation and thus might serve as a platform for neurotoxicity assay development. QRT-PCR analysis of the Nrf2 downstream target genes revealed increased expression levels with the progression of neuronal maturation (*Figure 4.16*).

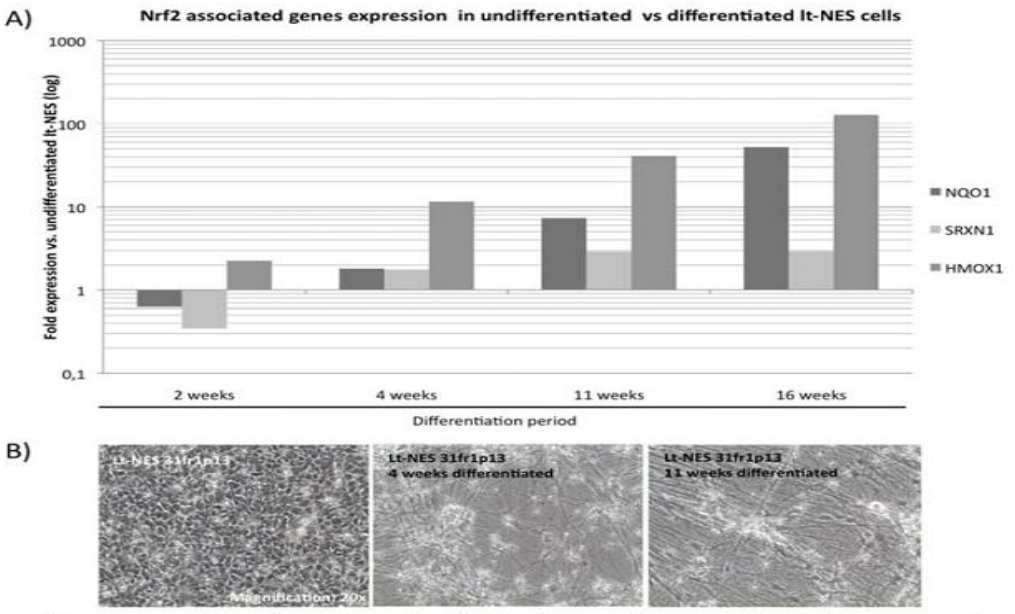

Figure 4.16 *Basal expression levels of Nrf2 downstream target genes increased with progressing neuronal maturation of lt-NES cells. 31fr1 lt-NES cells were differentiated by growth factor withdrawal from the culture medium for up to 16 weeks and RNA was collected at the indicated time points.* ***(A)*** *QRT-PCR analysis of the expression of the Nrf2 downstream target genes NQO1, SRXN1, and HMOX1 in comparison to undifferentiated lt-NES cells (equal to 1). Data are normalised to GAPDH RNA levels.* ***(B)*** *Representative bright field images of the cells at the indicated maturation stages.*

In accordance with the qRT-PCR results, dose-response curves for repeated-dose toxicity revealed an increased resistance of more mature neurons to rotenone as determined by Alamar Blue assay. Furthermore we could show that Nrf2 pathway activation is strongly increased in response to rotenone treatment upon complete removal of antioxidants from the cell culture medium. Co-culture experiments with our RGL-NPC-derived astrocytes indicated that the glia cells in lt-NES-derived neuronal cultures are a main contributor to the Nrf2-response (*Figure 4.17*).

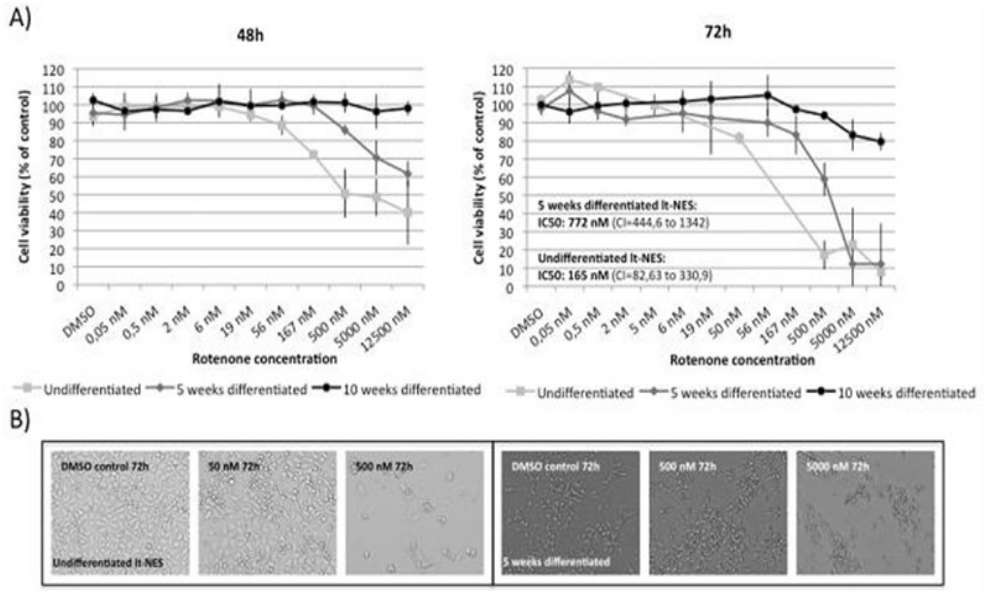

Figure 4.17 *Susceptibility to rotenone treatment decreased with increasing time of neuronal differentiation of lt-NES cells. Undifferentiated lt-NES cells and lt-NES cell-derived neurons differentiated for 5 and 10 weeks were treated with a rotenone concentrations between 0,05 nM and 12500 nM for up to 72h. The cells were treated with the corresponding DMSO concentration as a solvent control.* ***(A)*** *Cell viability was measured by Alamar Blue assay after 48h and 72h and is depicted as percentage relative to untreated cells (equal to 100%). Data are presented as means ±SD (n=3, consisting of 3 technical replicates each).* IC_{50} *values for 72h were calculated by performing dose-response analysis by nonlinear regression curve fit using GraphPad Prism 6.* ***(B)*** *Representative images after 72h of treatment under the indicated conditions. Images were acquired with a CellaVista imaging platform.*

In order to exploit the Nrf2 pathway as an indicator of cell stress-associated events we generated an lt-NES reporter cell line for Nrf2-mediated oxidative stress response. To that end, hiPSC-derived lt-NES cells were transduced with a lentiviral vector carrying a luciferase reporter gene under the control of a single copy of the ARE-element of the human AKR1C2 gene (ARE-Luc; Givaudan Schweiz AG).

Assay Development of Toxicity Tests

Several other *in vitro* cell models have been used in order to characterise responses of specific cell phenotypes to various toxicants. One model that was fully developed within the framework of the ***SCR&Tox*** programme used mesodermal precursor cells of the myogenic

lineage in order to explore simvastatin-induced toxicity to muscle cells. Employing mesodermal progenitor cells we tested several statins (cerivastatin, atorvastatin, pravastatin, fluvastatin, simvastatin and lovastatin) following acute dose toxicity experiments and based upon *in vitro* toxicity results and pharmacokinetic data, we selected simvastatin as a good representative of the toxicity of this pharmacological class.

In order to set-up an experimental model of repeated-dose exposure, mesodermal progenitor cells were exposed to simvastatin, renewed every other day in the culture medium, for 17 days. Three different doses of simvastatin were chosen based on the results of the 48 h acute toxicity dose-response curve: the highest dose that did not affect cell numbers (0.4 μM), the lowest dose that induced a cell loss at plateau level (2.5 μM) and an intermediate dose (1 μM; *Figure 4.18*).

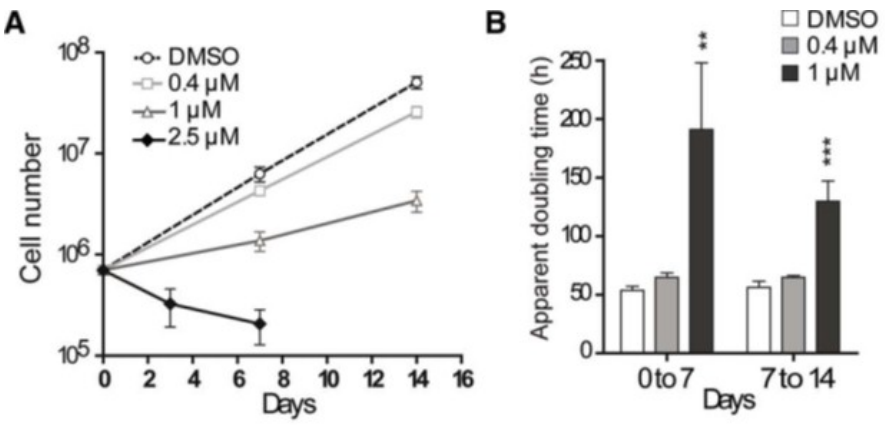

Figure 4.18 *Characterisation of simvastatin-induced acute and repeated-dose exposure responses in mesodermal precursor cells.*

The cellular mechanisms leading to the chronic reduction of cumulated MPC number under the 1 μM simvastatin treatment were explored by analysing in parallel cell death and cell proliferation at different time points following repeated-dose exposure. Once adjusted to the level of basal cell death occurring in control conditions, there was very little, if any, additional cell death under the 1 μM treatment. In contrast to the absence of direct induction of cell lethality, cell proliferation was significantly affected by simvastatin 1 μM at all time-points studied. The chronic cytostatic effect of simvastatin 1 μM was reversible when the drug was withdrawn from the culture medium after 8 days of exposure.

A whole genome transcriptomic analysis was performed on mesodermal progenitor cells treated with simvastatin 1 μM either for 48 h with a single dose exposure or with repeated-dose exposure for 17 days. A massive perturbation in gene expression followed the acute

single dose exposure, with a total of 3,740 differentially expressed genes when compared to DMSO treated controls (| FC | ≥ 2, p ≤ 0.05; *Figure 4.19A*). However, the prolonged drug exposure dramatically decreased the number of differentially expressed genes to 139 after 17 days of treatment (*Figure 4.19B*). After the repeated-dose exposure of 17 days, gene expression changes were mainly related to tissue morphogenesis, cell adhesion, and regulation of differentiation and of muscle development (*Figure 4.19C*).

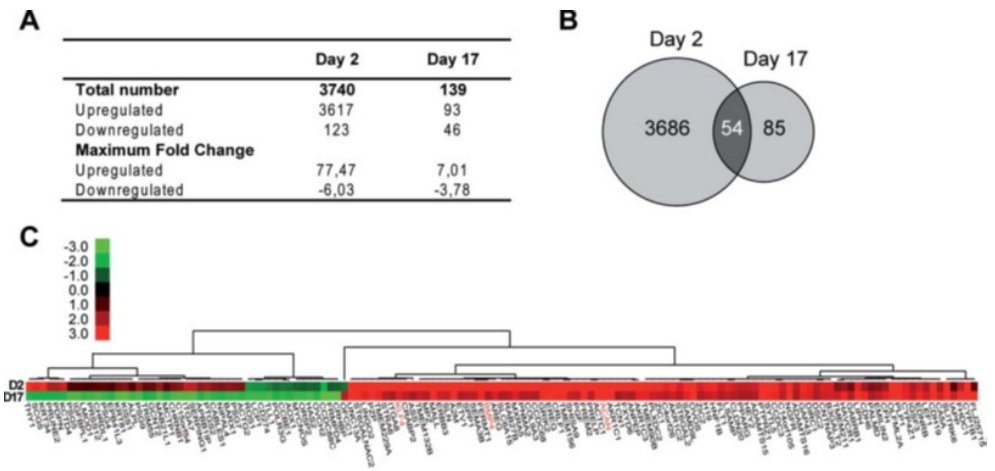

Figure 4.19 *Differentially expressed genes in mesodermal precursor cells in response to simvastatin 1 mM repeated-dose exposure. (A): Number of differentially expressed genes following treatment with simvastatin 1 mM as compared to DMSO (jFCj_2; p<.05). (B): Venn diagram comparing number and distribution of differentially expressed genes following treatment with simvastatin 1 mM during 2 and 17 days, respectively. (C): Heatmap representing color-coded hierarchical clustering of FC expression values from microarray analysis, after 2 days or 17 days of simvastatin 1 mM treatment, for the list of genes significantly modulated by the repeated-dose exposure of 17 days.*

Altogether, that study demonstrated the value of derivatives of human pluripotent stem cells by showing, in particular, that repeated-dose exposure to simvastatin may elicit an adverse cytostatic effect on cells of the myogenic lineage in the absence of cell death. This long-term effect was associated with molecular changes that strongly differed from those induced by acute exposure of cells to the same dose of the drug, thus revealing powerful adaptive mechanisms.

4.2.3.4 Technology Transfer to Industry Platforms

Standard Operating Procedures for Industrial Scale Assessment of the Selected Toxicity Test

SOPs have been transferred for several cell lineages. Human pluripotent stem cells differentiated efficiently into hepatocytes *in vitro*, but the resulting final cell population generated was considered to be still not functional as adult human freshly isolated hepatocytes.

In the case of cardiomyocytes, hPSC differentiated well into this cell *in vitro*. Although a relatively high yield of cardiomyocytes was generated with specific protocols, the resulting final cell population still contained a fraction of non-cardiomyocytes. A purification step for selecting the differentiated cardiomyocytes from mixed cell populations was suggested, in order to engineer cells expressing GFP under the control of a cardiomyocyte specific promoter, and subsequently use a FACS to purify the cells.

An SOP was successfully developed for the cultivation of It-NES cells (stable neuronal intermediates) in 96-well plates (which were then cryopreserved) in order to meet the challenges of industrial-scale neurotoxicity screening tests.

An SOP was first transferred for the manual production of keratinocytes after iPSC-differentiation, and another for keratinocyte amplification and the *in vitro* production of reconstructed 3D epidermis. Then SOPs have been established for industrial-scale automated differentiation and amplification of keratinocytes from iPS cells using the Compact SelecT automated platform. The efficacy of the keratinocyte production depends, however, on the iPS cell line.

Transfer of the Selected Toxicity Test to Industrial Scale Platforms

Technology Transfer of the Assay

The SOPs for seeding, differentiating iPS keratinocytes and culturing 3D epidermis for this cell line were transferred following successful training. Since the monolayer keratinocyte assay would be more applicable than the 3D epidermis it had been suggested that the assay on keratinocytes in 2D should be performed. As the KeratinoSens™ assay had already been validated in-house at CiToxLAB, Covance hosted I-Stem for a training programme on the assay procedure using the KeratinoSens™ assay. Bonn University transferred the It-NES iPS derived neuronal cells and protocols to Covance, who successfully performed the experiments according to the supplied protocol. No specific assay was transferred but University of Bonn had previously presented data on a number of genes downstream of Nrf2 pathway among which NQO1 and HMOX-1, analysed at Covance with iPS keratinocytes and primary keratinocytes and KeratinoSens cell line at CiToxLAB.

Protocols Refinement

Two additional protocol-endpoints were established with the It-NES iPS neuronal cells: the determination of relative LDH values in comparison to maximum LDH value (method of analysis reflecting the cytotoxic effect of the test chemical) and the total GSH quantification following exposure of the cells to chemicals.

Concerning the keratinocytes assay performed with the iPS keratinocytes, it was decided to investigate the contribution of inflammatory cytokine effects following the exposure of the cells to chemicals. Three cytokines were selected (IL-6, IL-8 and IL-10) and the results obtained demonstrated that the chemicals tested did not affect the release of those cytokines.

4.2.4 Potential Impact

The ***SCR&Tox*** Project was organised in order to offer a systematic scientific and technological approach towards improving our search for an efficient and reliable assessment of the toxicity of chemicals. This was deemed necessary within the general framework of a shift in the focus of regulatory toxicology from empirical assessment in whole animals and clinical trials to *in vitro* assays based on human cells. This shift in scientific paradigm can be associated with major foreseeable impacts, namely on the overall value of toxicity evaluation, on the price of drugs and cosmetic products and on the societal acceptance of activities in the health industry.

As concerns the value of toxicity evaluation, it is widely considered nowadays that whole animal testing is associated with a number of false negatives and false positives due to interspecies variations. False negatives, due to lack of sensitivity, is sending potentially valuable drugs to the waste basket, with social and industrial consequences. False positives, resulting from lack of specificity, may lead to hasty and costly withdrawal, and sometimes to human morbidity and mortality. The ***SCR&Tox*** Project was designed to bring the proof of concept at industrial scale of the possibility to overcome previous problems by turning to the most rigorous scientific approach of toxicity pathways. Even though the final demonstration could not be brought, due to unfortunate delays linked to the unexpected withdrawal of a key partner, the results obtained are highly consequential in scientific terms and will help promote the shift in focus in the entire toxicology community.

Predictive toxicology has become, over the past twenty years, one of the most expensive components in drug and ingredient discovery, and some analyses consider that it accounts for 20% of the entire cost of the final product. The continuous expansion of regulatory requirements and the pressure against *in vivo* animal testing are continuously increasing those costs. *In vitro* cell-based assays, because they rely on unlimited biological resources and large-scale technological approaches well before development of the products up to clinical trials, will allow for major reduction in this heavy bill. The versatility of the human cells and their theoretical ability to accommodate any functional read-out will be major advantages in that direction.

Last, but not least, the life sciences as a whole have faced strong attacks over the past decades from advocates of animal welfare, with particular focus on the industrial use of animals for toxicity testing, in particular in the cosmetic industry. Cell-based assays of toxicity pathways are no promise for a complete elimination of animal testing in the pharmaceutical industry – in particular because it is beyond reach to test in cells either ADME (absorption, distribution, metabolism and elimination) or DMPK (drug metabolism and pharmacokinetic). However, cell-based assays will unavoidably reduce considerably the number of animals as the brunt of the toxicity testing will be borne by *in vitro* assays and animal testing will come as a complement only for those issues when relevant.

SCR&Tox has met the strategic objectives that it had set more than 5 years ago. The depth and breadth of the consortium has enabled a substantial impact on developing the essential knowledge and platform technologies. A key feature of ***SCR&Tox*** was its industrial dimension by incorporating 6 biotech companies but also two CROs that are directly the main stakeholders of the programme in the large pharmaceutical and cosmetic industries. Emphasis to this connection was further given through the organisation of an 'industrial and regulatory consultative body' in which representatives of major companies were convened and invited to discuss, on a regular basis, concepts and results of the project. It is our contention that all the research carried out by ***SCR&Tox***, whether it be addressing basic questions or else promoting assay development to industrial scale, has provided the foundation for exploiting this area of biotechnology for wealth and job creation across Europe.

Even though the programme has not been able to complete the development of new designs for predictive toxicology at major stakeholder sites, as originally planned, it has delivered all the components of such a design. We have, indeed, established biological resources for those new designs. The five cell lineages along which human pluripotent stem cells have been differentiated either fully or up to a relevant intermediate precursor, are now available tools for *in vitro* drug testing. In parallel, we have developed technological means and methodologies to use those resources in assays that allow analysing toxicity pathways at the molecular level. Among them, a particular notice should be made of the bioelectronics approaches that have been developed in a quite original manner by the network. Bioelectronics would, in our collective view, be a major addition to the current array of methods that analyse cell responses to toxicants.

Using those biological and technological resources developed by the network, we have been able to demonstrate in several assays for specific toxicants applied to a variety of cell phenotypes that drug toxicity could be reproduced and quantified. We have shown that repeated dose toxicity elicited molecular changes that were very significantly different from those that accompany an acute response, even for the same dose of a toxicant. This result, by itself, is a major warning for the field and an important contributor to the trend toward a change, which has been promoted since the mid 2000's and has contributed to the launch of the call to which the **SEURAT-1** Research Initiative was a response. In addition, we have used

the paradigms and resources developed in the ***SCR&Tox*** Project to reassess the myopathic toxicity of statins and suggested that, contrary to what had been proposed before, direct cell lethality, a cytostatic effect of statins may be responsible for the chronic myalgias that affect many patients who take statins regularly. This result underscores the clinical relevance of the *in vitro* approaches that were at the core of the ***SCR&Tox*** Project.

During the elaboration of the programme, we had identified collectively four domains in which impact could be anticipated and we can now indicate for each of them the impact of the work performed during the ***SCR&Tox*** Project.

(a) Tangible advances in the scientific approaches to

i. pluripotent stem cells provision and differentiation into derivatives along different lineages of interest. This has been fully carried out and transfer of the SOPs to industry partners has demonstrated the value of this work for the two lineages tested;

ii. gene and protein engineering as a way to provide cells with additional properties in view of an industrial use. The techniques have been developed and demonstrated on the bench, although the unfortunate withdrawal of the partner in charge of that development (Cellectis SA) before the transfer of the technology was done did not allow us to establish its value at the industrial scale;

iii. gene and protein profiling as well as exploration of dynamic functions at those two same levels as well as using bioelectronics in excitable cells. This part of the programme has provided major improvement to the array of analyses that can be used for studying drug toxicity *in vitro*. As referred to above, a special mention is worth making for bioelectronics, which has revealed extremely informative.

(b) European industrial innovation and competitiveness *by bringing proof of concept of predictive toxicology testing at industrial scale on the basis of cell-based assays using human pluripotent stem cell derivatives*. Cell-based assays have been quite successfully developed up to transfer of resources and technologies to industry partners. This has clearly opened a path for reaching the goal that was originally set to the network. The delay introduced by the use of a contingency plan in order to overcome the difficulty related to the withdrawal of a key partner in the middle of the programme has not, however, allowed us to demonstrate fully the value of our paradigms. This will have to be completed beyond the time schedule of the ***SCR&Tox*** Project.

(c) European healthcare policy and regulation *by entering into the validation process in vitro testing to replace or reduce animal experimentations in predictive toxicology for pharmaceutical compounds and cosmetic ingredients*. For the same reason described in the previous paragraph, this impact could not be fully reached. One assay that was designed in order to meet all challenges of pre-validation by ECVAM has been partially developed, though, and only awaited the cell resources (the Nrf2-responsive engineered iPS cell line)

required for its full exploration. All preliminary analyses have been done, and the path toward pre-validation is now open with the delayed provision, at the end of the time schedule of the programme, of the needed cell line.

(d) Specific training experiences for the next generation of toxicologists *involved in regulatory toxicology*. It was quite important to us to make scientific advances usable at industry scale. Scientific progress by itself does not guarantee that impact will happen. For actual impact to occur, scientific advance must be coupled and integrated with an appropriate strategy to promote the new findings up to the appropriate setting, and, in the particular case of predictive toxicology, that means industrial platforms sufficiently scaled to welcome large libraries of compounds and ingredients as well as existing chemicals. The new findings and developments should be made understandable to the potential beneficiaries in ways that are normal and actionable to them. For this reason ***SCR&Tox*** has dedicated its training and dissemination activities essentially to 'technology transfer', in order to provide industrial R&D personnel with the necessary training to handle biological and technological resources required for implementing cell-based assays as developed by the consortium. A number of internal and external training sessions have been organised during the ***SCR&Tox*** programme, with specific emphasis placed on cell reprogramming for the production of iPS cell line (25 external teams trained), the differentiation of cells into phenotypes of interest for drug screening (4 internal teams trained, including those of the two CROs), and automation/miniaturisation of cell bioproduction (three platforms developed and the two CROs trained).

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4.3 HeMiBio: Hepatic Microfluidic Bioreactor

Catherine Verfaillie
on behalf of the HeMiBio consortium

4.3.1 Executive Summary

In ***HeMiBio***, the aim was to create a bioreactor culture system of hepatocytes alone or in combination with the non-parenchymal fraction of the liver (hepatic stellate cells (HSCs) and liver sinusoidal endothelial cells (LSECs)) to allow repeated toxicity testing of cosmetics and chemicals for up to 2-3 weeks *in vitro*.

In the project we made the following advances beyond the state of the art:

Cells: We characterised for the first time human primary *HSCs and LSECs* at the functional and transcriptional level; characterised transcriptomic as well as epigenomic processes that cause activation of HSCs, and developed methods to counteract this activation; while we demonstrated that LSECs very quickly de-differentiate in culture and developed medium that can delay this event for ± 2 passages; we developed *progressively improving methods to create hepatocytes from PSCs,* yielding cells that can be used to study the toxicity of chemicals. However, despite the significant improvement in hepatocyte progeny, the cells remain less mature than primary hepatocytes. Therefore, transcriptome, epigenome and metabolome studies were performed to understand hurdles in the differentiation process, insights which were and are continuously used to improve creation of mature hepatocytes. Likewise, cells with *HSC-like properties were created from hPSC*. Again, the progeny is not fully similar to quiescent HSCs from the liver. Transcriptome studies confirmed this and are now being used to further improve differentiation. A similar set of studies was also done to create *LSECs from either PSCs* or from *blood outgrowth endothelial cells*. In a third set of studies, the transiently immortalised UpCyte hepatocytes were fully characterised, and were shown to have functional properties that approach those of primary hepatocytes. These *UpCyte hepatocytes* (generated from 5 different donors) were suitable for toxicity testing.

Sensors: In a second major set of studies, sensors to be incorporated in cells or in the bioreactor were created and tested. For *cellular sensors*, a genome edited set of stem cells

was generated that now allows very fast recombination of any incoming cassette such as for instance the NFkB reporter. *For sensors within the bioreactor*, fully biocompatible microbeads equipped with an oxygen-sensitive, phosphorescent dye were incorporated within the bioreactor allowing real time detection of oxygen consumption. In addition, a novel ALT sensor was created, which can assess with high sensitivity the excretion of Alanine aminotransferase (ALT) from diseased liver cells. These sensors, combined with additional standard pH and glucose sensors were incorporated in two different switch boards/liquid handling units to allow intermittent sampling of culture fluid enabling assessment of the health of cells within the bioreactor for protracted times.

Bioreactors: Three different bioreactor designs were generated: (i) *An antibody-based, microfluidic system*, capable of patterning any biotin-conjugated set of antibodies using streptavidin-based surface chemistry, allowing the generation of arbitrary cell patterns from heterogeneous mixtures in microfluidic devices. (ii) *A flow-over bioreactor* made of stainless steel, which protects hepatocytes from shear forces while creating stable oxygen and nutrient gradients mimicking the *in vivo* zonated liver. We demonstrated that HepG2/C3A cells could be maintained for over 28 days *in vitro*, while displaying over 98% viability and high expression of liver specific markers including CYP450 enzymes. (ii) *A flow-through bioreactor*: although very challenging, significant progress was made towards producing a 3D COC-based bioreactor for liver cell culture, and most technological hurdles in producing prototype reactors were overcome. Further testing will be needed to ensure cell viability.

Toxicity Testing: The ultimate goal was to exploit the technologies, in toxicity studies. *UpCyte hepatocytes and PSC hepatocytes* were shown to be suitable for testing molecules shortlisted by the **SEURAT-1** Gold Compounds Working Group (see section 4.9.5). A significant amount of work has also gone *to develop an in vitro model for liver fibrosis*, using co-cultures of HSCs and hepatocytes. These cocultures can identify fibrosis inducing drugs, as deposition of cross linked collagens can be detected following e.g. repeat dose exposure to methotrexate, a premier liver fibrosis inducing drug, among others.

In conclusion, ***HeMiBio*** has developed numerous tools towards the creation of innovative bioreactors, including cells, sensors and the reactors themselves to allow 2-3 week culture of hepatocytes with or without non-parenchymal cells to study the effect of drugs from the gold-compound list of standard reference compounds defined by the **SEURAT-1** Gold Compounds Working Group and to allow for the first time assessment of liver fibrosis inducing drugs.

4.3.2 Project Context and Objectives

Refinement, reduction and replacement of animal usage in toxicity tests (the 3Rs principle) is of particular importance for the implementation of relevant EU policies, such as the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Regulation (EC1907/2007)

or the 7th amendment to the Cosmetics Directive (76/768/EEC). Although multiple projects had been funded by the European Commission aimed at decreasing the need for animals in toxicity testing before the start of the ***HeMiBio*** Project, the assessment of toxic effects of chronic exposure still requires a relatively high consumption of animals. Moreover, aside from these ethical considerations, there was also a great need for suitable human cells to be used for toxicity testing due to the often poor concordance seen between human and animal models.

HeMiBio proposed to generate a liver-simulating device mimicking the complex structure and function of the human liver. The device should reproduce the interactions between hepatocytes and non-parenchymal liver cells (hepatic stellate, sinusoidal endothelial, and Kupffer cells) for over one month *in vitro* in a high-throughput format. Such a Hepatic Microfluidic Bioreactor should serve to test the effects of repeated exposure to chemicals, including cosmetic ingredients.

We postulated that to create a liver-simulating device suitable for long-term toxicity testing, (i) the cellular components of the liver need to be viable for extended periods of time (more than one month), with appropriate metabolic and transport functions and physiology that is comparable to the *in vivo* liver; (ii) the device should allow fluid to flow over or even through the mixed cells that recreate a small liver section, (iii) the zonation of the hepatocytes (and some non-parenchymal liver cells) must be recreated; (iv) the role of the non-parenchymal cells on the function and downstream toxicity of hepatocytes, as is the case in liver fibrosis, must be assessed. The device should be able to (v) screen drug-drug interactions as well as long-term toxicity of chemical entities.

The underlying hypothesis for the successful creation of a 3D liver-simulating device suitable to test repeated dose toxicity was that: (i) *hepatocytes* and *non-parenchymal cells* need to be combined; (ii) both *homotypic and heterotypic* cellular interactions between the different components are required to maintain the functional, differentiated and quiescent state of each cell component; (iii) the *matrix* whereupon cells are maintained, *oxygenation*, and *nutrient transport* will need to be optimised to support long-term maintenance of hepatocyte and non-parenchymal cell function, in an environment where shear forces are kept at their *in vivo*-like levels; and (iv) the system needs to be built such that *repeated on-line assessment* of cellular integrity, as well as metabolic and transport function, and physiology of the different cellular components is possible.

Human livers, from which the different cellular components could be selected, are in general unavailable for studies in the cosmetic and pharmaceutical industry due to liver donor shortage. Therefore, we proposed to isolate the cellular components from differentiated pluripotent cells. Pluripotent cells are normally derived from blastocysts, as embryonic stem cells (ESCs). Alternatively, they can be created from mature terminally differentiated cells by the introduction of pluripotency genes, that leads to the generation of induced pluripotent

stem cells (iPSCs). Although the exact configuration of the different cell types (as shown in *Figure 4.20*) may not be required, the short distance cellular interactions shown between (A) hepatocytes-LSEC and (B) hepatocytes-HSC cells is required for maintaining the functional state of the three cell types, (C) and the presence of monocytes/Kupffer cells is required to fully assess drug toxicity.

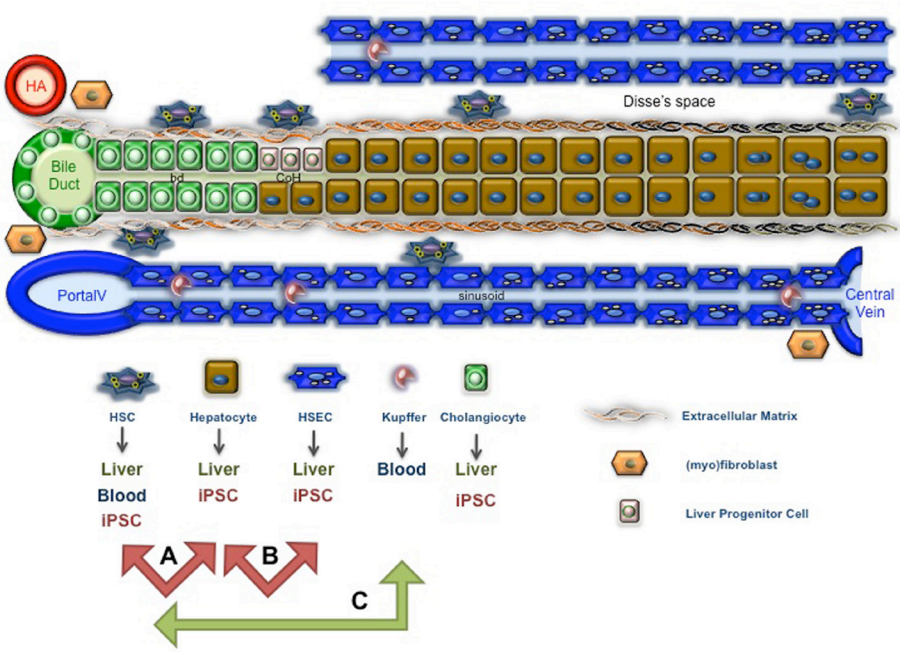

Figure 4.20 *Schematic representation of a liver sinusoid (adapted from: Dollé et al., 2010: J. Hepatol., 52: 117-129).*

To create a liver-bioreactor taking into account the hypotheses stated above, the specific objectives were:

- To develop tools to engineer the cellular components for the bioreactor to allow specific and spatially defined enrichment of the different cell components; this implies to non-invasively and in real-time assess the differentiation state of the parenchymal and non-parenchymal cells as well as cell damage;
- To incorporate *molecular sensors* and *electro-chemical sensors* that allow assessment of function and cell integrity;
- To develop a *2D-bioreactor* for the efficient isolation of differentiated iPSC mixtures by trapping different cell types on micropatterned surfaces;

➠ To generate a *3D liver-simulating device* by combining the above-mentioned engineered cells and sensors, which will allow dynamic monitoring of cellular function and health in a high-throughput format under various conditions;

➠ To provide proof-of-principle that a liver-simulating device can *recreate the toxicity profile in vitro* of toxins with a known *in vivo* toxicity profile over a minimum of one month, with specific emphasis on liver fibrosis;

➠ To assess the *molecular, functional and metabolic phenotype* of the hepatocellular, HSEC and HSC components at all stages of bioreactor development, and compare this with that of cells freshly isolated from human livers.

4.3.3 Main Achievements

4.3.3.1 Cells to Incorporate in the Bioreactor

Primary Liver Cells

Hepatic Stellate Cells

Isolations and culture: Hepatic stellate cells (HSCs) are the main effectors of liver fibrosis, independent of the etiology, they activate upon liver injury and inflammation and produce large amounts of extracellular matrix resulting in scar tissue formation and liver fibrosis. HSCs are routinely obtained from rodents by *in situ* perfusion of their livers with solutions containing pronase and collagenase, followed by cell separation through density gradient centrifugation (e.g. 8% Nycodenz®) and typically yields relatively pure qHSC populations (90-97%). This procedure may be completed by fluorescence-activated cell sorting (FACS) where HSCs can be further purified (up to 99%) based on a positive selection for ultraviolet-positivity (retinyl esters autofluorescence at 328 nm). In ***HeMiBio*** we optimised this procedure for human donor livers, by using UV positivity combined with a negative selection for CD32 and CD45 in FACS-based isolations of the non-parenchymal fraction (*Figure 4.21*). We used this procedure to obtain highly pure HSCs for gene and miRNA expression as well as DNA methylation studies comparing freshly isolated quiescent 'healthy' HSCs with fully activated 'fibrotic' HSCs.

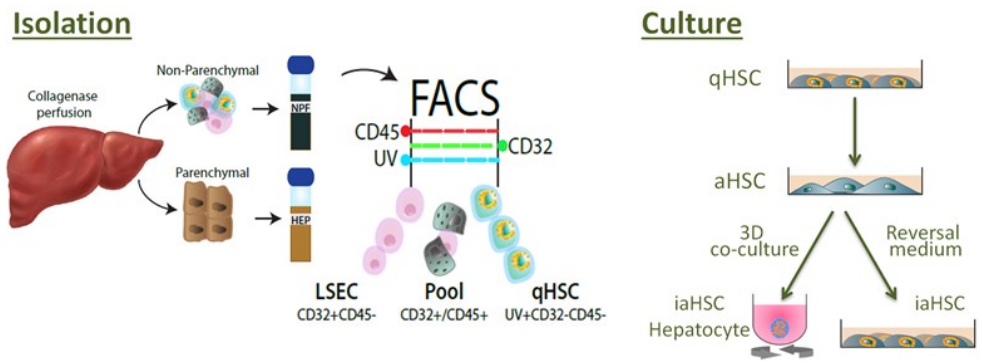

Figure 4.21 *Isolation and culture of human HSCs. Parenchymal cells were removed by low-speed (50 g) centrifugation steps. Enriched populations of human qHSCs were sorted out through a negative selection for CD32 and CD45 expressing cells and a positive selection for ultraviolet positivity (retinyl esters auto-fluorescence at 328 nm), using a fluorescence activated cell sorter (FACS). qHSC activate when culture in regular mono-layer 2D culture conditions. Activated HSCs exposed to reversal medium or co-cultured in 3D with HepaRG cells regain a quiescent like phenotype here referred to as inactivated HSCs (iaHSCs).*

Once HSCs are purified, culturing them on a stiff plastic surface leads to their transformation towards fibrogenic myofibroblasts. Rodent HSCs cultured on soft matrices or cultured in 3D spheroids show a lesser activation than cells cultured in traditional 2D cultures. During ***HeMiBio*** we developed culture conditions that can lead to a more quiescent phenotype of human HSCs. Firstly, by exposing 2D monocultures to a proprietary medium (Reversal medium) that can push the human HSCs towards an inactivated phenotype, a HSC cellular state that resembles quiescent HSCs but is more prone to activation was achieved. Secondly, through co-culture of human HSCs with differentiated human HepaRG® cells (hepatocyte-like) in 3D organoids, HSCs obtain and maintain a more quiescent-like state which is characterised by low secretion and deposition of collagen, but still keep their capacity to activate. These novel culture methods were used to test compounds for their pro-fibrotic capacity.

Transcriptome of fresh and cultured cells: We determined changes in the transcriptome associated with human HSC activation in culture. We compared freshly isolated, uncultured qHSCs with culture-activated HSCs (aHSCs). Over 2,000 genes are differentially expressed upon HSC activation (2,017/20,816 genes). This profiling allowed the confirmation of genes known to be associated with HSC activation, such as GREM1, LOX, TNC. This enabled identification of novel putative human *in vitro* HSC activation-associated genes. We find limited overlap (12-18%) between changes in gene expression underlying *in vitro* activation of mouse primary HSCs – the most common model used to study HSC biology – and human primary

HSCs, suggesting a different transcriptional cascade underpinning HSC activation in both species. For example, neurotrimin (NTM), a quiescence associated gene in mouse HSCs, is upregulated during human HSC activation. We furthermore compared the transcriptome of activated and *in vitro* reverted human HSCs (by reversal medium). Gene expression profiling experiments reveal that these *in vitro* reverted primary human iaHSCs display an intermediary phenotype that is distinct from qHSCs and aHSCs. Interestingly, this intermediary phenotype is characterised by the increased expression of several previously identified signature genes of *in vivo* inactivated mouse HSCs such as CXCL1, CXCL2, and CTSS, suggesting also a potential role for these genes in promoting a quiescent-like phenotype in human HSCs (*Coll et al., 2015; El Taghdouini et al., 2015a*).

miRNAs in fresh and cultured cells: Unveiling regulatory pathways of Hepatic Stellate Cells (HSC) quiescence and activation is essential to develop new therapeutic strategies to treat liver fibrosis. The aim of was to identify miRNAs regulating HSC activation by integrating HSC gene and miRNA profiling. miRNA analysis showed 215 up and 48 down-regulated microRNA in activated compared to qHSC. Transcriptome analysis identified 345 genes deregulated during HSC activation. Bioinformatic miRNA-mRNA integration revealed a set of deregulated miRNAs that presented a significant correlation with the expression of their predicted target genes. Expression of miRNAs regulating key genes involved in quiescence maintenance and in activation was confirmed in whole healthy and cirrhotic human liver. miRNA-inhibitors for miR-21 and miR-100, upregulated in aHSC, and mimic-miRNAs for miR-192, associated to qHSCs, were transfected in cultured HSC demonstrating their role in the regulation of key genes involved in HSC activation and fibrogenic phenotype. Integrated analysis of miRNA and gene expression profiles of qHSC and aHSC identified a miRNA signature of hepatic stellate cell activation and potentially liver fibrosis.

Epigenome of fresh and cultured hepatic stellate cells: To evaluate whether activation of HSCs can be correlated with methylome changes we compared changes in the promoter methylome of aHSCs vs qHSCs. MeDIP-chip reveals 5,862 methylated genes in qHSCs and 5,191 in aHSCs. We identified a core overlap of ~50% of all methylated genes in either qHSCs or aHSCs. We further noted a net reduction in promoter methylation in aHSCs, with demethylation of 3,102 promoters (53% of all methylated promoters before culture), including several different members of the collagen and lysyl oxidase gene families, the main constituents and enzymatic stabilisers of fibrotic scar tissue. 47% of all methylated promoters in aHSCs was *de novo* methylated. We furthermore investigated the correlation between DNA methylation and gene expression and found 416 genes with concordant changes in DNA methylation and gene expression upon HSC activation. For these genes, transcriptional upregulation correlated with abrogation or reduction in promoter methylation, as shown for *ACTG2*, *LOXL1*, *LOXL2* and *COL4A1/2*. Conversely, transcriptional downregulation among these 416 genes was associated with DNA hypermethylation (*APOB*, *ADAMTS9*, *MMP15* and *CXCL9*; *El Taghdouini et al., 2015a*).

Liver Sinusoidal Endothelial Cells

To provide a possible source of endothelial cells (ECs) for the bioreactor, we established three methods to isolate ECs from human liver biopsies. Two methods were based on FACS sorting using combinations of positive (Tie2 or CD32B) and negative (CD45, podoplanin and/or UV) selection markers and 1 was based on macromolecule uptake, a distinguishing functional feature of LSECs. While the FACS-based methods were suitable for (comparative) transcriptomic profiling, LSECs could only be cultured when isolated by the function-based method. The transcriptome of sorted LSECs was compared to that of stellate cells and hepatocytes (*El Taghdouini et al. 2015a*), as well as to ECs from two other organs in order to define a unique LSEC reference gene signature. Like for hepatocytes, a major bottle-neck in obtaining sufficient numbers of primary LSECs is their limited expansion capacity. To overcome this problem, functionally isolated LSECs were treated with the UpCyte® technology, which significantly prolonged their proliferation capacity (up to 40 population doublings). Quantitative analysis of bona-fide LSEC marker gene expression however revealed that their LSEC marker expression dramatically dropped, and that this loss could only partially be prevented by metabolic arrest. Also the use of epigenetic modifiers (*e.g.*, HDAC inhibitors) did not prevent dedifferentiation. As a means to restore the gene signature, a method based on chemical induction was tested, but this only partially reinduced the expression of some LSEC markers, including *STAB2* and *LYVE1*. Nevertheless, upon improving the function-based derivation protocol (*e.g.*, by shortening the procedure's duration), dedifferentiation was less dramatic and scavenger activity remained significantly higher than in the currently available human LSEC lines from commercial sources and these cells retained fenestrations, a morphological hallmark of LSECs.

Pluripotent Stem Cell Derived Cells

Hepatocytes

Progressively improved methods to create hepatocytes from PSCs were developed within ***HeMiBio***. As shown in *Figure 4.22*, this included optimising the culture medium, the type of growth factors added, and mimic the vena porta blood composition early after birth. Despite these optimisations, the final cells generated from PSCs did not attain the maturity of primary adult liver derived hepatocytes, for which we term the cells hepatocyte like cells (HLCs). Nevertheless, we demonstrated that cells can be used for toxicity studies including molecules shortlisted by the **SEURAT-1** Gold Compound Working Group (see section 4.9.5). In addition, we demonstrated that the HLCs can be infected with different hepatotropic viruses.

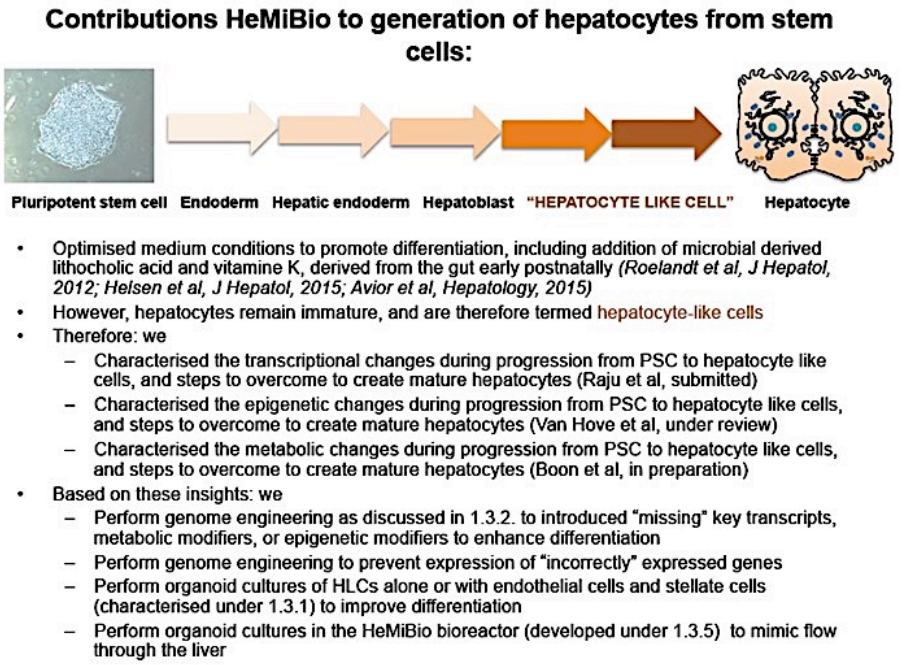

Figure 4.22 *Hepatocyte differentiation from PSCs: Maturity attained is not fully similar to primary hepatocytes. Hurdles in the differentiation process have been identified which are now being addressed using different tools developed under* ***HeMiBio****.*

In view of the fact that the HLCs were not fully comparable with primary hepatocytes, extensive '-omics' studies were done to characterise the progressively maturing hepatocytes and compare the final hepatocyte like cells with primary hepatocytes. Based on these insights ongoing studies are testing whether addressing the different hurdles present to create more mature functional hepatocytes can be addressed using for instance the genome engineering tools developed in ***HeMiBio*** (see section 4.3.3.2).

In addition, with the creation of the ***HeMiBio*** bioreactor, as well as the characterisation of liver sinusoidal endothelial cells and stellate cells (see above), ongoing studies are evaluating if co-culture of the HLCs with these non-parenchymal cells in 3D organoids, in the ***HeMiBio*** bioreactor (see below), will allow full maturation of HLCs.

Hepatic Stellate Cells

Differentiation of pluripotent stem cells to hepatic stellate cell-like cells: Pluripotent stem cells were directed to mesodermal cells, followed by the acquisition of a mesenchymal phenotype, mesothelial and finally the HSC-like phenotype. Flow-cytometry analysis showed that along differentiation, the percentage of PDGFR-beta cells (a good marker for HSCs) increased, reaching a maximum of ± 80% at day 12, suggesting that the majority of the differentiated cells have already acquired the HSC phenotype.

iPSc-derived HSC-like cells characterisation: iPSC-differentiated HSC-like cells expressed ACTA2, vimentin, PDGFRbeta, NGF and NCAM at a comparable level to primary HSC, as well as ALCAM, desmin, glial fibrillary acidic protein (GFAP), PDGFRα, PDGFRβ, peroxisome proliferator-activated receptor (PPAR)γ, synaptophysin than primary HSC. Interestingly, markers associated with HSC activation such as ACTA2 and COL1α1, where expressed at a lower level in differentiated HSC-like cells, suggesting that their level of activation may be lower and could better resemble an intermediate activated phenotype with more quiescent cell features.

Transcriptomic analysis of PSC-derived HSC-like cells: Transcriptomic analysis of primary fresh and activated HSCs and PSC-derived HSC-like cells were performed at passage 1. *Figure 4.23* shows a clear clustering of each group of cells. Moreover, PSC-derived HSC-like cells showed an intermediate phenotype between quiescent and activated HSCs, although they clustered more closely to cultured activated HSC, suggesting that this expression profile could be due to culture conditions. Hierarchical cluster analysis also demonstrated as from the hepatocytes described in the section above, that PSC-derived HSC-like cells differ from primary quiescent HSCs (data not shown). These differences may be related to an incomplete differentiation or to the culture effect, since quiescent HSC where analysed directly after isolation from human liver tissue.

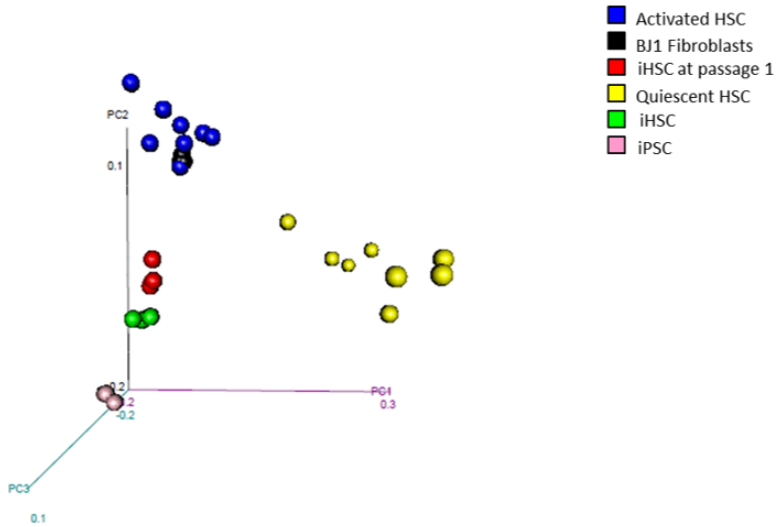

Figure 4.23 *Transcriptomic analysis of iPSC-derived HSC. 3D principal component analysis (PCA) showing distances between the transcriptomic profiles of different human cell types. Human samples are placed in a three-dimensional space according to their transcriptomic signature. Activated HSC are represented in blue (n=7), quiescent HSC in yellow (n=7), iPSC-derived HSC in green (n=3), cultured iPSC-derived HSC at passage one in red (n=3), iPSCs (n=3) in pink and parental fibroblast BJ1 in black (n=3).*

Liver Sinusoidal Endothelial Cells

We found that exposure of pluripotent stem cells (PSCs) to conditions favoring hepatocyte differentiation (*Roelandt et al., 2012*) also generated cells with expression of EC markers, including Tie2 and CD31. Characterisation of the EC component isolated from hepatic PSC differentiation cultures after 3-4 weeks (based on the FACS sorting protocols designed for primary LSEC derivation described above), revealed that the yield of ECs was low (not more than 1% of total cells was $Tie2^+CD31^+$). After 45 days, no $Tie2^+$ cells remained. The expression profile of the $Tie2^+CD31^+$ cell fraction was immature, as most of the LSEC markers (*e.g.*, *LYVE1*, *STAB1*, *STAB2*, *CLEG4G*, *L-sectin*) were not or only marginally expressed. The resulting EC component was also heterogeneous, with some cells occasionally expressing CD32B protein, a scavenger receptor uniquely expressed in LSECs. Those cells sorted based on CD32B protein surface expression had a different gene signature from those sorted based on the Tie2/CD31 combination. Whether this differential expression pattern represents the existence of LSEC zonation, a phenomenon well-documented for hepatocytes, remains to be determined. To improve the yield of ECs, therefore, an alternative differentiation method was designed wherein PSCs are committed to mesoderm and then to ECs. This resulted in a significant enrichment (up to 60%) of KDR^+ mesodermal progenitors, which then could be fated to $CD31^+$ ECs by exposure to EC growth factors, with a yield of up to 25% of total cells. Currently, the system is being optimised to limit contamination with fibroblasts that tend to overgrow the differentiated EC cultures. While this $CD31^+$ fraction already showed a significant induction in expression of bona fide LSEC markers (*e.g.*, *CD32B*, *L-SIGN* and *L-sectin*), currently a protocol based on forced transcription factor overexpression from the *AAVS1* locus (*Ordovas et al., 2015*) might boost the specification process.

Upcyte® Hepatocytes

Although upcyte® hepatocytes were already generated before the start of the project, in the course of the project the process to generate upcyte® hepatocytes has been improved and upcyte® hepatocytes have been generated from multiple donors. In addition upcyte® hepatocytes have been extensively characterised with respect to functional activities (phase I, phase II, transporter activities), epithelial markers and application in toxicity testing (*Levy et al., 2015*). It was shown that these cells exhibit similar metabolic functions to primary plated hepatocytes.

Upcyte® hepatocytes from different donors were generated at Medicyte produced as working cell bank and delivered to ***HeMiBio*** partners. The cells were further characterised with respect to the response to hepatotoxic chemicals from the **SEURAT-1** list of standard reference compounds (see section 4.9.5). Upcyte® hepatocytes derived from different donors differed only slightly in their response to the compounds. All compounds could be correctly classified as non-toxic, moderate and toxic compounds which is in accordance with *in vitro* data from primary hepatocytes.

Comparison of whole genome expression profiles of primary hepatocytes and upcyte® hepatocytes revealed difference in the range of 1-2% of genes that were found to be more than twofold up- or downregulated. A significant portion of the affected genes are involved in cell growth which is one of the main differences in primary versus upcyte® hepatocytes. Comparing upcyte® hepatocytes from the same donor cultured in two different labs revealed almost no statistical difference (0.25% of more than 2-fold differentially regulated genes).

Generation of Liver Sinusoidal Endothelial Cells from Other Cell Populations

Since we anticipated that using primary LSECs or LSECs differentiated from PSCs may be subject to practical difficulties, we also worked in parallel on exploring alternative sources to generate the EC component of the liver bioreactor. We optimised a protocol to derive endothelial progenitors from the mononuclear fraction of peripheral blood and cord blood, known as blood outgrowth endothelial cells (BOECs). These cells have very low expression levels of LSEC-specific markers and do not exhibit uptake of macromolecules nor do they have fenestrae. Although BOECs have a significantly increased expansion potential compared to primary ECs, the number of passages they can undergo is too limited to make the cells amenable to genome engineering to build in molecular sensors, as we planned in PSCs. Therefore, BOECs were successfully treated with the UpCyte® technology such that passage frequency was increased up to 40 without alteration of EC marker expression. Before using these cells as a substrate for LSEC generation, approaches to induce an LSEC gene signature were first tested in human umbilical vein ECs (HUVECs). The chemical induction protocol was slightly less efficient in inducing LSEC marker expression in BOECs as compared to LSECs treated with the UpCyte® technology (see above). A second approach based on forced overexpression of a set of transcription factors identified during the comparative transcriptomic profiling in different EC types from different organs could serve as an alternative.

4.3.3.2 Genome Engineering of iPSC

Tools for rapid and efficient transgenesis in ‘safe harbor’ loci in an isogenic context remain important to exploit the possibilities of human pluripotent stem cells (hPSC). We created hPSC master cell lines suitable for FLPe recombinase-mediated cassette exchange (RMCE) in the *AAVS1* locus that allow generation of transgenic lines within 3-4 weeks with 100% efficiency and without random integrations. Using RMCE, we successfully incorporated several transgenes useful for lineage identification, cell toxicity studies, and gene over-expression (*Figure 4.24; Ordovás et al., 2015*).

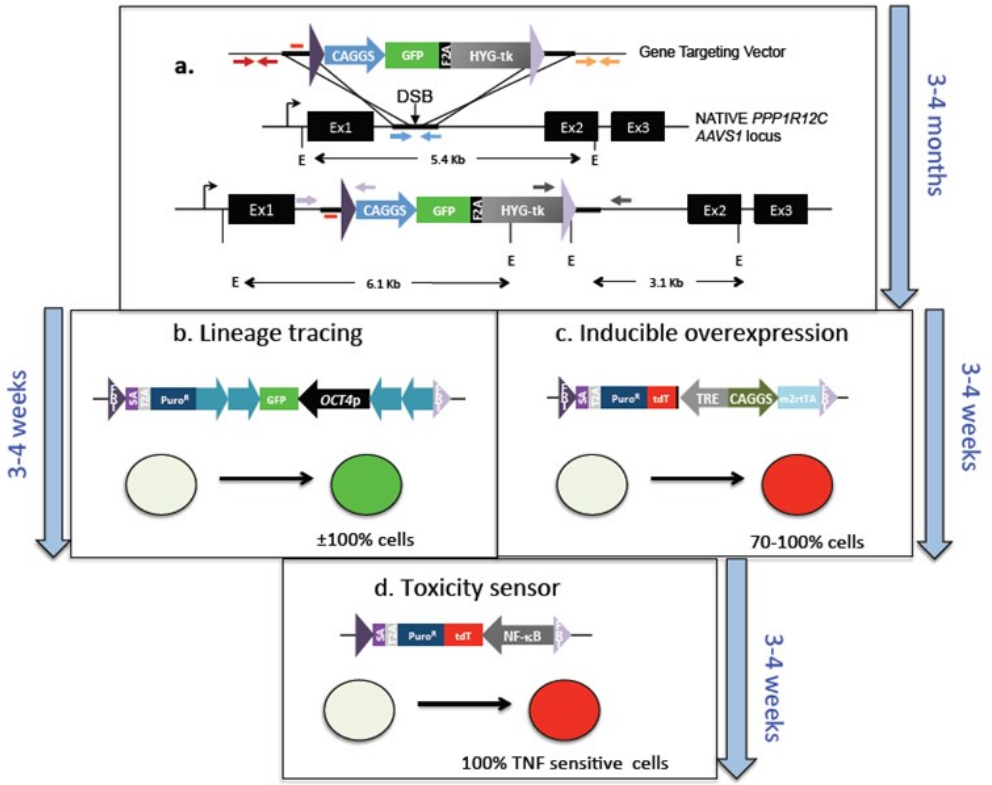

Figure 4.24 *Creation of master cell line as well as lineage tracing, inducible over-expression and toxicity sensor line by incorporation of a Flippase cassette in the AAVS1 line: (a) Creation of the master cell line using zinc finger nucleases and a FRT flanked cassette including a positive selectable cassette as well as the thymidine kinase cassette, (b) Incorporation of an Oct4 promoter by flippase recombination allows identification of ±100% of pluripotent stem cells, (c) incorporation of an inducible tDt cassette allows for induction of 70-100% of the included gene sequence, (d) Incorporation of an NfkB sensor allows faithful detection of TNFα toxicity.*

4.3.3.3 Electrophysical Sensors

O_2 Sensor

The integrated optical oxygen sensor is based on fully biocompatible microbeads of polystyrene with a diameter of 50 μm (CPOx-50-RuP, Colibri Photonics, Germany). The beads are equipped with an oxygen-sensitive, phosphorescent ruthenium-phenanthroline-based dye. As oxygen acts as a quencher and leads to a decrease in decay time and signal intensity with increasing concentration (*Figure 4.25A*), the measurement of the decay time allows the

determination of the ambient oxygen concentration. We chose to measure decay time rather than signal intensity, as the decay time is not sensitive to changes in probe concentration or excitation intensity over the course of the experiment, i.e. insensitive to loss of optical focus (*Figure 4.26*). To this end, the OPAL system (Colibri Photonics, Germany) was used for phase modulation, providing sinusoidal amplitude-modulated green light (532 nm) for excitation (*Figure 4.25B*). Emission light is shifted in phase due to oxygen quenching (*Figure 4.25B*). In-phase background signals were eliminated using two-frequency phase modulation, i.e. excitation was performed with two frequencies in superposition.

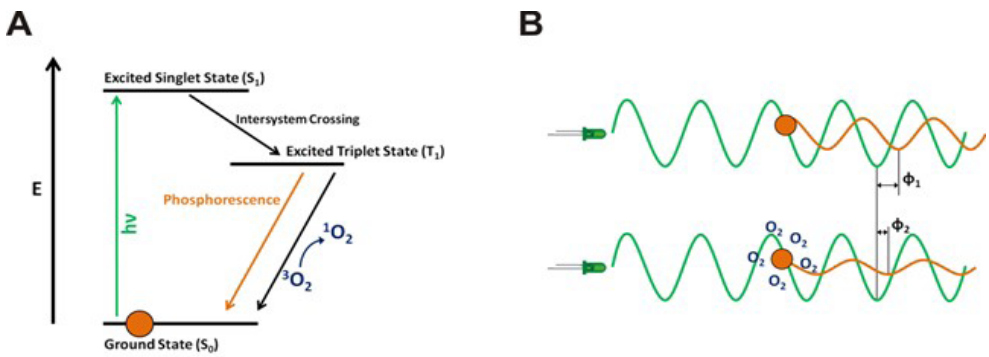

Figure 4.25 *Principle of CPOx-50-RuP-based phosphorescence under modulated excitation. (A) Jablonski Diagram describing the energetic pathway of phosphorescence generation. The phosphorescence signal of the probe is emitted with a delay given by the lifetime of the excited triplet state T_1 of the dye molecule. Oxygen acts as a quencher as it is converted from ambient triplet to singlet oxygen trough energy uptake. This leads to a decrease of the decay time and signal intensity with rising oxygen concentration. (B) Scheme of the sinusoidally intensity-modulated excitation signal (green) used in the presented system to determine the decay time in real time. The emission signal (orange) exhibits an oxygen concentration-dependent phase shift with respect to the excitation signal. The shift can be correlated to the decay time and, thus, is used to determine the ambient oxygen concentration.*

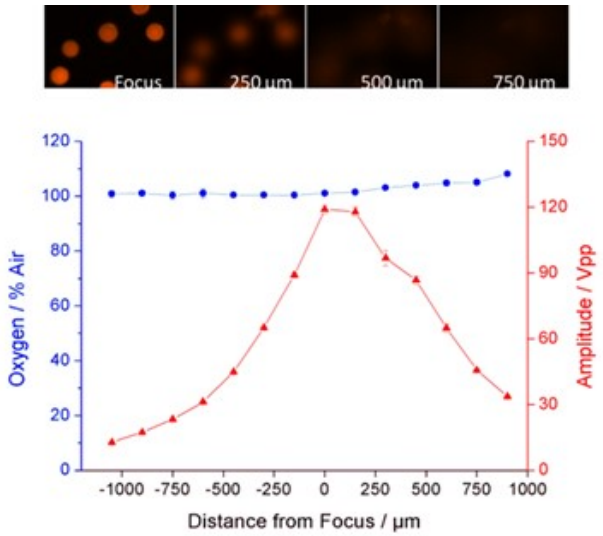

Figure 4.26 *Phosphorescence decay time-based oxygen measurement is independent of signal intensity. Stepwise changing of the optical focus, leading to consecutively less signal intensity, has no influence on the measured oxygen concentration (nominal oxygen concentration atmospheric, i.e. constant).*

ALT Sensor

An amperometric sensor has been developed for the detection and the quantification of ALT. The sensor's technology is based on screen-printed electrodes. The equations below report the principle of detection of the ALT sensor: ALT generates glutamate in presence of the L-alanine and α-ketoglutarate. Thus when L-alanine and α-ketoglutarate are added in the proper concentrations, this enables a linear relation between the activity of ALT and the concentration of glutamate. The detection principle relies on the detection of glutamate through the reaction catalysed by glutamate oxidase, thus making the ALT sensor an indirect sensor.

$$\text{L-alanine} + \text{α-ketoglutarate} \xrightarrow{ALT} \text{L-glutamate} + \text{pyruvate}$$

$$\text{L-glutamate} + O_2 + H_2O \xrightarrow{Glutamate\ oxidase} \text{α-ketoglutarate} + NH_3 + H_2O_2$$

ALT activity has been measured in HepG2 cell lysate as a function of cell concentration. As shown in *Figure 4.27*, it is possible to detect ALT from lysate for a concentration of cells of approx. 1.10^6 cells/mL, with a corresponding activity of ALT in the order of 1.5U/L. It has to be noted that the measured ALT concentration is low compared to most *in vivo* data found in literature. However, literature data on ALT concentration *in vitro* vary from units per liter to thousands of units per liter, making a significant comparison difficult.

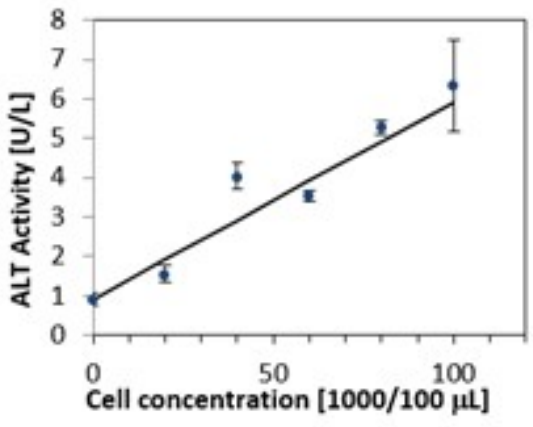

Figure 4.27 *ALT measurements in HepG2 cell lysate. ALT activity is plotted as a function of cell concentration (in thousands per 100 µL) in the lysate. All samples were tested in triplicates.*

Incorporation of Sensors

Sensors and Switchboard

The oxygen-sensing microbeads were integrated directly into the reactor-internal cell culture compartment. This was realised by employing an optimised protocol for cell seeding leading to a three-dimensional, collagen-based cell culture matrix. By this, real-time oxygen detection in the direct micro-environment of the cells was possible in a non-invasive fashion (*Figure 4.28A*). Commercially available electrochemical sensors for glucose and lactate detection in whole blood (BST GmbH, Germany) were adapted for the use in a cell culture environment by transferring them into a microfluidic flow cell (*Figure 4.28B*). The latter was addressed with cell culture medium using a fully automated fluidic system consisting of several solenoid valves and peristaltic pumps (*Figure 4.28C*). Thus, electrochemical signals were automatically recorded and stored for subsequent data processing steps.

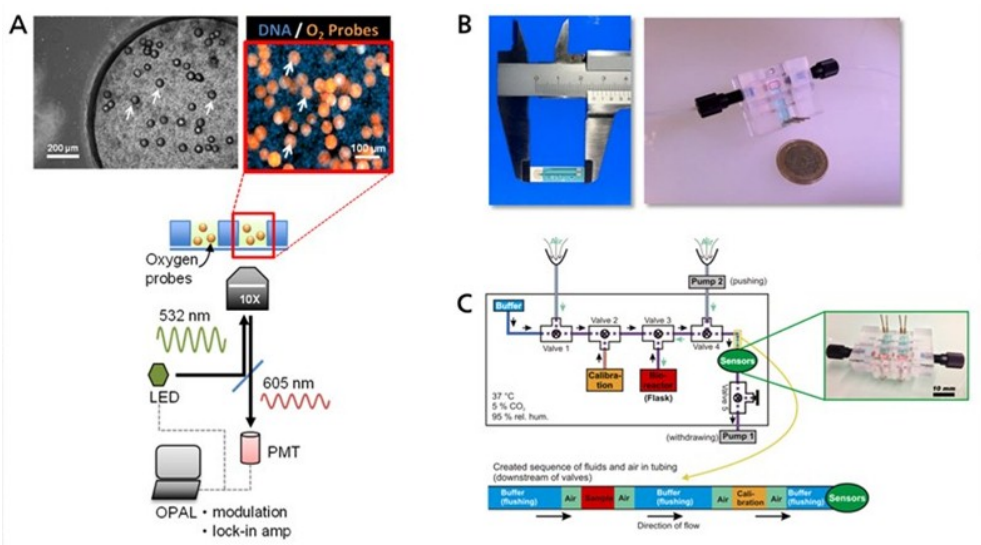

Figure 4.28 *Integrated sensors. (A) Scheme of the measurement setup connected to the microscope, comprising an electronic control unit for signal modulation and readout, a LED for excitation, an optical filter set (531/40, 555, 607/70 nm), and a detector unit containing a photomultiplier (PMT). (B) Electrochemical, enzyme-based sensors for glucose and lactate. (C) Automated fluidic system for cell culture medium sample handling and measurement.*

To overcome flow and shear fluctuation complicating sensor synchronisation, we designed an equi-pressure combinatorial mixer composed of a microfluidic switchboard and a passive mixer. The microfluidic switchboard consists of 11 inlets and a common outlet that are regulated by self-addressable micromechanical valves. The valves are controlled by one pressure manifold, delivering precise combinations of fluids to the mixer unit. Flow is driven by positive pressure provided by a second, independently controlled pressure manifold.

Using this setup we were able to automate sampling and calibration of a lactate electrochemical sensor and monitor hepatic toxicity, in real time. Human Huh7 cells were seeded in a microfluidic chamber, producing a confluent monolayer. Cells were exposed to 100 µM of rotenone. Perfusate was connected to a high resistance waste syringe and a single inlet of the microfluidic switchboard for automated sampling (*Figure 4.29a*). We used the microfluidic switchboard to perfuse a sequence of buffer, air, sample and air over an enzymatic-amperometric lactate sensor every 2 h (*Figure 4.29b-c*). Introduction of air acts as a diffusion barrier preventing sample contamination and providing zero-point calibration. Between each experiment a calibration medium was introduced to correct sensor drift. The electrochemical sensor was connected to an embedded potentiostat. A fully automated 16.5 h experiment was programmed allowing the derivation a time-of-death of 5.5 h (*Figure 4.29d*).

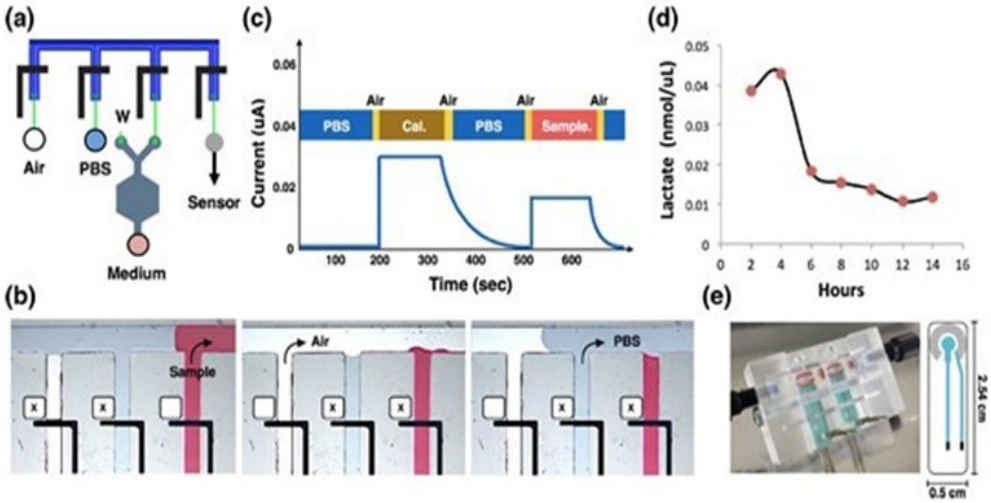

Figure 4.29 *Real time monitoring of hepatic toxicity using automated sampling and calibration of electrochemical sensors.* ***(a)*** *Flow system schematics: microfluidic switchboard (blue) connected to air (white), phosphate buffer saline (gray), single outlet of the liver bioreactor, and the electrochemical lactate sensor (green). The second outlet of the liver bioreactor is connected to waste (W). Control valves are shown in black.* ***(b)*** *Sequential addition of sample (red), followed by air purging (white), and washing buffer (blue). Bar = 200 µm.* ***(c)*** *Readout from the sensor for a sequence of calibration fluid, buffer, air and a sample. The output current is proportional to the fluid lactate concentration.* ***(d)*** *Real time measurement of lactate production in perfusate of liver bioreactor exposed to 100 µM rotenone. Huh7 cells die within 5.5 h of exposure to the pesticide.* ***(e)*** *Image and schematics of the microfluidic lactate sensor and its low-volume PMMA housing.*

In summary, we demonstrated the generation of complex perfusion sequences, by connecting an electrochemical lactate sensor downstream of a liver bioreactor exposed to the environmental toxin rotenone. Our system permits automated sampling, washing, and calibration of the electrochemical sensor, providing continuous real-time measurement of cell viability.

Automated Fluidic Handling Interface

A fluidic handling system capable of pumping, storing and delivering the sample automatically was fabricated and tested. The system also allows washing of the sensing module as well as calibration by means of respective solutions of standard reference compounds (*Figure 4.30)*. The interface was designed to handle small volumes of samples. Furthermore, compatibility with the use as part of the ***HeMiBio*** bioreactors (see below) was another important boundary condition.

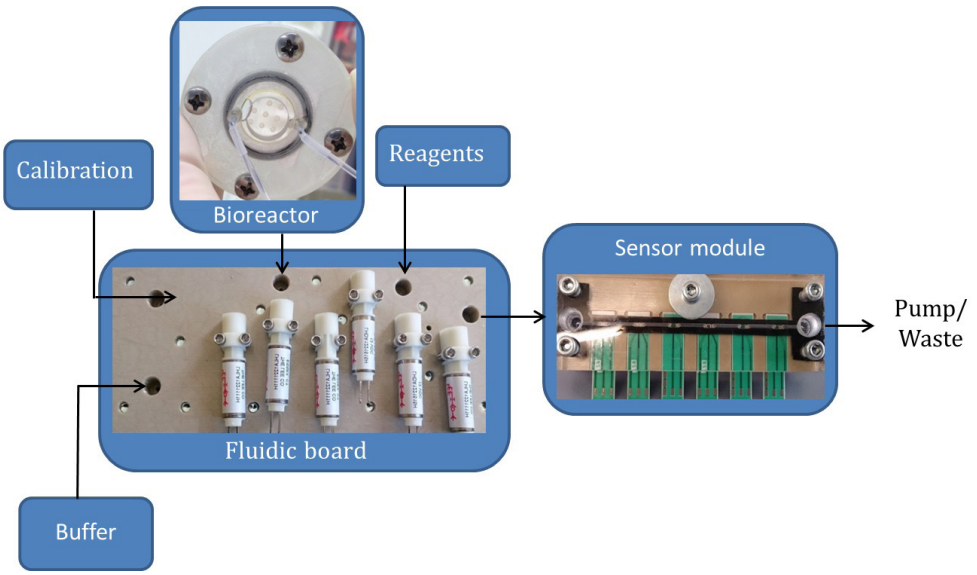

Figure 4.30 *Scheme of the automated bioreactor monitoring setup.*

The automated fluidic handling interface was used to monitor glucose, lactate and ALT in the supernatant flowing out of the ***HeMiBio*** flow-over bioreactor, where a HepG2 cell culture was exposed to rotenone at a concentration of 200 μM. Concentrations profiles of glucose, lactate, pO_2 and ALT were successfully recorded over five hours after the application of rotenone with a sampling rate of 1 sample/hour. Cell necrosis was clearly observed as indicated by the decreasing oxygen consumption of the cells. At the same time, the ALT concentration signal increased, indicating cell death (*Figure 4.31*).

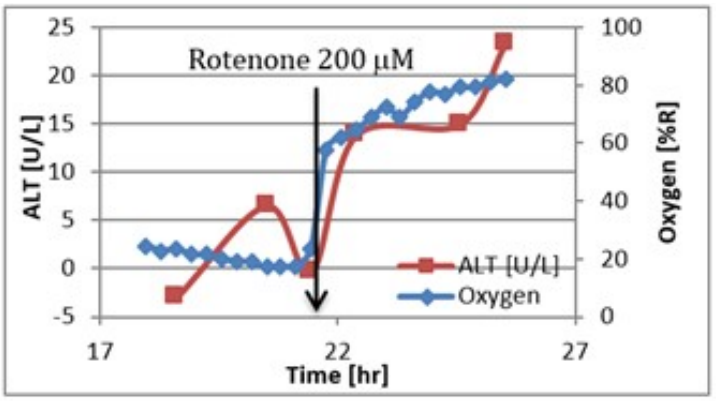

Figure 4.31 *Concentration profile of pO_2 and ALT in the **HeMiBio** bioreactor with a HepG2 cell culture before and after the exposition of the cells to 200 µM rotenone.*

4.3.3.4 Design of Bioreactors

Patterning Bioreactor

Cell-cell interactions play a key role in regeneration, differentiation and basic tissue function taking place under physiological shear forces. However, previous solutions to mimic such interactions by micro-patterning cells within microfluidic devices have low resolution, high fabrication complexity, and are limited to one or two cell types.

We designed and developed an antibody-based, microfluidic system capable of patterning any biotin-conjugated set of antibodies using streptavidin-based surface chemistry. The design permits the generation of arbitrary cell patterns from heterogeneous mixtures in microfluidic devices. This system is therefore capable of one-step cell isolation and patterning in microfluidic devices permitting the generation of tissue micro-patterns from complex cell mixtures with minimal sample preparation. Our approach can be broadly employed to combine cell isolation with micro-patterning. The significance of such a combination is the ability to directly inject a complex starting sample such as whole blood or tissue digest into a patterning device with minimal sample preparation, resulting in functional, perfusable tissue on a chip. Using this system we were able to demonstrate robust co-patterning of α-CD24, α-ASGPR-1 and α-Tie2 antibodies for rapid isolation and co-patterning of mixtures of hepatocytes and endothelial cells (*Figure 4.32*). In addition to one-step isolation and patterning, our device permits step-wise patterning of multiple cell types and empty spaces to create complex cellular geometries *in vitro*.

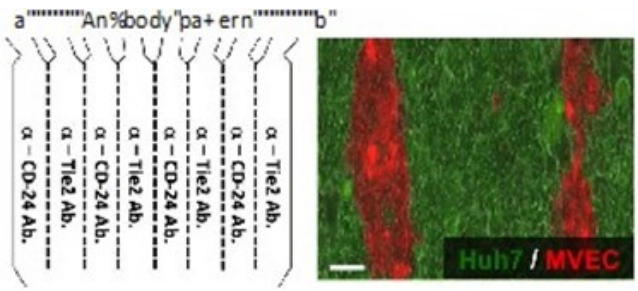

Figure 4.32 (a) *Schematic diagram of antibody pattern used to capture and co-pattern hepatocytes and endothelial cells under flow.* ***(b)*** *Capture and co-patterning of Huh7 and MVEC on an anti-CD24/Tie2 patterned device at shear stress of 0.8 Pa. Under these conditions Huh7 show preferential binding. Scale bar = 100 µm.*

Flow-over Bioreactor

Microfluidic liver-on-chip devices offer an alternative to animal experiments as they can mimic the native microenvironment and support long-term function under continuous perfusion. One

critical advantage of microfluidics is the ability to expose cells to a stable stimulation over time, eliminating the rapid loss of signal due to nonspecific adsorption and metabolism that characterises both static *in vitro* assays and *in vivo* experiments

Bioreactor Design and Characterisation

The liver is highly vascularised, delivering oxygen at rates of 0.9 nmol/s/10^6 cells, while at the same time protecting hepatocytes from the negative effects of shear forces. The gradient of oxygen that develops along the sinusoid is thought to induce a demarcation of function, termed *metabolic zonation.* To mimic this environment, we designed a stainless steel bioreactor that protects hepatocytes from shear forces while creating stable oxygen and nutrient gradients mimicking the *in vivo* zonated microenvironment (*Figure 4.33A-G*). HepG2/C3A cells were suspended in collagen gel prior to seeding directly in the microwell insert (*Figure 4.33C*). The small diameter of the microwells leads to rapid aggregation, creating liver spheroids after overnight incubation. The inserts were physically sealed in the bioreactor and continuously perfused thereafter. Computation fluid dynamic modeling (*Figure 12D-G*) showed physiological shear forces under 0.1 Pa inside the microwells for perfusion rates of 10 µl/min. The high flow rate resulted in similar oxygen concentration delivered to each well in the array (*Figure 4.33E, F*). Oxygen consumption caused a gradient to develop along the perfused spheroid mimicking the *in vivo* microenvironment.

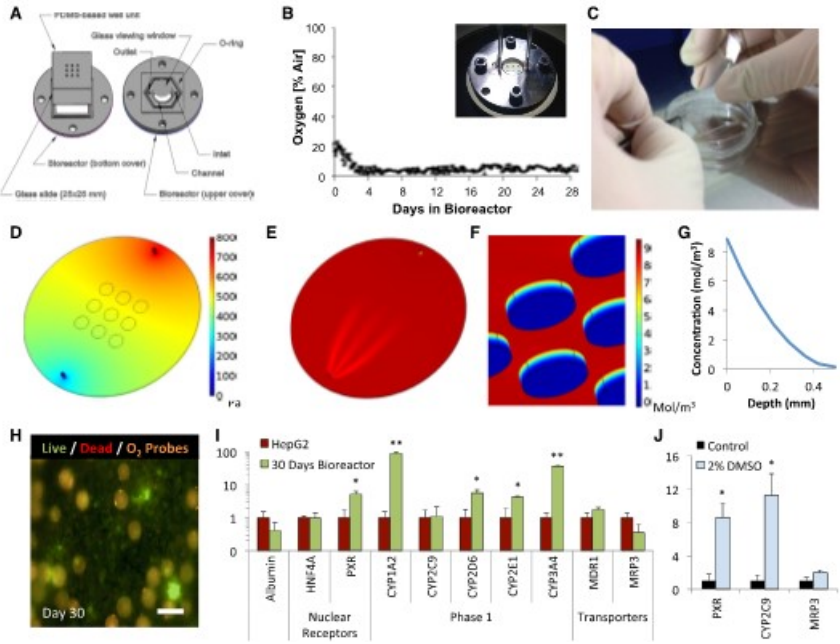

Figure 4.33 (A) *Bioreactor components in overview. Left bottom part with PDMS microwell insert on glass substrate; top view. Right top part with glass coverslip for optical accessibility also from top, recess for O-ring and perfusion inlet/outlet; upside down view.* ***(B)*** *Long-term oxygen measurement over 1 month in bioreactor perfused with fresh cell culture medium at 10 µl/min. 100 % air represents atmospheric dissolved oxygen concentration (21 % O2 = 210 µmol/l; no consumption).* ***(C)*** *Photograph taken during microwell insert seeding procedure.* ***(D)*** *Numerical simulation of the shear stress magnitude and* ***(E, F)*** *oxygen concentration along the bioreactor.* ***(G)*** *Oxygen concentration variations within the well (from top to bottom), mimicking the in vivo microenvironment.* ***(H)*** *Fluorescent staining for metabolically active cells (green) and dead nuclei (red) after 28 days of continuous perfusion in the bioreactor. Ru-CPOx beads emit orange. Bar 100 µm.* ***(I)*** *Log-scale gene expression analysis of HepG2/C3A cells in static culture compared with those perfused for 28 days. PXR and CYP3A4 expression shows a fivefold and 36-fold increase in the bioreactor, respectively.* ***(J)*** *Cells exposed to 2 % DMSO for 28 days show marked elevation of PXR and its target genes CYP2C9 and MRP3. Error bars indicate standard deviation for all panels. *p < 0.05, **p < 0.01 by student t test.*

Long-term Maintenance of Perfused Spheroids

HepG2/C3A cells were maintained for over 28 days *in vitro*, while displaying over 98% viability (*Figure 4.33H*) and high expression of liver specific markers including CYP450 enzymes as

compared to static culture (*Figure 4.33I*). Remarkably, CYP3A4 showed a 36-fold higher expression than in static culture, reaching 6% of primary human hepatocyte level (*Figure 4.33I*) Finally, continuous exposure of the HepG2/C3A spheroids to 2 % DMSO for 28 days resulted in a further eightfold increase in PXR expression, and an associated twofold and tenfold increase in its target genes CYP2C9 and MRP3, respectively (*Figure 4.33J*).

3D Bioreactor

In the framework of ***HeMiBio***, a 3D microfluidic bioreactor for hepatic cell cultures was developed. The bioreactor is constructed using Cyclic Olefin Copolymer (COC), since the material has good chemical resistance, low adsorption and good optical properties, including low auto-fluorescence. To guarantee these desirable properties for the finished bioreactor, after structuring the microchannels, the COC is bonded without adhesives. Two parallel approaches were developed for structuring the COC. In a first approach, mechanical micro-milling of the channels allows for extremely fast manufacturing of new design variations, at the expense of difficulties in scalability to mass-production and a channel surface that requires post-processing to achieve sufficient optical quality. In a second approach, hot embossing using epoxy molds allows for direct structuring of optical grade channels and is scalable to mass production, at the expense of longer cycle time in the development of new channel designs. In both cases, the structured COC is bonded using thermocompression to seal the channels. The design of the bioreactor was intended to maintain and expose pre-formed hepatic co-culture spheroids to chemicals for more than a week. Once seeded, spheroids rest on a polycarbonate porous membrane, allowing the medium to flow-through, while flow-over is maintained to avoid an excess pressure on the cells. In a single bioreactor, 9 wells are connected to a common inlet to provide the cells with fresh culture medium or test compounds (*Figure 4.34*). Due to the challenging nature of lab-scale structuring and bonding of COC, the testing of the 3D bioreactor experienced more delay than expected, and testing with hepatic cells was limited to some small scale initial trials. Significant progress has been made towards producing a 3D COC-based bioreactor for hepatic cell culture, and most technological hurdles in producing prototype reactors have been overcome. Further testing is needed to see which improvements to the reactor design or the flow conditions should be made to ensure cell viability.

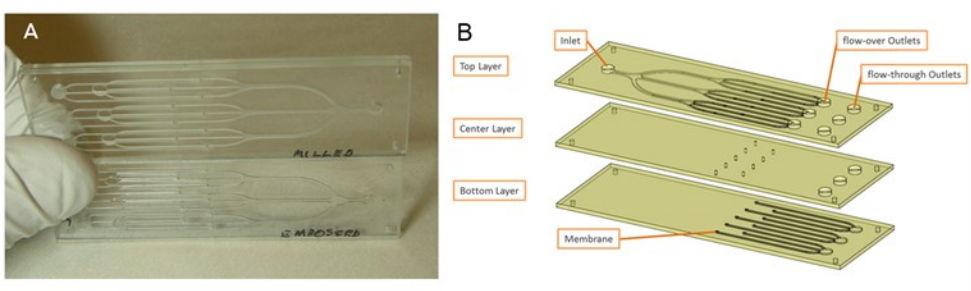

Figure 4.34 A *Camera picture of assembled milled (top) and embossed (bottom) 3D bioreactor.* ***B*** *Exploded view of the 3D bioreactor design.*

4.3.3.5 Toxicity Testing

Use of Upcyte® Hepatocytes for Toxicological Evaluation

As discussed above, hepatocytes treated with the UpCyte® technology are functionally polarised cells with albumin production, phase I and II gene expression, and CYP450 activity similar to those of primary human hepatocytes. These hepatocytes retain the ability to modulate CYP450 activity, and expression through nuclear receptor activation, with different isolates exhibiting variable responses.

To assess the ability to predict toxicological outcomes, we determined the concentration that causes 50% cell death (TC50) over a 24h exposure to known hepatotoxic compounds (*Figure 4.35a*). A permutation test revealed that the TC50 toxicity profile of hepatocytes treated with the UpCyte® technology was not significantly different from that of primary hepatocytes.

Second, we compared the toxicological profile of proliferating and differentiated hepatocytes of the same passage (*Figure 4.35b-h*). The TC50 of compounds clustered into three groups. One group showed no difference between proliferating and differentiated cells (*Figure 4.35b*). The second group showed significantly higher toxicity in differentiated hepatocytes than in proliferating hepatocytes, suggesting metabolic activation was required for toxicity (*Figure 4.35c*). Surprisingly, the three control compounds clustered into a group in which toxicity was higher in proliferating than in differentiated hepatocytes (*Figure 4.35d*). Proliferating hepatocytes were affected by concentrations as low as 200 µM for mannitol (*Figure 4.35e-h*).

To test for adverse outcome pathways (see section 4.3.4), we utilised TC20 concentrations of each drug (*Figure 4.35i*). We quantified bile secretion as a measure of cholestasis (*Figure 4.35j*). All three cholestasis drugs caused morphological changes leading to loss of bile secretion. Exposure to the steatosis-causing agents caused a 33–47% increase in intracellular lipids compared to control (*Figure 4.35k*). Finally, exposure of the cells to apoptotic drugs caused 3- to 6-fold increases in apoptosis compared to the control (*Figure 4.35l*). These data confirm the ability of hepatocytes treated with the UpCyte® technology to assess the toxicity of hepatotoxic compounds.

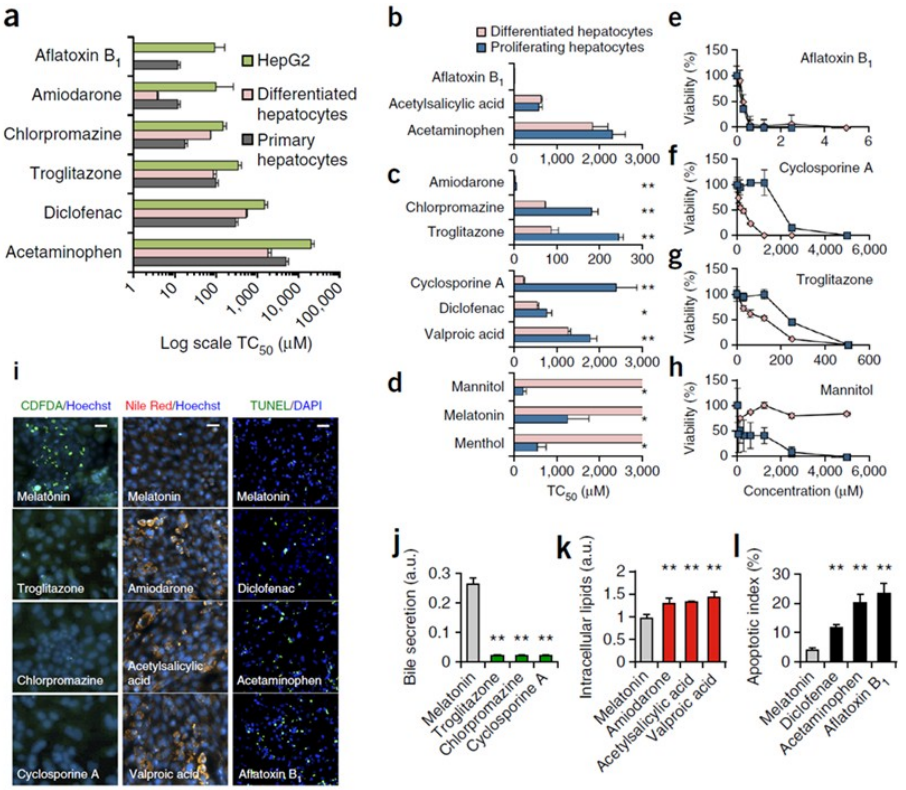

Figure 4.35 *(**a**) TC50 values of six chemical compounds obtained from 24-h dose-response studies in differentiated hepatocytes, HepG2 cells and cryopreserved primary human hepatocytes. The normalised TC50 toxicity profile of differentiated hepatocytes was not significantly different from that of primary human hepatocytes (P = 0.466; n = 4), whereas the HepG2 profile was significantly different (P = 0.030; n = 3). (**b**–**d**) TC50 values of metabolically functional differentiated hepatocytes and proliferating hepatocytes of the same genotype and passage number (n = 4). *P < 0.05, **P < 0.01 (**c**,**d**). (**e**–**h**) Dose-dependent toxicity curves of differentiated hepatocytes treated with various compounds, compared with proliferating hepatocytes of the same genotype and passage number. (**i**–**l**) Fluorescence quantification of adverse outcome pathway in differentiated hepatocytes. (**i**) Loss of bile acid production (cholestasis) evaluated by CDFDA staining, lipid accumulation (steatosis) by Nile Red staining and apoptosis by TUNEL labelling of nuclei. (**j**) Cholestasis in differentiated hepatocytes exposed for 24 h to cholestasis-causing drugs or melatonin (negative control) (**P < 0.01; n = 3). (**k**) Steatosis in differentiated hepatocytes after 48 h of exposure to steatosis-causing drugs or melatonin (**P < 0.01; n = 3). (**l**) Apoptosis of differentiated hepatocytes following 24 h of exposure to apoptosis-causing drugs or melatonin (**P < 0.01; n = 4). Data are from donor 653. a.u., arbitrary units; all n values represent the number of biological samples analysed per condition. All error bars indicate ± s.d.*

PSC Hepatocytes for ToxicityTesting

Human pluripotent stem cells (hPSCs) represent an excellent source of differentiated hepatocytes; however, previously developed protocols still produce fetal-like hepatocytes with limited mature function. Striving to increase maturity of hPSC-derived hepatocytes, and characterise their potential to fulfil different experimental needs, we took several avenues.

Interestingly, fetal hepatocytes acquire mature CYP450 expression only postpartum, suggesting that nutritional cues may drive hepatic maturation. We established a differentiation protocol, which mimics postnatal nutritional cues and we were able to show that vitamin K_2 and lithocholic acid, a by-product of intestinal flora, activate pregnane X receptor (PXR) and subsequent CYP3A4 and CYP2C9 expression in hPSC-derived and isolated fetal hepatocytes. Differentiated cells produce albumin and apolipoprotein B100 at levels equivalent to primary human hepatocytes, while demonstrating an 8-fold induction of CYP450 activity in response to aryl hydrocarbon receptor (AhR) agonist omeprazole and a 10-fold induction in response to PXR agonist rifampicin. Flow cytometry showed that over 83% of cells were albumin and HNF4α positive, permitting high-content screening in a 96-well plate format. Analysis of 12 compounds showed an R^2 correlation of 0.94 between TC50 values obtained in stem cell-derived hepatocytes and primary cells, compared to 0.62 for HepG2 cells (*Avior et al., 2015*). Finally, stem cell-derived hepatocytes demonstrate all toxicological endpoints examined, including steatosis, apoptosis, and cholestasis, when exposed to nine known hepatotoxins. This body of work provides fresh insights into liver development, suggesting that microbial-derived cues may drive the maturation of CYP450 enzymes postpartum. Addition of these cues results in the first functional, inducible, hPSC-derived hepatocyte for predictive toxicology.

Liver Fibrosis Model

One of the ***HeMiBio*** achievements is the development of an *in vitro* drug-induced liver fibrosis model. As mentioned earlier, traditional HSC 2D mono-cultures lead to the activation of the HSCs towards fibrotic myofibroblast and does not allow testing of compounds that need metabolisation by hepatocytes or hepatotoxicity to cause liver fibrosis. The developed model consists of human 3D hepatic organoids comprised of HepaRGs® and primary human HSCs. The organoids are generated by mixing the two single-cell suspensions in a ratio of 1Heps:2HSCs in non-attachment round-bottom 96 well-plate wells with orbital stirring. This allows for quick generation of spheroids with an optimal diameter of ≤200 μm, representing the maximum physiological distance between a cell and a blood vessel and precludes the formation of necrotic cores. *Figure 4.36* shows a schematic representation of such spheroids, in which after several days in culture HSCs arrange themselves in the core of the spheroid while HepaRGs concentrate more at the outside (*Figure 4.36*, lower panel).

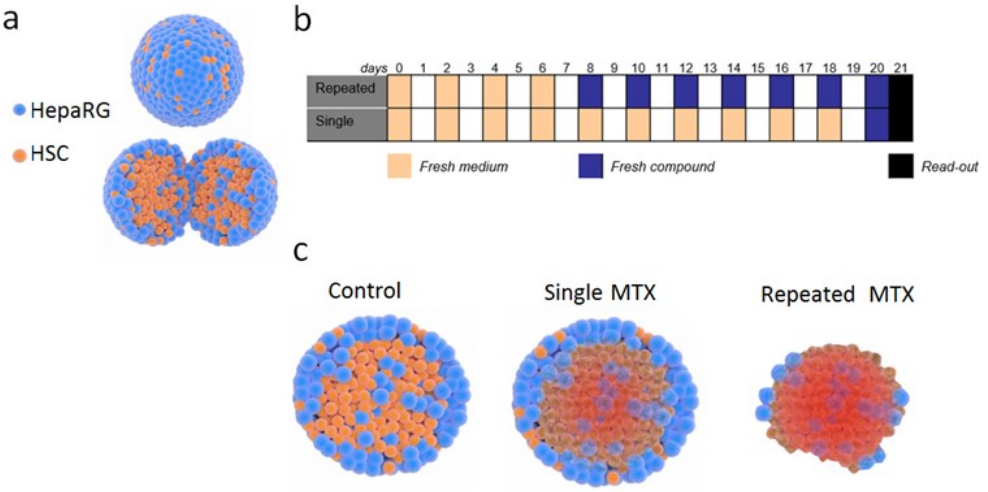

Figure 4.36 *Schematic representation of the 3D hepatic organoids organisation and read-out after exposure to pro-fibrotic compounds.* ***(a)*** *cellular distribution of HepaRG and Hepatic Stellate Cells (HSCs) within the organoid);* ***(b)*** *exposure scenario to compounds such as Methotrexate (MTX) and Allyl Alcohol during organoid culture time;* ***(c)*** *Schematic representation of HSC activation and collagen accumulation within the spheroid upon single and repeated exposure to MTX. Brown cells represent activated HSCs and the red color represents the accumulation of cross-linked collagen around the cells. Note that after repeated MTX treatment many hepatocytes have died and only the core remains which is mainly composed of activated HSCs that secrete and deposit high amounts of collagen. A single MTX treatment does not lead to hepatocyte death but also results in activation of HSCs in the centre of the spheroid (Leite et al., 2016).*

In these hepatic organoids both hepatocytes and HSCs maintain their cell-specific functions/characteristics for at least 3 weeks. This is the first *in vitro* culture system that can detect drug-induced hepatotoxicity as well as drug-induced liver fibrosis. Its long stability allows not only single but also chronic repeated-exposure to reference fibrosis compounds such as methotrexate and allyl alcohol. Organoids exposed to such compounds show an increase of collagen secretion and accumulation in the organoid, remeniscent of what happens in *in vivo* fibrotic livers. Even the hepatotoxic-independent HSC activation observed after repeated exposure to Allyl alcohol is hepatocyte-dependent since the effect is not observed in the HSC 3D mono-cultures (*Leite et al. 2016*). This highlights the potential of the hepatic organoids to not only 'just' identify hepatotoxic compounds that might cause indirect HSC activation (i.e. MTX), but also by compounds that are not hepatotoxic but need the presence of functional hepatocytes to indirectly activate HSCs (i.e. allyl alcohol at the tested concentrations). The relevance of this *in vitro* liver fibrosis model, developed entirely during the ***HeMiBio*** Project,

is that it allows the screening of pro-fibrotic drugs with human relevance before being tested in animals. Furthermore, this model can also contribute to the development of anti-fibrotic therapies, but might require further incorporation of liver cell types such as liver sinusoidal endothelial cells, Kupffer cells and Natural Killer cells.

The liver fibrosis model was considered as a **SEURAT-1** case study demonstrating how the theoretical adverse outcome pathway description 'from protein alkylation to liver fibrosis' was used to develop an *in vitro* test system addressing one particular key event of the AOP. Further contributions from the ***HeMiBio*** Project to the **SEURAT-1** case studies are discussed in the following.

4.3.4 Contributions to the SEURAT-1 Case Studies

Adverse Outcome Pathways

The adverse outcome pathway (AOP) approach has played a key role in the **SEURAT-1** Research Initiative, and hence it was fully adopted in ***HeMiBio***. Since cholestasis, or bile acid accumulation, is a major form of liver toxicity that can be potentially induced by cosmetic ingredients, we focused attention on the establishment of an AOP framework for cholestasis triggered by chemical-mediated inhibition of the bile salt export pump transporter protein. For this purpose, an in-depth survey of relevant scientific literature was carried out in order to identify intermediate steps and key events. The latter include bile accumulation, the induction of oxidative stress and inflammation, and the activation of specific nuclear receptors. Collectively, these mechanisms drive both a deteriorative cellular response, which underlies directly caused cholestatic injury, and an adaptive cellular response, which is aimed at counteracting cholestatic insults. AOP development was performed according to Organisation for Economic Co-operation and Development (OECD) guidance, including critical consideration of the Bradford-Hill criteria for weight-of-evidence assessment and the OECD key questions for evaluating AOP confidence. The postulated AOP is expected to serve as the basis for the development of new *in vitro* tests and the characterisation of novel biomarkers of chemical-induced cholestasis.

Read Across Case Study

HeMiBio participated actively in the read across study entitled 'Read-across of 90-Day Oral Repeated-Dose Oral Toxicity for β-Olefinic Alcohols: A Case Study of Compounds with Similar Metabolism' (see section 3.2.2, scenario II). The hepatic organoid cultures (HepaRG/HSCs) optimised during the ***HeMiBio*** Project were assessed for fibrosis using a selection of β-unsaturated alcohols. Based on the results with 2-propen-1-ol, the 3D HepaRG/HSC co-culture model was used to evaluate five other β-unsaturated alcohols. The data obtained using single and repeated exposures (14 days) of the β-unsaturated alcohols reveal that

while straight chain β-olefinic alcohols induce HSC activation (upregulation of mRNA levels of COL1A1, COL3A1 and LOXL), the vinylic methyl-substituted β-olefinic alcohols only weakly induce these markers. This *in vitro* data support the read across *ex vivo* and *in chemico* data in concluding that 2-propen-1-ol can be read-across to other primary and secondary β-alkenols but a subcategorisation is necessary. This work was discussed in the ECHA Workshop in April 18-20 2016 in Helsinki (see section 2.3.2).

Ab Initio Case Study

HeMiBio also participated actively in the *ab initio* case study by testing pyperonyl butoxide (PBO), the selected compound for this case study (see section 3.3). The compound was tested at different concentration in iPSC-derived hepatocyte like cells, in hepatocyte-like cells generated by using the UpCyte® technology, in HepG2 as well as in hepatic 3D co-cultures of HepaRG and HSCs. Depending on the culture used, different readouts such as mRNA levels of (hepatocyte) functionality or (HSC) activation markers were recorded and communicated to the coordinators of this case study.

4.3.5 Potential Impact

PSC-Derived Cells

Human pluripotent stem cells (hPSCs) represent an excellent source of differentiated hepatocytes. The newly established hPSC differentiation protocol towards hepatocytes produces cells that are capable of demonstrating all toxicological endpoints examined, including steatosis, apoptosis, and cholestasis, when exposed to nine known hepatotoxins. Mimic TC_{50} values of drugs with an R^2 correlation of 0.94 as compared to primary cells, and recapitulate the Hepatocyte ability to modulate CYP450 activity in response to transcriptional regulator agonists. This body of work provides means of producing *functional, inducible, hPSC-derived hepatocyte for predictive toxicology*.

Nevertheless, as has also been described by many other teams, although progress is being made in creating more mature hepatocytes with increased CYP levels among others, the cells still do not attain all functional, metabolic and transcriptional characteristics of primary mature hepatocytes. Future studies will still be needed to further optimise the differentiation to create fully mature hepatocytes, even if the studies during the ***HeMiBio*** Project have made significant progress towards this goal.

One of the main goals of ***HeMiBio*** was to engineer different liver cell type generated from induced pluripotent stem cells (iPSC) to be used in a liver-simulating device (Hepatic Microfluidic Bioreactor) mimicking the complex structure and function of the human liver. We have developed for the first time a protocol for generating hepatic stellate cell (HSC)

derived from human iPSC. The iPSc derived cells have been functionally characterised. Generated HSC-like cells accumulate vitamin A in lipid droplets, up-regulate activation markers in response to pro-fibrogenic and pro-inflammatory stimulus and as the primary HSC, differentiated cells have the ability to migrate when they are activated. The potential applications of iPSC-derived HSC-like cells to assess a fibrogenic response or toxicological assays are now being investigated

Bioreactors

The developed 'flow-over' or 2D bioreactor system for physiologically relevant cell culture and advanced metabolic real-time monitoring, by means of the integrated sensing technologies was shown to be of high relevance for the field of microfluidic liver equivalent devices and mechanistic, *in vitro* toxicology. The US and EP-patent applications entitled 'Method and System for Continuous Monitoring of Toxicity' were filed for this invention. The developed system provides the means to culture cells in microbioreactors for extended periods of time during which they can be exposed to various compounds in either single or repeated doses, all the while being monitored, in real time, for their oxidative and glycolytic metabolism, in order to assess their response. The mechanistic studies enabled by this system exceed the possibilities of *in vivo* experimentation, as the effects can be observed as they develop, i.e. kinetic data on drug-induced liver injury can be obtained. In contrast to experimentation in animals and conventional *in vitro* assays, that both represent the analysis of endpoints, the pathogenesis can be monitored and studied in real-time. This offers detailed mechanistic insights into the intracellular molecular events ultimately leading to the endpoint of liver injury. As an added benefit, the developed bioreactor gives the opportunity to design studies in a much faster and more cost-efficient way as compared to conventional assays. The intended exploitation comprises the sale of devices, the provision of services for in-house measurement series in the partners' labs and licensing.

With respect to the developed 'flow-through' or 3D bioreactor, the development was still ongoing at the end of the project. The establishment of robust fabrication routines and reliability tests for leakage-free use required more time than initially anticipated. This impeded the regular use for larger scale cellular experiments and the generation of toxicological data using the 3D bioreactor. However, these steps would be the next to take at the current time point.

Liver Fibrosis and Primary Hepatic Stellate Cell Cultures

To date there are no approved direct anti-fibrotic agents for the management of liver fibrosis. Hepatic stellate cells are the major collagen producing cells during conditions of sustained hepatic injury (either metabolic, cholestatic, viral or toxic) leading to liver fibrosis, when hepatocyte damage triggers a cascade of events leading to activation of quiescent HSCs

into a myofibroblastic (activated) HSC state. HSC activation is mediated by a plethora of pathways that finally result in increased secretion of extracellular matrix proteins, such as collagens, that accumulate as scar tissue (fibrosis) within the liver parenchyma and to liver cirrhosis in a later stage. Before the ***HeMiBio*** Project, the best *in vitro* fibrosis models consisted of mono-layer cultures of freshly isolated rodent HSCs in regular tissue culture dishes which leads to 'spontaneous' HSC activation. Obvious limitations of these cultures are the rodent background and the un-controlled and hepatocyte damage-independent activation of the HSCs, making these cultures less suitable for pro- and anti-fibrotic compound testing translatable to humans. During ***HeMiBio*** we developed a novel three-dimensional (3D) human co-culture model where both hepatocyte functionality and HSC quiescence can be maintained for at least 21 days. Unlike many currently used HSC cultures, the HSCs used are primary human and not cell lines. During ***HeMiBio***, human HSCs from multiple donors were isolated and characterised at the genomic and methylome level as well as functional level. Optimisation and characterisation of human HSCs from multiple donors were used in these organoid cultures. This novel system allows hepatotoxicity testing as well as drug-provoked and hepatocyte-dependent HSC activation and fibrosis, a drug-induced liver injury (DILI) rarely addressed *in vitro* unlike steatosis, choleastatis and phospholipidosis. This is a big step forward from the regular 2D HSC cultures that are generally used and served as basis for a patent application (PCT/EP2015/062551).

These hepatic organoids represent a substantial improvement when screening for drug-induced liver fibrosis in terms of cost, animal use and prediction of liver fibrosis in human. Exploitation of these results comprises the provision of services for in-house measurements of fibrotic capacity of chemicals and potential screening of anti-fibrotics in specific fibrosis settings. Further development of more complex organoid cultures including also LSEC or even imflammatory cell types could expand the applicability of the organoid cultures (incl. clearance studies, infection etc.). Furthermore, this technology could stimulate the development of culture models representative of fibrosis in other organs such as lung and kidney, since these share common mechanisms.

Toxicity Testing

The established AOP on cholestasis may have a considerable impact on the areas of toxicology and human risk assessment. Indeed, it may serve a number of purposes, such as the development of innovative *in vitro* and *in silico* tests, elaboration of prioritisation and tiered testing approaches, and chemical categorisation. Following generation of the cholestasis AOP, this conceptual tool has been tested for its predictivity, reliability and robustness in one of the **SEURAT-1** case studies conducted. The results confirmed the relevance of the existing elements of the AOP and identified potentially new ones, which in turn may lead to novel biomarkers of cholestatic injury. This AOP will be further tested in a number of recently started

national and European research projects. This will contribute to the overall implementation of the 3Rs concept and thus will reduce the number of animal tests, which was a main driver of the **SEURAT-1** Research Initiative.

Several toxicity assays were optimised and form the basis for the outcome of the project. Know-how associated with these assays might also be used to provide scientific and technical support to third parties, such as other research centres or private entities, in the framework of joint R&D projects or contractual research and consultancy services. Since the liver is the most important site of xenobiotic metabolism in the body, *in vitro* evaluation of metabolism and liver-related effects is an important stage in the development of new pharmaceuticals, cosmetics, chemicals and alike. Accordingly, companies operating in these sectors, as well as CROs performing toxicity testing, might be potential beneficiaries and adaptors of this particular foreground created within the ***HeMiBio*** Project. With respect to commercial exploitation, no IPR protection will be possible or commercially viable, as all of the methodologies have already been described in scientific and technical literature. As such, they have only been adapted and optimised for the purpose of ***HeMiBio*** and other **SEURAT-1** projects.

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El Taghdouini, A., et al. (2015): Development of culture conditions to revert the activated phenotype of cultured human hepatic stellate cells. Poster award at the SEURAT-1 5th Annual Meeting 2015, 21–22 January 2015, Barcelona, Spain.

The prizes consists of 500€ voucher contributing to travel expenses to attend a Conference related to **SEURAT-1** research fields.

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4.4 DETECTIVE: Detection of Endpoints and Biomarkers for Repeated Dose Toxicity Using *in vitro* Systems

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4.4.1 Executive Summary

As one of the building blocks of the **SEURAT-1** Research Initiative, the goal of the **DETECTIVE** Project was to identify robust, sensitive and specific, human relevant *in vitro* biomarkers and surrogate endpoints that can be used for safety assessments of chronically acting compounds. To this aim, the **DETECTIVE** Project has set up a screening pipeline composed of functional and '-omics' technologies, including high content and high throughput screening platforms. Multiple data streams derived from '-omics' readouts evaluated along with traditional toxicological and histopathological endpoints were analysed using integrative statistical analysis, systematic verification and correlation with *in vivo* data. The identification and statistical selection of highly predictive biomarkers in a pathway- and evidence-based approach constitutes a major step in this integrated approach towards the replacement of animal testing in human safety assessment.

The work undertaken in **DETECTIVE** covered liver toxicity, renal toxicity and cardiotoxicity, as well as cross-organ strategies. The 'Biomarker Repository' is one of the major outputs of **DETECTIVE**, which in turn is a substantial input for the ToxBank database *(http://toxbank.net/;* see also section 4.7), into which all **SEURAT-1** projects fed their data. The 'Biomarker Repository' is a database detailing biomarkers (functional and '-omics') together with the experimental details (*in vitro* models from which they have been retrieved, compounds and relevant concentrations). This is one of the major achievements of **DETECTIVE** as it is a concise database based both on literature searches and contributed to by all **DETECTIVE** partners with their experimental results. Integrated analysis of '-omics' datasets from multiple

platforms have been conducted identifying both organ-specific and generic biomarkers. Biomarkers selected for further validation were commonly associated with metabolism, development, protein degradation, stress response and energy metabolism as well as with few other biological functions.

Other significant achievements of **DETECTIVE** include amongst others a newly developed liver-based *in vitro* system, namely hepatic cells differentiated from human skin-derived precursors, source for *in vitro* screening of compounds that induce liver steatosis, a GFP-BAC toxicity pathway reporter platform for the imaging-based chemical safety assessment, as well as the accomplishment of three **SEURAT-1** case studies:

- Study 1: 'Omics-based *in vitro* verification of an adverse outcome pathway (AOP) of cholestatic liver injury', which not only achieved a proof of concept of a previously proposed AOP of cholestasis, but also allowed to identify new key events.
- Study 2: 'Valproic acid case study: Detection and verification of biomarkers by using a read across approach', which addressed the increasing need to advance the integration of animal free alternative methods, such as read across.
- Study 3: 'Common stereotypic and non-stereotypic transcriptome biomarkers for verification of toxicant compounds' that has identified and further classified toxicity biomarkers.

The successful completion of the **DETECTIVE** Project advances our understanding of repeated dose toxicity testing methods. This will lay the foundation for subsequent efforts in follow-up activities at the completion of the **SEURAT-1** Research initiative. Indeed, many of the collaborations established between **DETECTIVE** members are ongoing and will continue to advance the topics covered by the **DETECTIVE** Project, including in the new EU-project 'EU-ToxRisk'. Furthermore, development of the **DETECTIVE** cell systems into more physiologically relevant models, including complex cell systems, 3D, advanced micro-bioreactors for cultivation under flow with real time monitoring of essential physico-chemical parameters and organ-on-a-chip technologies are anticipated. This expansion will be highly relevant to establishing a solid and reliable basis on which the future *in vitro* test systems, employed by industry, can be based.

4.4.2 Project Context and Objectives

As one of the building blocks of the **SEURAT-1** Research Initiative, the **DETECTIVE** project focused on a key element on which *in vitro* toxicity testing relies: the development of robust and reliable, sensitive and specific *in vitro* biomarkers and surrogate endpoints that can be used for safety assessments of chronically acting toxicants relevant for humans.

Emphasis was given to systematic exploitation of a battery of complementary functional and '-omics' readouts, including high content and high throughput screening platforms to identify and investigate human biomarkers in cellular models for repeated dose *in vitro* testing. While functional parameters give more insights into the effects of toxicants on specific cell functions of interest, '-omics' techniques deliver data on the entire cellular situation at molecular level. Importantly, **DETECTIVE** has performed for the first time an in-depth investigation of repeated dose effects on epigenetics and microRNA (miRNA) expression, thus exploring whether such analyses deepen our understanding of toxic modes of action. In the last years, these two parameters have been identified as critical for cell behaviour and it has been a challenging task to determine whether long-term application of chemicals will affect cells at this level.

Upon combination and subsequent integration of the various readouts, biomarkers for predicting human long-term toxicity *in vitro* were obtained. Based on integrative statistical analysis, systematic verification and correlation with *in vivo* data, relevant, specific, sensitive and predictive biomarkers were selected.

DETECTIVE concentrated on hepatotoxic, cardiotoxic and nephrotoxic effects representing three target organs of repeated dose toxicity. Ultimately, developed concepts will also be applicable to other organs or organ systems affected by systemic toxicants such as the nervous system. Furthermore, **DETECTIVE** was able to define both generic and organ-specific human toxicity pathways.

The overall objectives of the **DETECTIVE** Project were:

- To conduct functional and '-omics' experiments under optimised protocols with repeat dose exposures and recovery periods;
- To analyse data-rich '-omics' data;
- To define relevant biomarkers and adverse pathways with predictive values;
- To prepare a road map, including proof-of-concept case studies, in close collaboration with the other **SEURAT-1** projects.

DETECTIVE, as a multidisciplinary collaboration of European researchers, has achieved its key scientific objectives by:

- Establishing a successful private-public partnership addressing an important societal need;
- Developing new tools and technologies together with a smart integration approach;
- Creating a screening pipeline for identifying biomarkers and surrogate endpoints relevant for assessing human long-term organ toxicity;

➠ Compiling a repository of

- verified, stable and easy-to-measure functional and '-omics' biomarkers of different organs, including GLP-compliant SOPs for selected, most relevant biomarkers;

- human toxicity pathways relevant for different organs (in cooperation with ToxBank and NOTOX);

- concepts for translating the knowledge gained to toxicity in the entire organism;

- adverse outcome pathways (AOPs) for cardiotoxicity;

➠ Novel public toxicogenomics directory of chemically exposed human hepatocytes (146 compounds);

➠ New live cell imaging pathway-based toxicity reporters for the identification of hepatotoxicant-induced cellular stress responses;

➠ Developing human skin-derived precursors (hSKP) as a novel cell source for *in vitro* screening of compounds that induce liver steatosis;

➠ Completion of 3 case studies at the **SEURAT-1** level.

4.4.3 Main Achievements

4.4.3.1 Strategy

The primary goal of **DETECTIVE** during the project duration (2011-2015) was the identification of robust, sensitive and specific, human relevant, *in vitro* biomarkers and surrogate endpoints that can be used for safety assessment of chronically acting toxicants. During this period, the goal was achieved through a series of defined milestones. To recapitulate, initially the target organs involved in repeated dose toxicity studies for 154 cosmetic ingredients were analysed and the most frequently affected organs by cosmetic ingredients were identified. The analysis revealed that liver and kidney were the primary target organs. Furthermore, as the mandate of the **SEURAT-1** Research Initiative was not restricted to cosmetics, it was also relevant to examine common toxicity effects relating to, for example, pharmaceuticals. In this context, the cardiovascular system is also one of the most commonly affected targets associated with attrition during drug development, representing a common cause of drug withdrawal from the market. In the following years, experimental work was pursued bringing together complementary research in the fields of alternative testing for toxicity in liver, heart and kidney cell models using various resources: functional technologies (imaging, impedance measurement, electrical activity), '-omics' technologies (metabolomics, transcriptomic, epigenetics, proteomics), statistics (modelling) and bioinformatics. Integrated analysis of '-omics' datasets from multiple platforms have been conducted identifying both organ-specific

and generic biomarkers. Biomarkers selected for further validation were commonly associated with metabolism, development, protein degradation, stress response and energy metabolism as well as with few other biological functions. The scientific and technical outcomes of the **DETECTIVE** Project are detailed in the following sections.

4.4.3.2 Organ Based Repeated Dose Toxicity Testing Models

Liver

Assessment of repeated dose toxicity of valproic acid, aflatoxin B1 and cyclosporine A in the human liver using integrative '-omics' data analyses

This strategy focused on delivering biomarker data based on challenging liver models with valproic acid (VPA), aflatoxin B1 (AFB1) and Cyclosporin A (CsA). The study proceeded with the hypothesis that the most interesting biomarkers would be the toxicant-induced changes in molecular networks which persist after terminating repeated dosing *in vitro*. This was revealed through cross-omics analysis after the repeated dose exposures on primary human hepatocytes (PHH), pooled from 3 donors, had been washed out for three days. Firstly, in response to VPA the generated data led to insights that could be mapped on the AOP construct of steatosis in order to provide information on the mode of action of VPA-induced steatosis. This will lead to the identification of new biomarkers and provide insight into the molecular mechanisms of VPA on the level of the epigenome and transcriptome. Next AFB1, a naturally occurring, but highly hepatotoxic and carcinogenic mycotoxin was examined. Cross-omics analysis identified modulated genes in response to two doses of AFB1 revealing persistent, reversible and newly expressed differentially expressed miRNAs (DE-miRs). A whole series of new genes were identified, which in general, related to pathways of cell cycle and DNA response, transcriptional regulation. Specific pathways within these AFB1-induced biological processes appeared involved in signal transduction cascades for liver toxicity. A final aspect was the assessment of repeated dose toxicity of CsA, an immunosuppressant drug widely used in organ transplantation to prevent rejection. Adverse side effects of CsA include cholestasis. Transcriptome and the miRNA analysis found the persistence of differentially expressed genes (DEGs) and DE-miRs after CsA exposure.

Overall, by applying integrative cross-omics analyses to an innovative cell model in a repeated dose regime, the molecular networks persistently affected by prototypical toxicants – VPA, AFB1, and CsA in the liver have been unravelled. In the course of this work promising biomarkers for repeated dose toxicity in humans have been identified. Follow-up studies are now required that take into account larger numbers of chemicals for training and validating the predictive models. It will also be necessary to use more physiologically relevant doses and to better explore the transition to human disease signatures.

Biomarker study to predict hepatotoxic blood concentrations

The aim of this study was to identify biomarkers which predict human hepatotoxic blood concentrations from publically available genome wide expression data of 150 compounds tested in primary human hepatocytes (PHH). The genes selected were altered by several compounds, overlapped with genes deregulated in human liver disease and covered the most relevant toxic mechanisms. Statistical analysis determined that a large set of compounds could be 'captured' by a relatively small set of genes ultimately represented by a list of the top seven potential biomarker genes that fit all the criteria. These genes are all upregulated in liver disease and reflect metabolic, cell cycle, cytoskeleton and protein degradation processes. To evaluate the ability of the biomarker genes to predict hepatotoxicity, a set of compounds that do or do not cause hepatotoxicity were identified and applied to HepG2 or PHH cells and analysed for biomarker RNA induction and cytotoxicity. The work carried out in this strategy has established a human-derived *in vitro* model based on biomarkers which predict human blood concentrations which cause hepatotoxicity. The novel prediction system might provide a promising tool to identify hazardous compounds during early screening processes in drug development.

A High Content Imaging-based Toxicity Pathway Reporter Platform for Chemical Safety Assessment

Drug induced liver injury (DILI) remains a major concern for drug development and in clinical practice. At the moment primary human hepatocytes are regarded as the gold standard for DILI toxicity testing. However, problems with the availability, inter-donor variability and stability remain critical issues. A BAC-GFP reporter platform has been developed, in which the activation of maladaptive stress pathways, which are typically activated by chemical-induced cellular injury, is monitored (*Figure 4.37; Wink et al., 2014*).

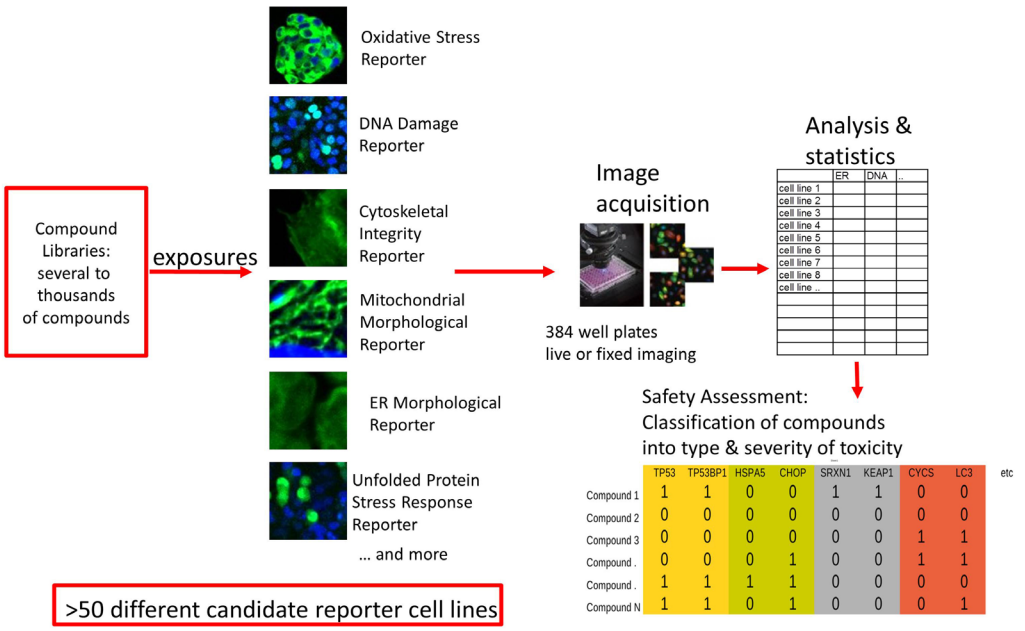

Figure 4.37 *Concept of the pathway of toxicity reporter platform.*

This system has enabled the establishment and characterisation of reporters for oxidative stress, ER stress, and DNA damage, allowing single cell time-resolved and quantitative analysis of the toxicity pathway activation (*Figure 4.38*). Exposure of these individual reporters to a library of more than 150 DILI compounds was followed by mapping of the dynamic activation of these toxicity pathways in a 24 h time period at a range of concentrations. By applying bioinformatics tools the entire time-concentration HepG2-BAC-GFP reporter response profiles of all compounds could be generated. Using this approach allows the clustering of similar mode-of-action compounds. Moreover, this screening strategy enriches for compounds with severe DILI drug labelling. It is anticipated that the cellular stress response reporters may play a key role in future safety assessment of DILI as well as other toxicity liabilities.

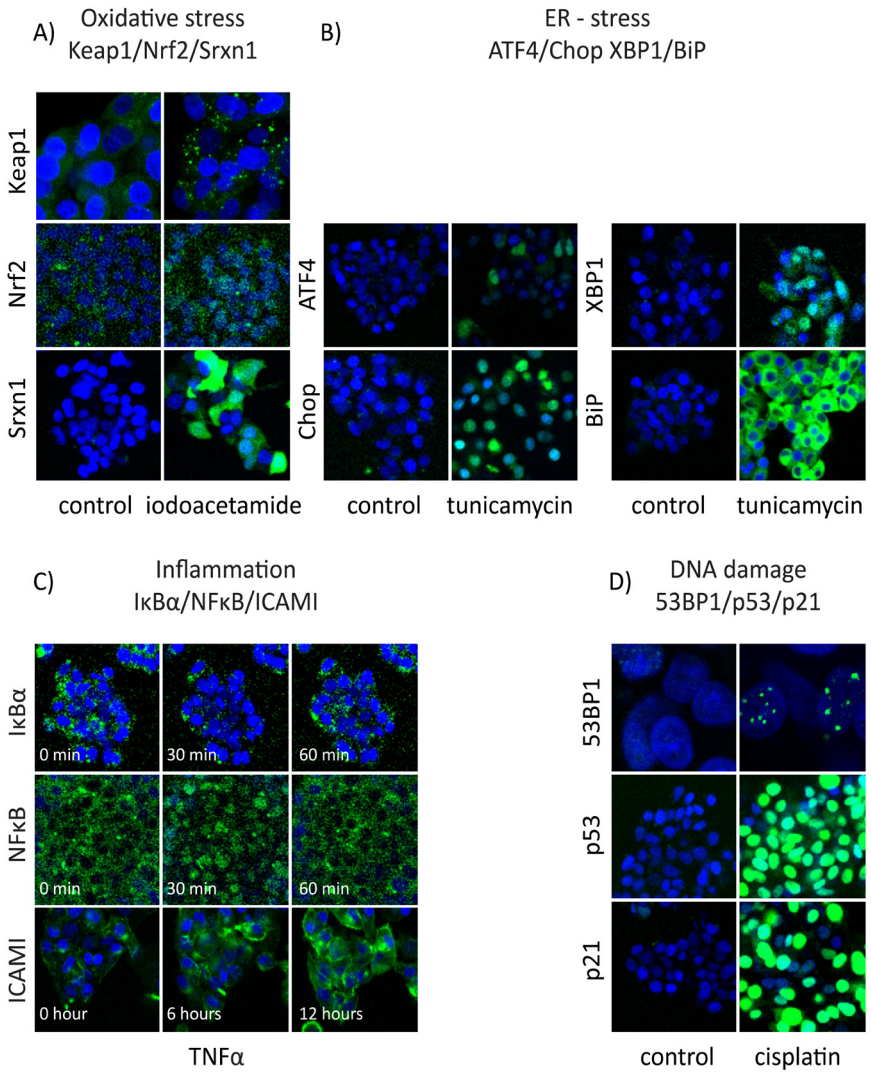

Figure 4.38 *Examples of BAC reporter cell lines of toxicity pathways in HCI. (A) Oxidative stress signalling. (B) ER-stress signalling. (C) Inflammation signalling. (D) DNA damage response. (Data source: Wink et al., 2014).*

Kidney

The mycotoxin ochratoxin A (OTA), a contaminant in foods and beverages, a renal carcinogen in rats and a suspected carcinogen in humans, was investigated for its previously reported impact on epigenetic mechanisms such as histone acetylation. The effects of OTA on the renal proximal tubule cell line RPTEC/TERT1 was examined using a repeated exposure protocol

over five days followed by a three-day washout recovery. Potassium bromate (KBrO3) was used as positive control for oxidative injury. This study revealed a strong impact of OTA on the transcriptome (*Figure 4.39*) that has been previously described (*Jennings et al., 2013*).

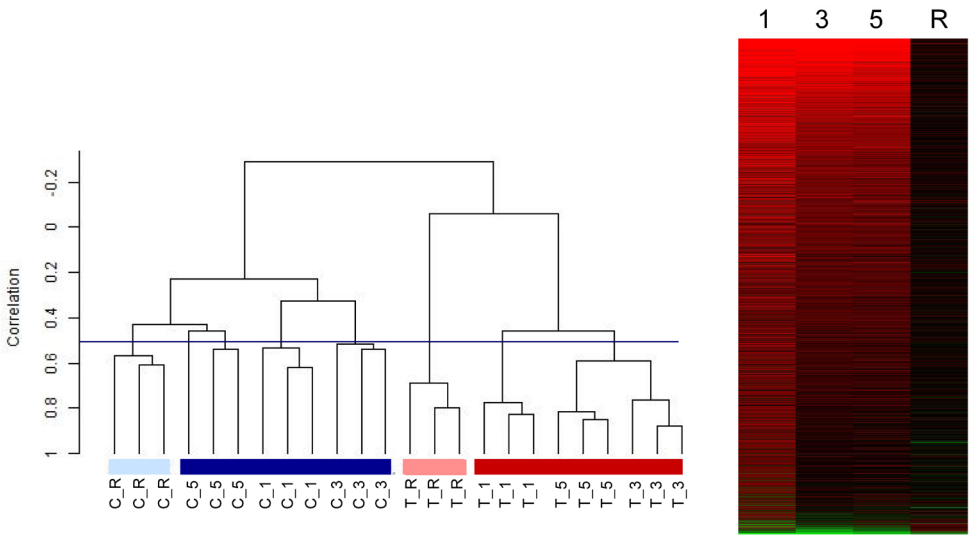

Figure 4.39 *Unsupervised hierarchical clustering of transcriptomic data (left) and a representative heat map of deregulated genes (right). Data source: Innsbruck Medical University, Austria.*

The contributions of epigenetic modifications and miRNA expression to the modulation of several protective stress responses, notably the Nrf2 response to oxidative stress (*Limonciel & Jennings, 2014*) were also investigated (*Figure 4.40*). The alterations identified by metabolomics were particularly interesting, especially as they have high potential for translation as clinical urinary biomarkers.

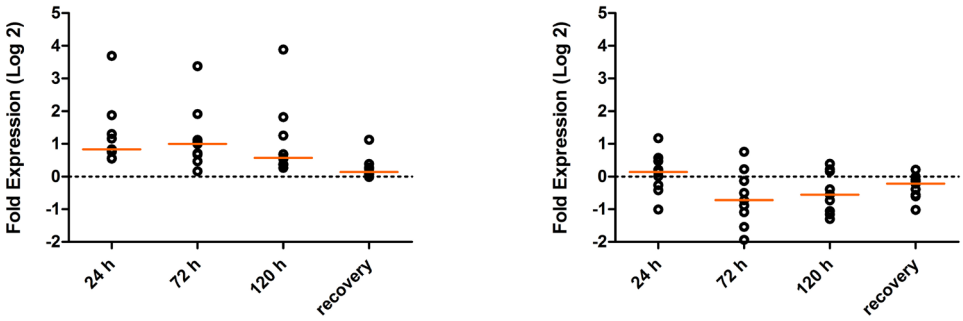

Figure 4.40 *Effect of compound exposure on the deregulation of Nrf2-dependent genes. The circles represent specific Nrf2-regulated genes. The solid line is the median. Data source: Innsbruck Medical University, Austria.*

Using iCluster+ software, comprehensive maps of regulated networks in response to KBrO3 and OTA were made whereby top key genes, and their dynamic changes over the course of repeated dose treatment and recovery wash out, could be described. This strategy has provided a global and comprehensive view of toxicological mechanisms for KBrO3 and OTA. Moreover potential new biomarkers for KBrO3 and OTA have been discovered in the context of renal toxicity.

Furthermore, the effect of a second repeat dose exposure was investigated, to see whether cells truly recover or if there is a 'cellular memory' of the first exposure. RPTEC/TERT1 kidney cells were exposed to a high, but non-cytotoxic concentration of cyclosporine A every 24 h for 5 days. Cells were allowed to recover for 8 days (24 h feeding) and treated again every 24 h for 5 days. Experimental readouts included indicators of primary pharmacology, metabolomics, transcriptomics and epigenomics. The results are currently being analysed, but hint to the possibility that indeed there is a 'cellular memory' of the first exposure.

Heart

Identification of Molecular Biomarkers for Cardiotoxicity

Cardiotoxicity is a well-known side effect of several cytotoxic drugs, especially of the anthracyclines in cancer patients. Anthracyclines are anti-cancer agents with a dose dependent cardiotoxicity that has strong impact on the quality of life and patient survival. This cardiac related side effect limits its use in cancer patients. In this context, a biomarker identification initiative focused on the identification of cardiotoxicity biomarkers in *in vitro* systems using different '-omics' technologies. The human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) have already shown their applicability in various *in vitro*

drug screening tools. The purpose of this study was to develop an *in vitro* repeated exposure toxicity methodology allowing the identification of predictive genomics biomarkers of functional relevance for drug-induced cardiotoxicity in hiPSC-CMs. The cells were incubated with doxorubicin, a well-characterised cardiotoxicant, followed by washout of the test compound with further incubation in compound-free culture medium (*Figure 4.41*). A panel of 35 genes was deregulated by all three anthracycline family members and can therefore be expected to predict the cardiotoxicity of compounds acting by a similar mechanism as doxorubicin, daunorubicin or mitoxantrone (*Chaudhari et al., 2015*). The identified gene panel can be applied in the safety assessment of novel drug candidates as well as available therapeutics to identify compounds that may cause cardiotoxicity. This study has demonstrated that doxorubicin-induced adverse effects on cardiac function can be detected at the genomic level, even before cytotoxicity and arrhythmia are observed. The developed methodology can allow for first-line *in vitro* preclinical tests and, reduce animal usage in drug safety studies and the costs of safety evaluations.

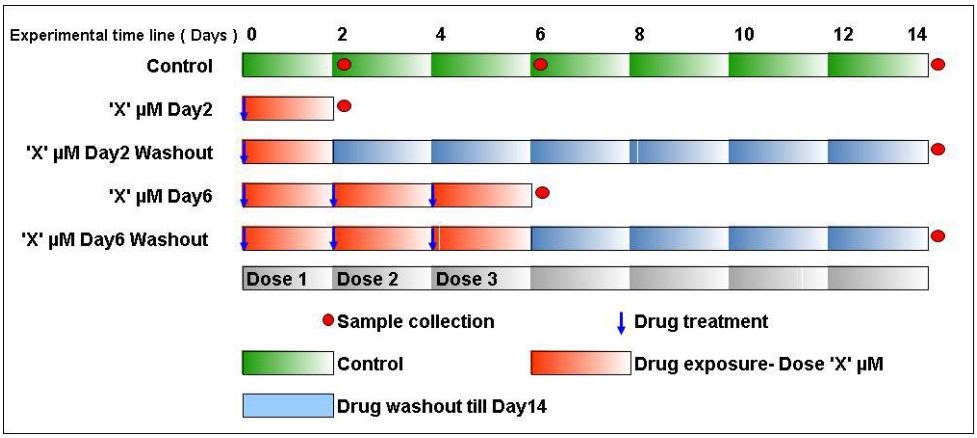

Figure 4.41 *The experimental design and timeline for repeated dose toxicity studies in human iPSC-derived cardiomyocytes. Data source: University of Cologne, Germany.*

Following the use of anthracyclines to identify biomarkers (*Chaudhari et al., 2015*), the next step was to validate these potential biomarkers with different cosmetic ingredients and one known liver toxicant, bosentan. The cosmetic ingredients chosen were kojic acid, triclosan and 2,7-naphthalenediol which are used in commercial cosmetics products. All three cosmetic ingredients and bosentan at low and middle concentrations showed no significant effect on the expression of the 35 key predictive biomarkers. From these results it was concluded that the panel of 35 genomic biomarkers is suitable to predict cardiotoxicity in humans and could also be applied in the safety evaluation of drug candidates and cosmetic ingredients.

Some further studies are needed to understand the precision of those biomarkers and further streamline the set of biomarker genes.

Predicting Drug-induced Cardiotoxicity by means of Electrophysiological Data

Drug-induced cardiotoxicity takes two primary forms: electrophysiological (electrical activity) and biochemical. Electrophysiological toxicities arise when compounds interact with ion channels or transporters to create a pro-arrhythmic condition in which patients are at increased risk for developing arrhythmias including life-threatening ones such as torsade de pointes. iCell cardiomyocytes from CDI (Cellular Dynamics) were exposed to repeated doses of reference compounds such as Doxorubicin, Lidocaine, E4031 and Quinidine at different concentrations for short (2 days) and long period up to 14 days. Data were recorded using xCELLigence impedance system (for 2D monolayer approach), Multi-electrode array (for 3D approach) and patch clamp (for single cell experiment) technologies. The results revealed that long-term exposure of cells to those compounds induced beating abnormalities in dose-dependent manner. It is clearly known that any alterations in ionic currents through ion channels (which contribute to the cardiac action potential) of the cell membrane is the main cause of beating abnormality of the cardiac cell. This study demonstrated that cells do not all respond at the same time or at the same dose of a pharmacologic agent due to cell-to-cell variability. Understanding the determinants of this variability will aid the development of multi-target treatment strategies for many diseases. Data generated in this study provides evidence that a pharmacologic agent may disrupt the expression level of genes and proteins, potentially by molecular and/or functional alterations.

4.4.3.3 Cross-Organ Strategies

Proteomics

The proteomic approach focused on early/immediate biological responses due to phosphorylation of proteins, which are not detectable by transcriptomics technologies. Protein phosphorylation has a direct impact on enzyme activities and protein-protein interactions. Proteomics has the potential to identify early molecular events following exposure to toxic model substances and to provide kinetic details of affected pathways. Usually the first response after stimulation of cells is the phosphorylation of heat shock proteins and other components of stress responses. The **DETECTIVE** Project delivered comprehensive relative proteome and phospho-proteome data for human *in vitro* culture models of heart, liver and kidney cells exposed to sub cytotoxic concentrations of relevant selected compounds using an iTRAQ-based LC-MS approach (e.g. *Dietz & Sickmann, 2015*). Furthermore, an LC-MS/MS-based targeted proteomics assay for the verification and validation of potential renal biomarker candidates in the chemical-induced RPTEC/TERT1 cells was developed.

Metabolomic Responses to Toxicity *in vitro* – Extracellular versus Intracellular Measurements

An objective of **DETECTIVE** was to explore the relationship between the metabolome of human *in vitro* cell systems and exposure to chemicals that cause repeated dose organ toxicity. In this strategy, a number of protocols (NMR, GC-MS and LC-MS) for *in vitro* models of toxicity including RPTEC, hiPS-CMs, HepaRGTM, primary hepatocytes were developed. Firstly, the response of hiPS-CMs to doxorubicin was analysed. Secondly, the metabolic response of RPTEC-TERT1 kidney epithelial cells to ochratoxin A and KBrO3 was assessed. Finally, the exposure of cholestatic toxicant bosentan to HepaRGTM cells indicated that low dose exposure to bosentan can induce mitochondrial dysfunction.

Common Renal/Hepatic Strategy to Identify Cell-specific and Generic Transcriptomic Signatures

While renal and hepatic *in vitro* systems are often run within the same project umbrella, they are not usually challenged with the same compounds at the same concentrations. To this end we conducted a focused study to challenge RPTEC/TERT1 and HepaRGTM to the same six compounds at the same concentrations, measuring the same end-points (impedance, glycolysis rate and targeted transcriptomics). The data is currently being analysed, with a view to identify tissue specific sensitivities, tissue specific biomarkers and common mechanistic biomarkers.

Delineation of the Nrf2 Pathway in Transcriptomic Datasets

Oxidative stress is a major factor in the development of chemical-induced injury and associated diseases. The identification of the up-regulation of Nrf2 associated genes in *in vitro* and *in vivo* systems is becoming an attractive method for classifying compounds with oxidative potential (*Wilmes et al., 2011*). However, there is still a gap of knowledge regarding the time course of events, key pathway signatures and overlaps with other pathways. In this strategy large transcriptomic data sets were examined with a focus to the Nrf2 pathway and associated pathways in liver and kidney toxicological contexts. The analysis indicates that certain associated pathways closely correlate with Nrf2 hubs, while others do not. The establishment of robust and rigorously selected transcriptomic signatures for these and other transcription-driven mechanisms is a promising avenue to provide deep mechanistic information from transcriptomic data (*Limonciel et al., 2015*). Beyond hazard identification, the study of concentration- and time-ranges of activation could also provide a means to quantify the activation of these responses for implementation as key events in quantitative AOPs.

4.4.3.4 Integration of Biomarker Identification Strategies

Approach

For the selection of biomarkers, a number of preparative activities were performed. Firstly the establishment of the biomarker repository, including both functional and '-omics' based readouts. Based on literature searches, and contributed to by all **DETECTIVE** partners, the repository is a database detailing biomarkers together with experimental details (the *in vitro* models in which they have been retrieved, compounds and relevant concentrations). This is considered one of the major outputs of **DETECTIVE**, which in turn is a substantial input for the ToxBank database *(http://toxbank.net/)*. In addition, some **DETECTIVE** partners have contributed to the generation of gene lists, specifically of transcriptomics biomarkers. This served as the basis for the generic biomarker identification strategy which allowed the identification of potential biomarkers that are liver, kidney and heart specific, as well as those that appear more generic and are detectable in multiple tissues in cases of toxicity. It is important to highlight that at the start of the project, there was no single specific strategy in place. Instead, several biomarker identification strategies progressed in parallel. Based on the expertise of the different partners, these strategies comprised different target organs (liver, kidney and heart), different set-ups of *in vitro* models and compounds and different functional and '-omics'-based read-outs. The scope of these strategies also varied, with some designed to run only within the context of **DETECTIVE** while some have relevance to other projects of the **SEURAT-1** Research Initiative or even extend beyond the borders and time constraints of the **SEURAT-1** Research Initiative. Following the generation of biomarker data, a number of steps were required in order to make a final selection of biomarkers. Firstly, statistical analysis was highlighted as the primary criterion for a putative biomarker to be selected. Secondly, central storage capacity was required for the collection of raw and processed data. From here a compendium of biomarkers (from all partners) was prepared and the output was the publication of scientific papers and, where possible, the dissemination and sharing of the data with ToxBank. The different steps of this process is described in more details in the following sections.

Statistics in DETECTIVE: Can We Get By with t-Tests?

Following the identification of a set of biomarkers, extensive statistical analysis was conducted to evaluate their inclusion in the compendium as a biomarker for toxicity in humans. To extend the analysis beyond Student's t-tests required the evaluation of all time points and dose levels in one Analysis of Variance (ANOVA) tests. In addition information can be borrowed about variation across features using Linear Models for Microarray Data (LIMMA). A critical aspect in the identification of biomarkers for treatment effects was the joint evaluation of '-omics' technologies. iCluster+ was used to find common patterns in data from multiple '-omics' technologies and discriminant features.

Integrative visualisations were generated, including heat maps, which show correlations between ordinal and quantitative values, and correlations as annotated scatterplots. Another aspect was data mining for biological information. Here, interactions between transcriptomic features by Gene Network Analysis and identified tissue-specific transcriptomic features in data from TG-Gates and the European projects carcinoGENOMICS and Predict-IV were investigated. Using Functional Data Analysis for time course data, it was determined how different a response was in comparison to control, and patterns in the compound space were discovered. Overall, advanced statistical methods were successfully employed and the visualisation of statistical results was often the key to interpretation.

Data Storage and Management

The data storage solution for the **DETECTIVE** consortium was provided by a central platform that stored all raw data from '-omics' experiments, their accompanying metadata and protocols produced by different partners within the **DETECTIVE** Project. The **DETECTIVE** database *(http://detective.quretec.com/)* holds data for more than 500 datasets across all the '-omics' readouts and target organs. The database was available for all the project partners for querying, comparing and receiving the conducted experiments throughout the **DETECTIVE** Project. The central data warehouse of the **SEURAT-1** Research Initiative was organised by the ToxBank Project (see section 4.7), which required all the data obtained across the data producing projects, like **DETECTIVE**, to be uploaded in a specific ISA-Tab format. For all the data uploaded to the **DETECTIVE** database, the metadata were automatically formatted to the ISA-Tab format and uploaded to the ToxBank data warehouse for long-time archiving. This ensures that the valuable datasets produced in this consortium will be available in a structured manner to the broader research community.

4.4.4 Contributions to the SEURAT-1 Case Studies

Challenging the Predictive Power and Robustness of an Adverse Outcome Pathway Construct from Bile Salt Export Pump Inhibition to Cholestatic Injury

This case study was focused on the *in vitro* verification of an adverse outcome pathway (AOP) construct, from bile salt export pump inhibition to cholestatic injury, in order to confirm established key events and identify new ones. Cholestasis accounts for about half of the cases of Drug-Induced Liver Injury (DILI) and is caused by an accumulation of bile in the liver due to inhibition of the bile salt export pump (BSEP). BSEP inhibition leads to several severe effects which are depicted in the AOP of cholestasis (*Vinken et al., 2013*). An *in vitro* model of cholestatic liver injury was developed by exposure of HepaRGTM cells to bosentan, a known inhibitor of BSEP. A collaborative '-omics' approach generated transcriptomic, proteomic, metabolomic and epigenomic experimental readouts. Preliminary data suggest

the identification of some key events of the AOP. Furthermore, transcriptomics and proteomics analysis identified genes and proteins that could represent potential new cholestatic biomarkers. The newly identified biomarkers could potentially contribute to the refinement of the AOP, either as individual biomarkers or as general key events.

Detection and Verification of Biomarkers by Using a Read Across Approach

Valproic acid (VPA) was selected as a data-rich compound and served as the basis for the verification of the established knowledge regarding gene changes and a critical steatosis effect. Ten structurally similar branched and unbranched carboxylic acids were selected. Five of them induced steatosis in repeated dose toxicity studies in rodents (termed '*in vivo* positive') while the remainder did not affect the liver (termed '*in vivo* negative'). The aim of this study was to predict systemic toxicity of VPA by using biomarkers and to show that the identified biomarkers discriminate '*in vivo* positive' from '*in vivo* negative' VPA analogues. From a list of the 150 highest up-regulated genes by VPA, ten candidate biomarker genes, representing seven typical cellular reactions, were selected. Key toxicity pathways were investigated: oxidative stress, endoplasmic reticulum stress and DNA damage. Three genes were identified that discriminated between *in vitro* positive and negative compounds while, in contrast, genes associated with energy and lipid metabolism were not able to discriminate the partly active from inactive compounds. In conclusion, the read across approach is a promising concept for biomarker detection and validation.

Screening of Perturbed Toxicity Pathways by Transcriptomics Fingerprinting of Data Poor Substances

The study 'Common stereotypic and non-stereotypic transcriptome biomarkers for verification of toxicant compounds' has identified and further classified toxicity biomarkers and has established a toxicogenomics directory of chemically exposed human hepatocytes on a basis of the gene array data for 151 compounds available from the TG-Gates database and other publically available resources. The highest number of deregulated genes has been revealed at the highest tested compound concentration after 24 hours of exposure. Approximately one third of the compounds were responsible for the strongest effects while a large fraction of compounds had a weak effect independent of the time and concentration. In follow-up studies a number of up-regulated genes per compound at highest dose and at 24 hours were studied. Only 32 of the analysed compounds were responsible for the strongest (100-fold) up-regulations within the transcriptome. Furthermore, the SV20 parameter was introduced which represented genes that were significantly modulated by at least 20 compounds. Based on this criterion, all deregulated genes could be separated as providing stereotypic and more compound-specific gene expression responses. Therefore, stereotypic genes could be more

relevant when selecting for liver toxicity biomarkers. SV20 genes were commonly associated with metabolism, development, protein degradation, stress response and energy metabolism as well as a few other biological functions. Down-regulated SV20 genes were associated with cell-cycle control, DNA synthesis and repair and immune response as well as a few other biological functions. A broader transcriptomics analysis revealed a significant number of SV20 genes that were strongly modulated, including in liver samples from patients with non-alcoholic steatosis, hepatocellular carcinoma and other liver diseases. Therefore, a range of the biomarkers that were revealed based on the current study of hepatotoxicity could also be potentially applied to the detection of liver diseases. Furthermore, a comparison of SV20 genes with genes modulated in human cardiomyocytes under exposure with cardiotoxicants has revealed both tissue-specific and generic transcriptomic responses.

Additionally, 35 transcriptomic biomarkers established in the initial **DETECTIVE** cardiac *in vitro* toxicity study (see section 4.4.3.2 'Heart' above) were selected for prediction of toxicity of 3 cosmetic ingredients and bosentan. The cosmetic ingredients were selected from 220 ingredients described in the SCC(NF)P opinions issued between 2000 and 2009 as those of potential cardiotoxicity concern based on repeat dose toxicity animal studies (*Vinken et al., 2012*), while bosentan was chosen as cholestasis inducing compound. The working concentrations chosen were at least 10-100 fold in excess of the one reported in the animal plasma. No cardiotoxicity was observed under any compound concentrations tested *in vitro*. Importantly neither cosmetic ingredients tested nor bosentan showed any effect on the expression of 35 predictive biomarkers at low and middle compound concentrations. While at highest concentrations of the tested compounds an expression of sub-fraction of tested genes was affected, these highest compound concentrations were not achievable *in vivo*.

4.4.5 Potential Impact

Overall, the **DETECTIVE** consortium can list a number of achievements as contributing to the legacy of the consortium. The establishment, selection and verification of highly predictive biomarkers in a pathway- and evidence-based approach constitutes a major building block in an integrated approach towards the replacement of animal testing in human safety assessment. Major advancements have been made in the field of integration of *in silico* methods with *in vitro* toxicity cell systems for compound hazard and risk assessment. In addition, the screening of toxic compounds in *in vitro* human cell systems combined with mechanistically relevant biomarkers of toxicity, under either acute or chronic exposure scenarios, will contribute to better safety assessment in humans. The AOP on cholestasis was further completed and can be used for risk assessment, tiered testing approaches, prioritisation, test development and for chemical categorisation (*Vinken et al., 2013; Vinken, 2015*). Another major advance is the use of human skin-derived precursors as a cell source for *in vitro* screening of compounds that induce liver steatosis (*Rodrigues et al., 2014*). Technology including fluorescent cell

sensors for pathway-specific toxicity screening has been developed, suitable for adversity detection in both 2D cell cultures and in 3D spheroid systems (*Wink et al., 2014*). The data output has been handled in the form of a toxicogenomics directory, with a database of global transcriptomics data for 146 hepato-toxicants with a new biomarker classification (*Grinberg et al., 2014*). These new biomarker classifiers can be used for hazard prediction and toxicity risk assessment based solely on *in vitro* data. **DETECTIVE** has also identified organ-specific and generic toxicity biomarkers. These diverse datasets, acquired during the study, will be stored with ToxBank and be publically accessible. These are considerable resources that provide an invaluable depth and breadth of knowledge in the area of repeated dose toxicology.

The successful completion of the **DETECTIVE** Project advances our understanding of repeated dose toxicity testing methods. This will lay the foundation for subsequent efforts in follow-up activities at the completion of the **SEURAT-1** Research initiative. Indeed, many of the collaborations established between **DETECTIVE** members are ongoing and will continue to advance the topics discussed in this report. Future activities are envisaged to address the limited scope of **DETECTIVE** / **SEURAT-1** which focused on the use of a limited number of human cellular systems and test compounds. The knowledge generated through the detection of endpoints and biomarkers of repeated dose toxicity, by the **DETECTIVE** Project, will also contribute to the foundation of future research initiatives, such as for example the recently started research consortium EU-ToxRisk *(http://www.eu-toxrisk.eu/)*. This programme focuses on the integration of new concepts for regulatory chemical safety assessment with the ultimate goal to develop reliable, animal-free hazard and risk assessment strategies.

Furthermore, the advancement of the **DETECTIVE** cell systems into more physiologically relevant models, including complex cell systems, 3D, advanced micro-bioreactors for cultivation under flow with real time monitoring of essential physico-chemical parameters and organ-on-a-chip technologies, is anticipated. This expansion will be highly relevant to establishing a solid and reliable basis on which the future *in vitro* test systems, employed by industry, can be based. Ultimately, **DETECTIVE** results will contribute to the reduction of animal experiments and a more efficient and reliable safety assessment.

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4.5 COSMOS: Integrated *in Silico* Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimise Safety

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4.5.1 Executive Summary

The cosmetics industry is vitally important to the economy of the European Union (EU) and its products are innovative as well as being essential to improve the quality of life. The safety, to consumers, of the final products, which may include varied combinations of many different ingredients and materials, is paramount; potentially harmful cosmetics products could have a deleterious effect not only on individual consumers, but also on the commercial viability of the company that produces it. Traditionally, the safety of chemicals has been assessed from the results of testing on animals combined with a knowledge of the level of exposure to that chemical. However, legislation within the EU coupled with, and as a response to, public opinion, means the testing of cosmetics ingredients is no longer possible. This change in policy has come at the same time as revolutions in computational, molecular and biological sciences along with a modern perspective on toxicology i.e. that we are able to move away from testing relatively high doses on animals, to considering more realistic exposures to human pathways, cells or even tissues. What is termed '21st Century Toxicology' relies, therefore, on alternative approaches that utilise computational modelling and results from *in vitro* tests.

The **COSMOS** Project was initiated to support the computational modelling of toxicity and specifically to address the assessment of safety of cosmetics ingredients. The **COSMOS** Project was co-ordinated by Liverpool John Moores University and brought together a further twelve partners from the EU and two from the USA. Specifically the partners brought together expertise in toxicity data compilation and evaluation, threshold of toxicological concern (TTC), modelling of toxicity and pharmacokinetics and relevant informatics. The philosophy of the **COSMOS** Project was to ensure a reliable database and robust computational workflows would be freely available to aid safety assessment.

The proper harvesting, curation and presentation of toxicological data and information is a pre-requisite for modelling. The COSMOS Database (DB) is a freely available legacy from the **COSMOS** Project (available from *https://cosmosdb.eu*) that incorporates an inventory of over 5,000 chemical structures which are known cosmetics ingredients; toxicity data for over 1,600 chemical substances including the results of over 12,500 toxicity studies were compiled in the database. These data proved to be a rich source of information to enable extraction of chemically based toxicological knowledge. In addition, safety data (the concentration at which no significant adverse effect was observed), derived from the repeated dose toxicity test results, enabled a thorough reanalysis of pre-existing TTC levels. The TTC approach was supported by a scheme that allows for route-to-route extrapolation (i.e. from oral to dermal exposure) of toxicological information. Models to predict activity of chemicals, solely from their structure, were developed for effects such as binding to DNA, protein and nuclear receptors, as well as predicting effects associated with liver toxicity. The modelling approaches were developed in concert with on-going research into relevant adverse outcome pathways (AOPs). Biokinetics and the distribution of compounds in humans, following dermal and oral exposure were also modelled. The full suite of models for biokinetics and toxicity prediction is freely available as a series of KNIME workflows *(http://www.cosmostox.eu/what/knime/)* with accompanying web-tutorials.

There is a significant legacy from the **COSMOS** Project, which includes not only COSMOS DB, KNIME workflows, webinars, web-tutorials, a large number of publications and conference presentations but also the development of new approaches to asses cosmetic safety.

4.5.2 Project Context and Objectives

Society expects high quality, safe products. To ensure the safety of cosmetics ingredients, as well as many other types of chemicals, testing on animals has normally been performed. The effects seen in animals were extrapolated up to those that may occur in man and a decision made regarding the safety of a particular ingredient. However, decades of public opinion has been against animal testing on finished cosmetics products and their individual ingredients. This stimulated changes to legislation in Europe and elsewhere, and ultimately resulted in the European Union's Cosmetics Regulation which required cessation of all animal testing, for toxicological purposes, of cosmetic ingredients marketed in the EU from 2013 onwards.

To assess the safety of cosmetics ingredients, knowledge is required about their effects following long-term use. To determine this experimentally, repeated dose experiments were performed. The goal therefore is to be able to predict the effects from such an experiment, without recourse to using animals. Prediction of repeated dose toxicity has posed a real challenge to computational modelling to provide a viable alternative to animal testing. To understand this challenge, it must be appreciated that toxicity resulting from long-term exposure to a chemical is a consequence of perturbing a biological system at the cellular,

tissue and organ (or multiple organ) level. Several factors can influence the outcome including toxicokinetics and physiological adaptive response mechanisms. Repeated dose toxicity testing provides a No Observable (Adverse) Effect Level (NO(A)EL) which is used in quantitative risk assessment of chemicals; data from 28-day or 90-day rodent oral toxicity assays are typically used. Because of these factors, added to the fact that there are many potential mechanisms and may be multiple (interacting) organ systems involved in eliciting the toxicity, *in silico* models have previously been considered too simplistic to model such complex interactions.

There are a number of requirements for the development of models for repeated dose toxicity. It is fundamental that models are based on reliable information. Thus, access to high quality toxicity data, on which to extract knowledge and develop models, is required. These data need to be stored in a manner that allows relevant details (such as experimental protocol, organ level effects, pathology, etc.) to be readily accessed. There is currently no single source of repeated dose toxicity data; in addition, many data may be available within industry with no means of extracting them. What is required is a comprehensive, flexible and reliable database, tailored for repeated dose toxicity, from which useful predictive models can be developed.

Project Objectives

It is clear that to meet the needs of 21st Century Toxicology, computational modelling must move away from being a retrospective, and often academic, analysis of data and information which has often been performed in isolation. There is now a paradigm-shift towards developing models based on an understanding of the underlying mechanisms involved in eliciting an adverse effect. Development of new models will require the integration of several approaches, such as category formation and read-across, *in vitro* methods, *in vitro* to *in vivo* extrapolation (IVIVE) models, etc. The aim is to develop flexible and usable models that may achieve the ultimate goal of predicting NO(A)EL values using entirely non-animal, alternative methods. Such an aspiration requires a multinational effort to pool expertise and resources into a unique project entirely focused on addressing this challenge. The **COSMOS** Project was developed to meet several of these challenges by tackling some of the problems outlined above.

Specifically, the aim of the **COSMOS** Project was to develop tools for the retrieval of data and to support the prediction of repeated dose toxicity to humans for cosmetics-related chemicals. **COSMOS** was at the centre of efforts to integrate reliable and open access toxicity data, greater application of the TTC approach, grouping for read-across, (Q)SARs and modelling of biokinetics, with the opportunities offered by informatics and the toxicity pathway approach. This was in line with the current paradigm-shift in toxicology towards developing models based on an understanding of the underlying mechanisms involved in eliciting an adverse effect. The tools developed include adaptable workflows that form a set of building blocks allowing users to incorporate their own data and search existing data compilations. The specific objectives of the **COSMOS** Project were:

➠ To collate and curate toxicological data with an emphasis on repeated dose exposure. The COSMOS database of toxicological information for cosmetic ingredients (and beyond) was intended to provide the backbone to the development of alternative models and forms a robust platform to collect, organise and mine highly curated and quality assured *in vivo* and *in vitro* toxicity data. It was designed to have the capability of contributing to the development of alternatives in the other projects of the **SEURAT-1** Research Initiative by providing access to high quality data as well as to the **SEURAT-1** case studies.

➠ To create an inventory of known cosmetic ingredients and associated quality controlled chemical structures. The COSMOS Cosmetics Inventory was developed to define the chemical space of cosmetics ingredients within an informatics environment. This enabled the analysis of the chemical space of cosmetics ingredients and provided a basis on which to determine the need, or otherwise, to extend the existing TTC (Munro) dataset.

➠ To extend the TTC approach and assess its applicability to cosmetics. The **COSMOS** Project developed TTC approaches better suited to classes of cosmetic ingredients in order to support efficient safety assessment. The TTC approaches have updated current knowledge and data sets and involved considerable quality assurance by external expects. Further analysis and effort were also placed into providing better route-to-route extrapolation facilitating the use of a TTC value, based on oral dosing, to dermal exposure.

➠ To develop innovative toxicity prediction strategies based on chemical categories, read-across and (Q)SARs for organ level toxicity and relate these to key events in adverse outcome pathways (AOPs). The **COSMOS** Project aimed to provide a number of innovative computational tools for organ-level toxicity prediction, which were built around the COSMOS database and Cosmetics Inventory. In particular, chemical categories were developed from knowledge derived from AOPs. These were extended into more quantitative approaches to predicting toxic potency, e.g. (Q)SARs and refined to incorporate kinetic and metabolic studies to permit quantitative interpretation of results in terms of consumer risk. The AOP approach provided a transparent link from chemistry to toxicological effect. **COSMOS** supported the development and promotion of AOPs, in particular by providing a framework for organising the chemistry involved in the processes.

➠ To develop a multi-scale modelling approach including cell-based and physiologically-based kinetic (PBK) models to predict target organ concentrations and extrapolate from *in vitro* to *in vivo* exposure scenarios. Models for toxicodynamics and toxicokinetics were developed within the

COSMOS Project which extended capabilities for *in vitro – in vivo* extrapolation (IVIVE), allowing for the better application of results from cell-based assays to perform human safety assessment. Research included kinetics modelling (e.g. through PBK models); a better understanding of the effect of the test system (e.g. sorption) and chemicals' (e.g. volatility, stability) properties relating to extrapolation; and modelling and prediction of metabolism. The intention was that these models could be used to determine the internal exposure (dose at target organ level) necessary to elicit the effect.

➠ To use KNIME technology to integrate access to data and modelling approaches into adaptable and flexible computational workflows that would be publicly accessible, providing transparent methods for use in safety assessment of cosmetics. The **COSMOS** Project aimed to utilise the highly adaptable KNIME computational workflow technology as a platform to create and distribute models. The KNIME web-server was used as a means to distribute the models to stakeholders.

4.5.3 Main Achievements

4.5.3.1 Data Collection

The COSMOS Cosmetics Inventory

The COSMOS Cosmetics Inventory is a compilation of cosmetics-related ingredients incorporating information from the European Commission's Cosmetic Ingredients (CosIng) database and the US Personal Care Products Council (PCPC) lists, including over 19,000 unique International Nomenclature of Cosmetics Ingredients (INCI) names and over 9,000 unique CAS numbers (*Figure 4.42*).

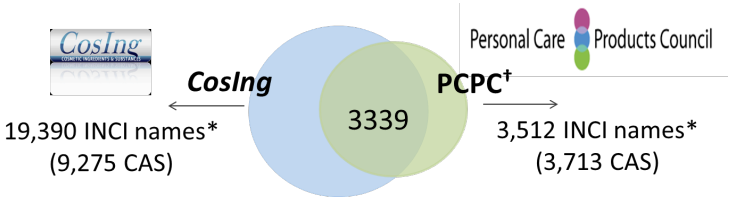

Figure 4.42 *Source and details of the COSMOS Cosmetics Inventory. *INCI: International Nomenclature for Cosmetics Ingredients; † in COSMOS DB v2.0, the US list (voluntary cosmetic registration program) is provided by CIR (Cosmetic Ingredient Teview). The COSMOS DB v1.0 counts are from 'Compilation of Ingredients Used in Cosmetics in the United States', 1st Edition, J.E. Bailey, Ed. The Personal Care Products Council, Washington D.C. 20036-4702. Therefore, the COSMOS DB v1.0 and 2.0 counts will be different.*

The COSMOS Database (DB)

Linked to the Cosmetic Inventory is the COSMOS DB (see *Figure 4.43*). The COSMOS DB is a chemo-centric system which provides chemical and toxicological data to support the data needs of the **COSMOS** Project, as well as safety assessors in public and private organisations. COSMOS DB a high quality web-based database completely based on open source technology which links chemical structures to repeated dose toxicity and other endpoint data. In total, COSMOS DB contains more than 12,000 toxicity studies across 27 endpoints for over 1,600 compounds and more than 80,000 chemical records with more than 40,000 unique structures.

Figure 4.43 *COSMOS DB (https://cosmosdb.eu).*

Data Content

COSMOS DB integrates data from various sources into a unified data model:

1) Chemistry

Chemistry data have been collected from various sources. Special emphasis was put on cosmetics related chemicals. The European Union Inventory of Cosmetic Substances and Ingredients (CosIng, *http://ec.europa.eu/consumers/cosmetics/cosing*), and the US Personal Care Products Council (PCPC) inventories have been parsed into COSMOS DB (forming the COSMOS Cosmetics Inventory). **COSMOS** partners harvested oral toxicity data from sources including the US FDA (Food and Drug Administration), PAFA (direct food additives and colourants) and OFAS (food contact substances) databases, US EPA (Environmental

Protection Agency) ToxRefDB, Opinions from the European Commission's Scientific Committee on Consumer Safety (SCCS) and the scientific literature. In addition, skin permeability data were donated from the EDETOX Database and **COSMOS** partners.

2) Toxicity Information

The COSMOS DB contains 12,538 toxicological studies for 1,660 compounds. Within US FDA PAFA there are 12,198 studies for 27 endpoints. The oral repeat dose toxicity database (oRepeatToxDB) contains 340 studies for five endpoints. Rat is the single most tested species and liver is the top target organ (see *Figure 4.44*). Most common pathological findings include steatosis phenotypes.

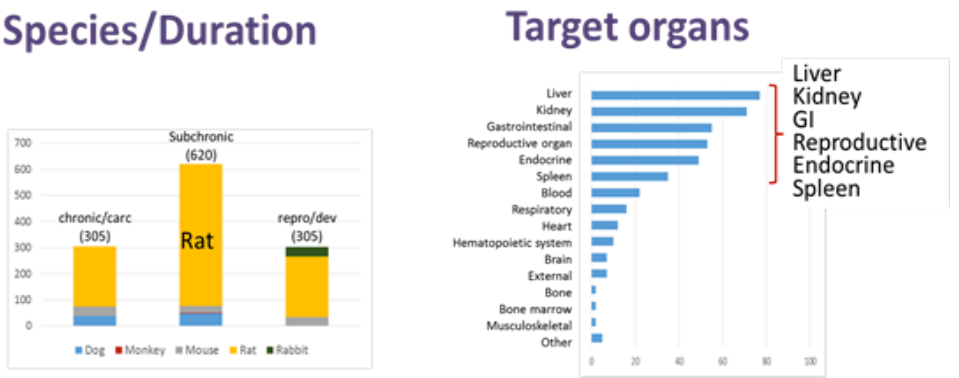

Figure 4.44 *Profile of in vivo study types in COSMOS DB.*

Components of COSMOS DB

The structure of the COSMOS Database is illustrated in *Figure 4.45.* The various components are discussed in the following.

1) oRepeatToxDB

A subset of the COSMOS DB with full-dose level toxicity information forms the oral repeated dose toxicity database (oRepeatToxDB, left panel in *Figure 4.45*). The oRepeatToxDB is enriched with 230 cosmetics-related chemicals, for which 340 oral studies (sub-acute (duration ≥ 28 days), sub-chronic, chronic, carcinogenicity (non-neoplastic lesions), reproductive-developmental toxicity, neurotoxicity, and immunology studies) were harvested from available regulatory and literature sources (such as SCCS opinions, NTP reports) as well as primary literature publications. The oRepeatToxDB includes ontology for phenotypic effects at each dose level using controlled vocabulary. Toxicity effects observed at target organ sites have been organised hierarchically to relate organs to tissues to cells. Species were limited to rat/mouse for target organs, rat/mouse/rabbit for reproductive-developmental toxicity, and dog/monkeys for all studies except neoplastic effects.

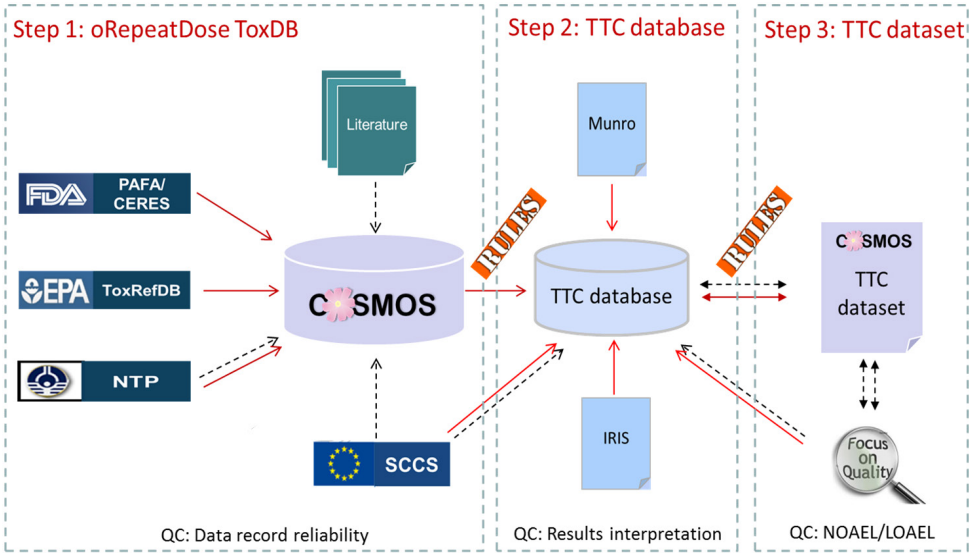

Figure 4.45 *Components of COSMOS DB. Red arrows indicate automatic processes, black dotted arrows indicate manual harvesting processes. The TTC database (right panel) stores the full history of NOAEL decisions and rationales.*

2) Threshold of Toxicological Concern (TTC) Database

The COSMOS TTC DB was extracted from the oRepeatDose ToxDB by applying a set of study selection criteria relating to following parameters: study type, species, duration, route of exposure, dose levels and range, effects and references (*Figure 4.45*, right panel).

Briefly, the COSMOS TTC DB contains 552 chemicals with critical information, including critical study information, critical effects and Point of Departure (POD) values. This new database consists of No Observe Adverse Effect Level (NOAEL) / Lowest Observed Adverse Effect Level (LOAEL) data of cosmetics-related chemicals along with information of oral toxicity studies selected as 'critical' by COSMOS Criteria and the review sessions.

3) COSMOS Safety Evaluation Database

The COSMOS Safety Evaluation Database was constructed in order to make the new TTC information available in a database format. Information includes: study design, NOAEL/LOAEL (values and owners), critical NOAEL, critical effects at LOAEL, quality control (rationales and quality control (QC) owners; see *Figure 4.46*).

The COSMOS Safety Evaluation Database includes all updated Margin of Safety data from SCCNFP/SCCP/SCCS as well as Margin of Exposure (MOE) and Acceptable Daily Intake (ADI) available from other regulatory bodies.

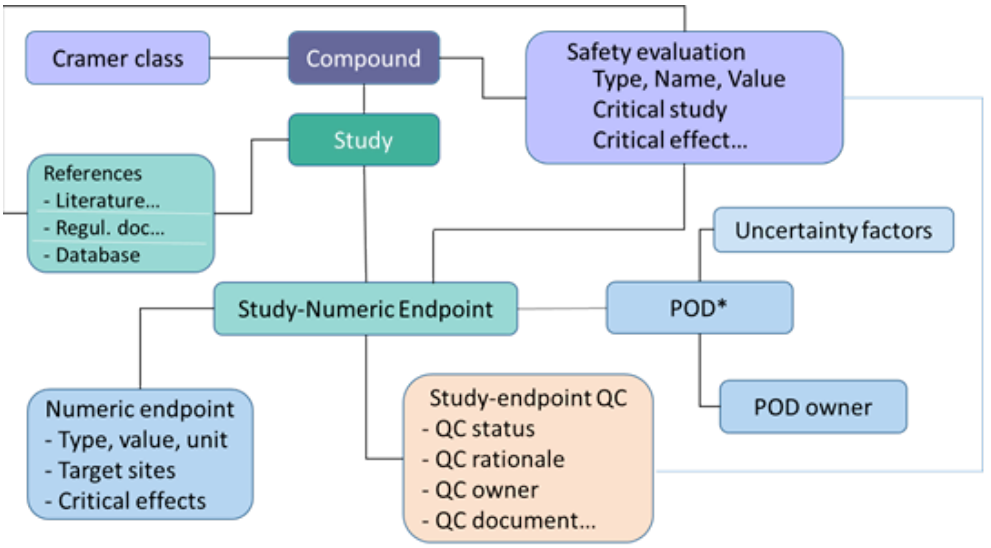

Figure 4.46 *Simplified COSMOS Safety Evaluation data model.*

Data Curation

A data curation strategy was developed in order to provide oral repeated-dose toxicity data. The process includes both manual harvesting and existing data merge.

The high quality of chemical records/structures and toxicity data within COSMOS DB was assured via formal QC and Quality Assurance (QA) procedures. Toxicity data were assessed for their quality and this information is also available in COSMOS DB. The data record reliability was rated objectively by applying COSMOS MINImum Study (MINIS) criteria which represent the set of required and recommended experimental parameters. Data acceptance was assessed by toxicologists using Klimisch scores.

Searching and Exporting Data from COSMOS DB

COSMOS DB supports data retrieval via a user-friendly web interface, which allows querying by chemical, toxicological or both types of data. The chemical search can be carried out by name, registry numbers (CAS RNs) or other identifiers (e.g., COSMOS IDs, DSSTox IDs) provided for a single structure or for a list of chemicals, as well as by structure (sketched or provided as SMILES string). Exact, substructure and similarity structure search types are possible. The scope of the toxicological queries can be defined in detail by the users with respect to the endpoint (study type) and endpoint-specific parameters (e.g., species, strain, sex, route of administration, cells/cell lines, test calls, target sites) as well as data source. The toxicological data of interest can be retrieved for all relevant compounds included in the database (if no query chemicals are defined) or retrieved just for the specified compounds

of interest. The search can be also carried out by inventories, e.g., cosmetics inventories (CosIng + PCPC).

COSMOS DB also includes a web-based application allowing for the export of data and predefined datasets. As shown in *Figure 4.47*, the workflow 'Export structures and data' allows exporting of single or multiple structures in an SD file or saving compound-related data in a flat file. The workflow 'TTC export' allows downloading of the pre-defined COSMOS non-cancer and Munro TTC datasets. The content of the first version of COSMOS DB v1.0 was also made downloadable from COSMOS Space as a SD file and Excel table.

Figure 4.47 *Web application of workflows within COSMOS DB.*

Sustainability and Legacy of COSMOS DB

COSMOS DB will remain available after the end of the project and access to COSMOS DB will remain free upon registration with an email address. The COSMOS DB effort will be continued and the database will be updated by an international public data-sharing initiative led by LJMU along with other partners from the **COSMOS** consortium. COSMOS DB v2.0 will continue the effort toward one centralised database for public projects. COSMOS Data SharePoint will house the v2.0 content, and will be maintained and managed by Molecular Networks-Altamira *(https://cosmosdb.eu/cosmosdb.v2/)*.

COSMOS Space

COSMOS DB is supported by COSMOS Space which facilitates sharing of predictive toxicology resources (data sets, models, workflows, documentation, meta-data as wikis editable by the data owners). COSMOS Space is a publicly available resource based on a free-registration process with minimum user information requirements. It was designed, implemented, tested within the **COSMOS** Consortium and with external users and released publicly during this reporting period.

COSMOS Space *(http://cosmosspace.cosmostox.eu)* manages the user interface to COSMOS Share (a public pool of resources within the COSMOS Space community), COSMOS DB and the COSMOS KNIME WebPortal (see *Figure 4.48*). COSMOS Space facilitates sharing of predictive toxicology resources (data sets, models, workflows, documentation, meta-data) as uploads within the user's own account. A basic scoring system to follow-up a community feedback on shared resources, as a validation flag of interest on resources, has been introduced. Additionally, COSMOS Space facilitates links to COSMOS DB and supports the dissemination of the COSMOS KNIME workflows. In particular COSMOS Space links to all COSMOS online resources, makes the documentation of the COSMOS KNIME workflows publicly available to external users, provides a list of all available workflows as well as a standardised online template for the workflow developers to compile the documentation.

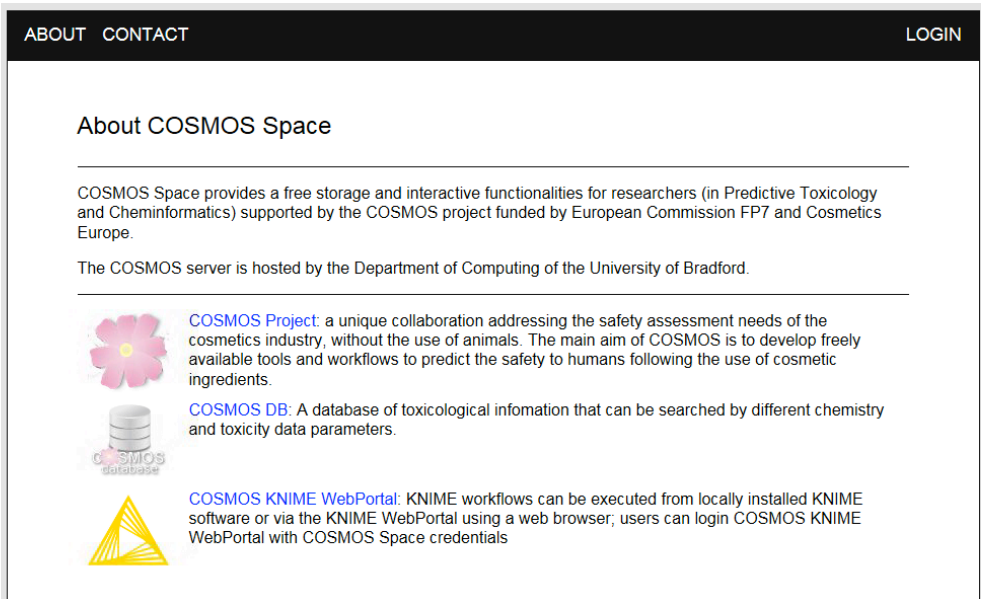

Figure 4.48 *COSMOS Space.*

4.5.3.2 Threshold of Toxicological Concern (TTC)

COSMOS TTC Database

As the basis for the evaluation of the TTC approach to cosmetics-related chemicals, a new oral repeated dose toxicity database, oRepeatTox DB (included in COSMOS DB), was compiled to be used as a resource to build the new COSMOS non-cancer TTC database of NO(A)ELs, enriched with cosmetics ingredients. This general oral repeat-dose toxicity database was the key in the process of compiling the appropriate studies to be used in construction of the TTC dataset. These studies provided the underlying data to be evaluated for an appropriate

NOAEL and LOAEL decision. It was also important to house the NOAEL/LOAEL values in a separate simpler database with critical effects as well as the sources of the decisions and their rationales. Therefore a database with reliability scores for methods and results was built (see section 4.5.3.1 above).

The final TTC database contains 556 chemicals. Most of these chemicals (495) belong to cosmetic inventory.

Curation Strategy of COSMOS TTC Database

To ensure a reliable, transparent database, much effort was devoted to the selection of data for chemicals and toxicity studies. Chemicals were selected when their toxicity data originated from regulatory sources including EU SCC, SCF, FDA, EPA, and JECFA.

The curation process for the COSMOS TTC dataset involved the following three stages (*Figure 4.49*).

➠ Implementation of study inclusion criteria to all studies included into the toxicity database:
- oral repeat dose (>=28 days) toxicity studies;
- reproductive and developmental toxicity studies.

➠ Implementation of data reliability and NOAEL selection criteria:
- reliability criteria;
- chronic NOAELs preferred;
- lowest NOAEL with clear LOAEL selected;
- free standing NOAELs excluded whenever possible.

➠ Final assessment by toxicological experts of the remaining data in terms of:
- toxicological meaningfulness;
- human relevance;
- known mechanism.

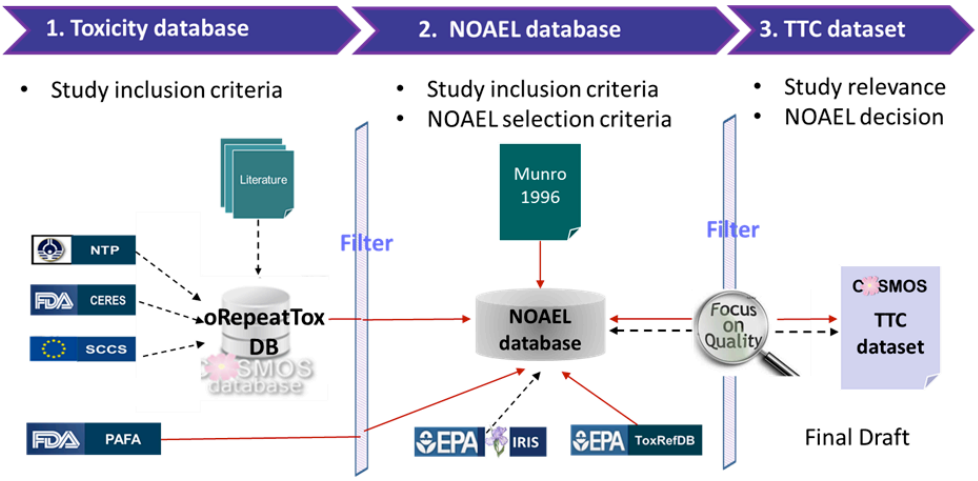

Figure 4.49 *The curation process for the COSMOS TTC dataset.*

It was not possible to evaluate all studies (around 500), however approximately 25% of the existing studies underwent the QC process. During that process, nine cycles of dataset evaluation and four separate QC sessions were undertaken.

Profiles of the Chemicals

The majority of chemicals (85%) in the COSMOS TTC database are found in the Cosmetics Inventory defined by COSING or PCPC databases. The biologically active lipid-soluble vitamins and essential amino acids were excluded from TTC database.

The largest sources of the COSMOS Non-Cancer TTC database were the EU Scientific Committee of Consumer Safety (EU SCC), US FDA PAFA and CFSAN documents, EPA ToxRefDB, and Munro. There were many overlaps between sources, although the critical studies and the point of departure decisions may vary widely.

'Munro' is the database from which the current non-cancer TTC thresholds were derived (Munro et al., 1996). There are 190 test substance names (178 unique chemical structures) overlapping between the COSMOS and Munro Non-Cancer TTC databases. The analysis of Cramer classification using the Toxtree v2.6 application showed that greater fraction of chemicals in the COSMOS TTC database (37%) belongs to Cramer Class I than in Munro dataset (less than 25%). Conversely, Cramer Class III is wider represented in Munro dataset (73%) than in the COSMOS TTC database (55%). Some chemical classes, such as nutrients were removed from the COSMOS TTC database, as the TTC concept is not applied to this group of compounds by regulatory bodies. However, retinol and phenyl alanine are still present in Munro dataset. Review of Cramer Classes showed 31 cases where Munro and Toxtree assignments were in disagreement. These conflicts were manually reviewed by **COSMOS** chemistry partners to assign the Cramer Classes for each case.

Chemical space was characterised with a new categorisation method using ToxPrint chemotypes instead of using Cramer Classes. ToxPrint chemotypes grouped chemicals based on types of atom, bond, ring, functions and connectivity, which are coded in the Chemical Substructure Representation Mark-up Language (CSRML) format. The comparative analyses using both Cramer Classes and ToxPrint Chemotypes also confirmed that the chemical space of the new COSMOS Non-Cancer TTC database is significantly different to the current Munro dataset. The COSMOS TTC dataset is missing natural products (steroids), in turn Munro is lacking organosilicon compounds and cationic surfactants. This confirmed the enrichment of the TTC database with cosmetics-relevant substances as compared to the original Munro dataset: The chemical space of surfactants, organosilicon and hair dye compounds was significantly increased.

Toxicity Study Profile

Although the COSMOS non-cancer TTC database used chronic NOAELs by preference, the most abundant studies in the database are rat sub-chronic / short-term studies (≥28 days). Therefore, duration adjustment factors of 3-fold and 6-fold were applied to sub-chronic and short-term (≥28 days) studies respectively, to derive the point of departure for chronic NOAEL values. In addition, for studies giving rise to Lowest Observed Adverse Effect Level (LOAEL) values alone, an adjustment factor of 3-fold was applied. No duration adjustment factors were applied to reproductive/developmental studies. These factors were applied to the original NOAEL or LOAEL values to calculate the point of departure of the chemicals for this database.

Moreover, the COSMOS TTC database is expected to be less potent than that of the current Munro database since the content was enriched with cosmetics-related chemicals. The median NOEL for the whole Munro database is 20.5 mg/kg-bw/day with a mean of 222 mg/kg-bw/day (for 613 test substances), whilst the median of the whole COSMOS TTC database is two times higher (*Figure 4.50*).

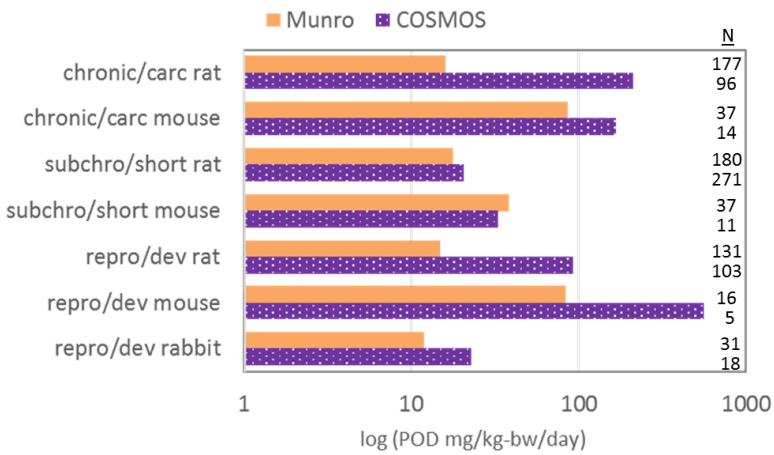

Figure 4.50 *Comparison of POD profiles of the two TTC database via study/species.*

Figure 4.50 illustrates that across the various study types /species, the Munro database is indeed more potent than COSMOS TTC database (a shorter bar denotes a smaller median POD value, and hence more potent).

Data Distribution and Thresholds

To calculate the point of departure (POD), the following Duration Adjustment Factors were applied:

- uncertainty factor of three for extrapolating of sub-chronic NOAEL to chronic NOAEL;
- uncertainty factor of two for extrapolating of short term NOAEL to sub-chronic NOAEL;
- no uncertainty factor was applied to reproductive, developmental, or multigeneration studies.

Analysis of the data in the COSMOS TTC dataset revealed that the 5th percentile in the cumulative probability distribution of NOAEL values for Cramer Class I and III cosmetics is higher than the corresponding 5th percentile in the Munro dataset. Conversely, the 5th percentile in the cumulative probability distribution of NOAEL values for Cramer Class II in COSMOS TTC database is much lower than the corresponding 5th percentile in the Munro dataset. This is caused by presence of two significant outliers: allyl heptanoate and 3,5-di-tert-butyl-4-hydroxyhydrocinnamate.

In turn, the analysis of the data in the combined (COSMOS + Munro) dataset showed that the 5th percentiles in the cumulative probability distribution of NOAEL values for Cramer Classes are similar to Munro dataset, especially for Cramer Class II (Munro: 0.15 mg/kg-bw/day). In the case of Cramer Class I, the 5th percentile in the cumulative probability distribution of NOAEL values in the combined dataset is slightly higher than in Munro dataset (Munro: 3.0 mg/kg-bw/day). For Cramer Class II, the 5th percentile in combined data set is slightly lower (Munro: 0.91 mg/kg-bw/day).

Tiered Decision Tree

In order to address the application of TTC values derived from oral data to use cases relevant to cosmetics, a tiered decision-tree approach was developed to assess the chemicals' bioavailability, which takes into account the absorption/permeability via dermal or oral routes as well as metabolism differences between skin and liver (see *Figure 4.51*). Dermal absorption was calculated using an established predictive algorithm (Potts and Guy equation) to derive the maximum skin flux adjusted to the actual 'dose' applied. The predicted systemic availability (assuming no local metabolism), can then be ranked against the oral TTC for the relevant structural class. The predictive approach has been evaluated by deriving the experimental/prediction ratio for systemic availability for 22 cosmetic chemical exposure scenarios. These emphasise that estimation of skin penetration may be challenging for penetration enhancing

formulations, short application times with incomplete rinse-off, or significant metabolism. While there were a few exceptions, the experiment-to-prediction ratios mostly fell within a factor of 10 of the ideal value of 1. It can be concluded therefore, that the approach is fit-for-purpose when used as a screening and prioritisation tool. The details on the development of this tiered decision tree were published. The algorithms for tiered decision tree and skin permeability models were coded into KNIME workflows.

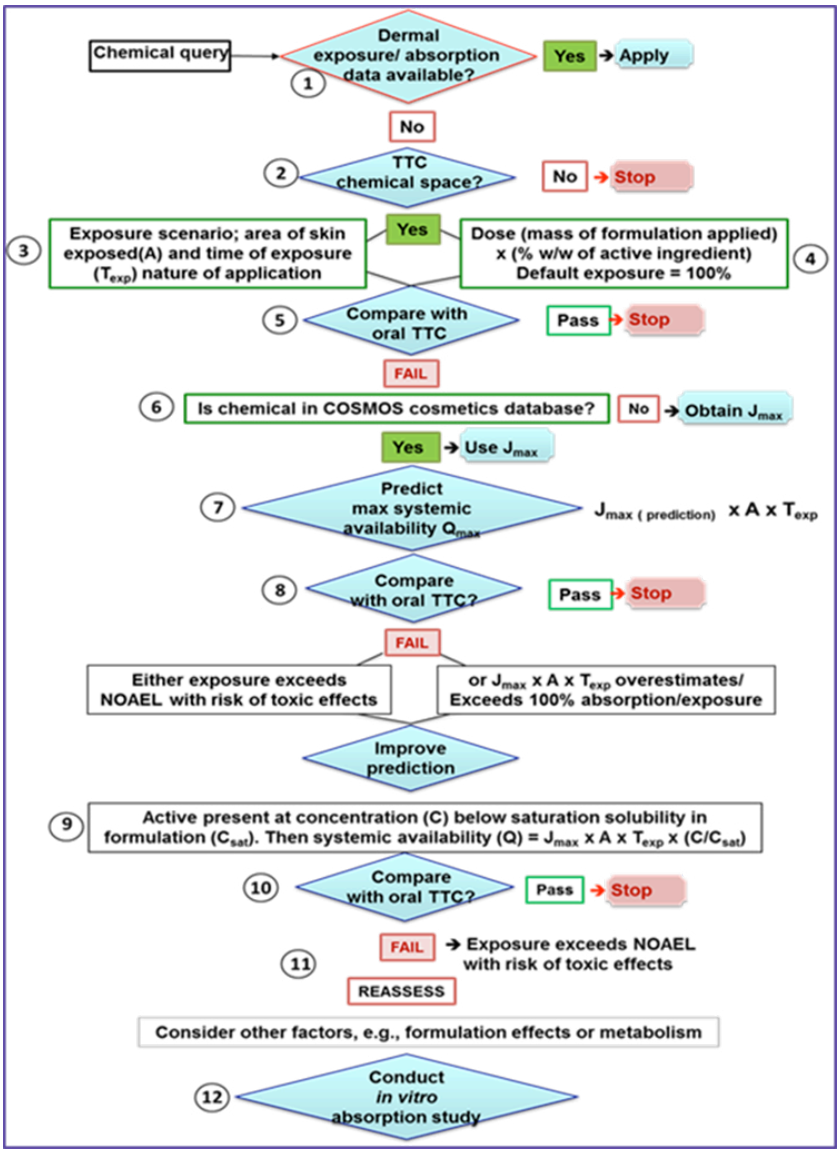

Figure 4.51 *The tiered tree approach for extrapolating oral TTC to dermal exposure (Williams et al., 2016).*

Software Tools

The non-cancer TTC databases of COSMOS and Munro have been successfully imported into the COSMOS DB v2.0 *(https://cosmosdb.eu)*. A web-based workflow service was established to provide a set of software tools to facilitate use of the TTC method.

Three workflow tools have been implemented:

➠ 'Export structure and data': Structures and other compound data can be exported from the database. A text file containing CMS ID, names, or CAS numbers can be selected and searched within this workflow. The resulting list of hits can be selected and exported either as SD file or flat file as illustrated in *Figure 4.52*.

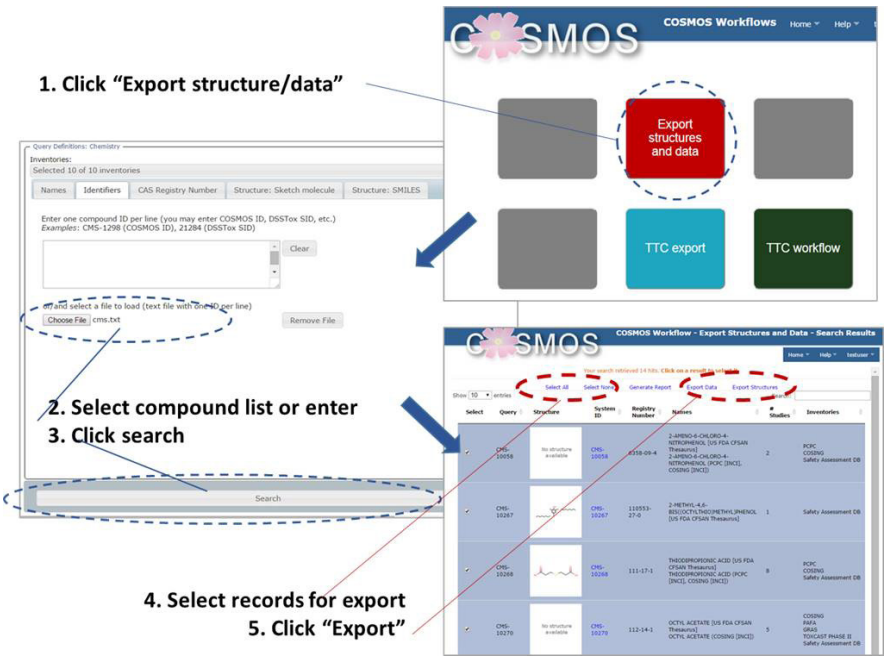

Figure 4.52 *Steps to export structures/data from COSMOS DB.*

➠ 'TTC Export': The full COSMOS TTC and Munro datasets as well as a combination of both as the 'Munro expanded by COSMOS' can be exported as MS Excel file containing the following columns: chemistry; study info: test substance; study info: study design; study results; POD calculations; critical effects; data quality – QC results; data quality; study info: data quality; study info: references and citations. The functionalities are demonstrated in the screenshots depicted in *Figure 4.53*.

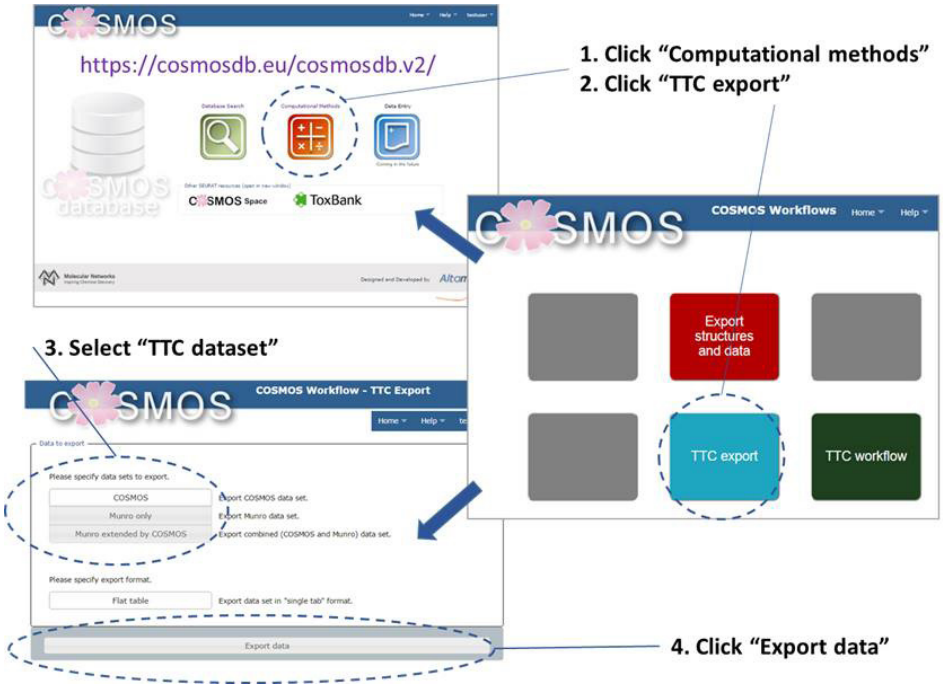

Figure 4.53 *Steps to export TTC dataset.*

➠ ‘TTC workflow’ to run decision tree: The workflow is to enter a compound (SMILES or draw molecule) along with the exposure value and retrieve the assessment from the system based on the Cramer Classification (using Toxtree implemented in COSMOS DB) and the current thresholds. Although **COSMOS** was well aware of the problems encountered in Toxtree results for Cramer Classifications, it was decided not to create another source of potential problems by implementing yet another Cramer Tree application until the problems could be solved. For this reason, the user is warned when the application encounters known problems in Toxtree.

When there are more than one study reported with different NO(A)EL/LO(A)EL values, an algorithmic selection of studies with minimum NO(A)EL values was performed. These methods can be applied to select studies from US FDA PAFA or US EPA ToxRefDB databases. This functionality is provided as ‘export structure and data’, not as a TTC export tool.

4.5.3.3 Innovative Chemistry Approaches

Computational Tools for Toxicity Prediction

In order to develop new models for toxicity prediction, an evaluation of QSAR models to predict the chronic toxicity endpoints that are important for TTC thresholds, e.g. repeated dose toxicity and selected target organ/tissue toxicities, was undertaken. Then a number of computational (so-called *in silico*) models were developed and evaluated within the **COSMOS** Project.

Modelling of the Binding to Nuclear Receptors (NRs) Involved in the Development of Liver Steatosis

In order to develop an *in silico* strategy for the evaluation of potential binding to, and possibly potential activation of, nuclear receptors involved in the development of liver steatosis, a number of computational approaches have been applied and integrated. A variety of nuclear receptors can play a role in liver steatosis, including LXR (liver X receptor), PXR (Pregnane X Receptor), AhR (Aryl hydrocarbon receptor), ER (estrogen receptor), PPARα and PPARγ (peroxisome proliferator-activated receptor isoforms α and γ). The focus of the **COSMOS** *in silico* studies has been mainly on LXR and PPARγ receptors.

1) Partial Least Square – Discriminant Analysis classification models for LXR binding prediction

A QSAR classification model for the prediction of potential LXR binders was developed based on Partial Least Square – Discriminant Analysis (PLS-DA) classification method using MOSES molecular descriptors. The model is based on three latent variables derived from seven MOSES 2D descriptors which encode basic electronic properties, hydrophobicity, molecular shape and complexity. Moreover, two related PLS-DA models based on freely available molecular descriptors, namely PaDEL and RDKit descriptors were developed. The PLS-DA classification models were implemented in KNIME.

2) Molecular Modelling methods for LXR binding prediction

Available crystal structures of human LXR (α and β isoforms) complexes from the Protein Data Bank (PDB, *http://www.rcsb.org*), in addition to the available experimental data on LXR binding affinity and activation from literature and available databases (e.g., ChEMBL), were collected. Different molecular modelling approaches, including both ligand- and structure-based methods (i.e., ensemble docking, e-pharmacophore, and fingerprints-based similarity), were used in order to characterise the ligand binding domain of LXR and to define the essential features leading to LXR binding (*Al Sharif et al., 2016*). A validation dataset, including known LXR binders, was assembled to assess the ability of the developed methods to identify LXR binders. The molecular modelling approaches developed were then integrated by means of data fusion/consensus modelling with the aim of optimising the predictive performances by taking advantage of the different modelling methodologies.

3) Nuclear receptor ligand screening

Structural and physico-chemical features of nuclear receptors ligands (e.g., ligands of RAR, RXR, LXR and PPAR etc.) have been examined using data (e.g. Ki and EC50) from ChEMBL database and data (i.e. ligand-protein-interactions) from PDB database (*Steinmetz et al., 2015; Mellor et al., 2016*). The gathered information was used to select relevant chemical features of potential NR ligands. For each NR, the information on relevant substructures (SMARTS strings of scaffolds and functional groups) and physico-chemical features, e.g. ranges for molecular weight, log P, vertex adjacency information magnitude, topical polar surface area, number of hydrogen bond donors and rotational bonds, are checked and if suitable, the compound is assigned as a potential ligand. For NR ligands which are associated with hepatosteatosis, an additional filter based on structural alerts for liver steatosis is applied.

Models for Gastrointestinal Absorption (GIA) and Skin Permeability Prediction

The estimation of bioavailability after oral and dermal administration is of key importance in the prediction of the chronic toxicity of cosmetic-related ingredients. In fact, whilst cosmetics are usually applied dermally, the majority of available repeated dose toxicity data are obtained from oral administration. The extrapolation of chronic toxicity data (e.g. NOEL/NOAEL) from oral to dermal exposure routes, i.e. the 'oral-to-dermal extrapolation', is one of the issues addressed within the **COSMOS** Project in order to extend the current TTC approaches to cosmetic substances.

To support bioavailability estimation after oral and dermal exposure, several scenarios for use of NOAEL data have been proposed and *in silico* models predicting GIA and skin permeability were developed.

1) PAMPA permeability prediction

The PAMPA (parallel artificial membrane permeation) assay is a high-throughput *in vitro* assay for the prediction of oral passive absorption. Permeability constants obtained from the double sink PAMPA assay were used to develop a multiple linear regression (model, as an improvement of existing model (*Nakao et al., 2009*). The new QSAR model implemented in KNIME uses log D and TPSA/MW ratio as descriptors and a data set of 276 compounds from the Database of Double-Sink PAMPA log P_0, log P_m at pH = 6.5 and logP_m at pH = 7.4. Because of the lack of freely available tools for log D estimation, two implementations of the model were produced based on log D estimations readily obtainable through free online services ChemSpider.com (calculated by ACD/Labs tools) and chemicalize.org (calculated by ChemAxon tools).

2) Skin permeability prediction

The QSAR developed by *Potts and Guy* (*1992*) to predict skin permeability was modified by incorporating a larger dataset and a statistical tool to assess data quality (*Steinmetz et al., 2014*). The QSAR model consists in a multivariate linear regression based on two descriptors, i.e. molecular volume and lipophilicity, and using confidence score as weights.

Chemical Space Analysis

A computational procedure for explorative analysis of the chemical space of datasets/chemical inventories was developed combining multiple approaches and descriptors. The procedure for chemical space analysis includes the following three main analyses.

1) Physico-chemical space analysis

The physico-chemical space of the dataset was analysed by means of distribution analysis of individual physico-chemical properties and Principal Component Analysis based on physico-chemical properties. A sub-set of descriptors accounting for physico-chemical and electronic properties were calculated using the PaDEL-Descriptors software and used for the physico-chemical space analysis as part of the Chemical Space Analysis Workflow.

2) Structural space analysis

The structural space of the dataset was analysed by means of Principal Component Analysis based on 1D-2D molecular descriptors. A sub-set of molecular descriptors accounting for mono- and bi-dimensional structural features were calculated using the PaDEL-Descriptors software and used for the structural space analysis as part of the Chemical Space Analysis Workflow.

3) Functional groups profiling

The functional group profiling of the dataset was performed by means of frequency rate analysis based on defined molecular feautres (e.g., chemotypes, fingerprints, functional groups, etc.). An example of the output is provided in *Figure 4.54*. Substructure fingerprints calculated using the PaDEL-Descriptors software were used for the functional group profiling as part of the Chemical Space Analysis Workflow.

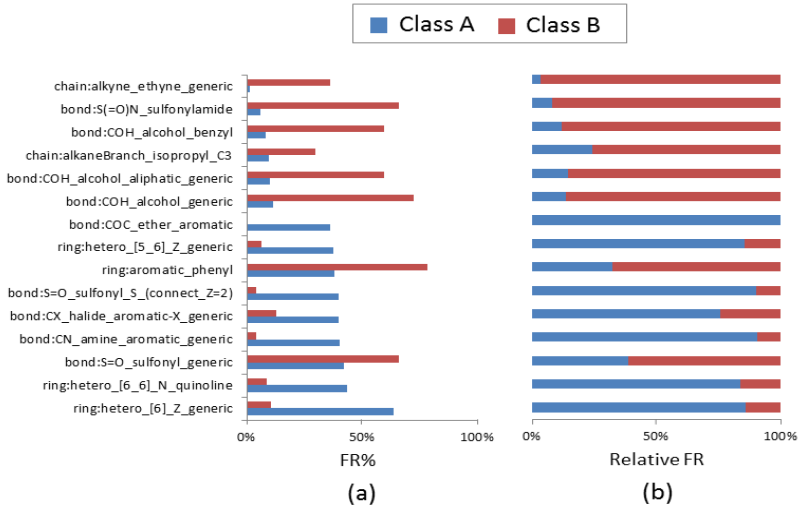

Figure 4.54 *Frequency rate bar graphs: a) frequency rate (FR%) of features within each class; b) relative frequency of features among the classes.*

Structural Alerts

The fundamental requirement for the development of a category suitable for predicting toxicological endpoints is the ability to group chemicals together based on a common molecular initiating event (MIE). The MIE is the interaction between a chemical and biological system that results in the initiation of the biological cascade leading to adverse outcome. Structural alerts define the key futures of a molecule that are required for commencing the MIE and provide the mechanistic knowledge about MIE. A collection of structural alerts that induce the same MIE are considered to be an *in silico* profiler.

1) In silico profiler for covalent DNA binding

It is well known that for both mutagenicity and carcinogenicity one of the fundamental steps is the formation of a covalent bond between a nucleophile and an electrophile. The mechanistic importance of this chemical reaction makes the mechanistic alert approach the natural choice for developing the profiler for DNA binding. Moreover, the assessment of the mechanistic domain overlap between corresponding structural alerts in the literature compilations has been investigated. This analysis ensured that for a given structural alert the maximum mechanistic information (and thus domain) was extracted. A total of 111 structural alerts crossing six broad organic chemistry mechanisms (domains): acylation, Michael addition, Schiff base formation, unimolecular aliphatic nucleophilic substitution, bimolecular aliphatic nucleophilic substitution and radical mechanism have been created and defined as SMARTS patterns (*Enoch et al., 2010*).

2) In silico profiler for covalent protein binding

The *in silico* profiler for covalent protein binding was developed based on the review of current scientific knowledge on structural alerts relating to a number of toxicity endpoints. There are several publications in which structural alerts for direct acting and indirect acting electrophiles have been published. The existing structural alerts have been mapped in terms of their relationships with mechanistic organic chemistry (i.e. identify alerts from the published compilations related to covalent protein binding). The mapping was performed to achieve maximum overlap and usability whilst restricting redundancy in the alerts, and to ensure that the alerts are related to the molecular initiating event of covalent protein binding.

As a result, a total of 108 structural alerts covering five broad organic chemistry mechanisms (domains): acylation, Michael addition, Schiff base formation, bimolecular aliphatic nucleophilic substitution and aromatic nucleophilic substitution have been created. The identified mechanistic structural alerts were defined as SMARTS patterns (*Enoch et al., 2011*). Also the detailed mechanistic chemistry reaction associated with each of the alerts has been compiled. The mechanistic information is intended to outline how the alert can act as a direct electrophile or how it can be converted into an electrophile. Therefore, the important consideration within the mechanistic chemistry framework is the inclusion of potential metabolic activation.

3) In silico profiler for hepatotoxicity

The ability of a compound to cause adverse effects to the liver is one of the most common reasons for drug development failures and the withdrawal of drugs from the market. However, the complexity and diversity of hepatotoxicity, the limited (if any) mechanistic insight, the lack of large high quality liver toxicity datasets and the role of metabolism, ensure that hepatotoxicity is one of the most difficult endpoints to model.

The *in silico* profiler for hepatotoxicity was developed based on generating 16 chemical categories and then developing structural alerts able to identify potential hepatotoxins (*Hewitt et al., 2013*). This was achieved by grouping chemicals based upon their structural similarity, and then the mechanism(s) by which these compounds cause hepatotoxicity were investigated and mechanistic rationale was proposed, where possible, to yield mechanistically supported structural alerts. Alerts of this nature have the potential to be used in the screening of compounds to highlight potential hepatotoxicity. The structural alerts identified were defined as SMARTS patterns and implemented in KNIME.

However, taking into account the complexity of hepatotoxicity, the structural alerts are not limited to a single mechanism of action. Indeed, it is probable that many hepatotoxic compounds elicit their toxicities via multiple mechanisms of action. Therefore, it is also possible that the alerts within this profiler may possess the ability to initiate multiple adverse outcome pathways leading to hepatotoxicity.

4) In silico profiler for mitochondrial toxicity

The ability to predict organ-level toxicity is becoming increasingly important to the long term goal of replacing animal use in determining a Lowest Observed (Adverse) Effect Level (LO(A)EL). The toxicity induced by mitochondrial dysfunction has been linked to a variety of organ toxicities within kidney, liver and nervous tissues. Therefore, there is an urgent need to develop models able to early detect potential mitochondrial toxicants. The structural alerts demonstrate such ability for identifying chemicals which can disrupt mitochondrial functionality.

21 Structural alerts for mitochondrial toxicity were developed based around clearly defined mechanistic information (*Nelms et al., 2015a, b*). This was achieved by grouping chemicals based upon their structural similarity, followed by a literature search to elucidate mechanistic information for the chemicals in categories associated with toxicity to mitochondria. The identified structural alerts were defined as SMARTS patterns and implemented in KNIME.

Ranking Methods and Ranking Models

A priority setting procedure was developed by means of innovative *in silico* approaches and chemometric tools to allow for the screening and ranking of chemicals according to their

toxicity profiles. Ranking is equivalent to sorting chemicals according to their relative levels of concern, thus providing the basis for analysing trends across multiple endpoints; additionally ranking may lead to the identification of different profiles of toxicological behaviour, which might also be regarded as different subcategories.

Different methodologies (e.g., consensus modelling, data fusion and total order ranking methods) have been employed, compared and integrated to develop an *in silico* procedure for ranking chemicals. A number of case-studies were carried out to exemplify the use of different ranking approaches that combine results from different sources:

- Ranking chemicals based on their potential binding to Liver X Receptor (LXR);
- Ranking chemicals across multiple endpoints, including LXR binding potential and liver toxicity potential.

4.5.3.4 Toxikokinetics in the Safety Assessment of Cosmetic Ingredients

Development of PBK models

Physiologically-based kinetic (PBK) models are crucial models to extrapolate from *in vitro* measurements to *in vivo* predictions. In the context of the ban of animal testing in cosmetics hazard assessment, these models have to be calibrated based on *in vitro* data and mathematical models.

The Virtual Cell Based Assay (VCBA) (see Figure 4.55)

The purpose of this model is to simulate the fate of a chemical and the intracellular concentration leading to cell perturbation. The VCBA comprises five interconnected sub-models:

- The fate and transport model that takes into consideration evaporation, partitioning of chemicals from the dissolved phase to serum proteins and lipids, adsorption onto the plastic, and also degradation and metabolism;
- The cell partitioning model that considers partitioning of the chemical between four compartments: one aqueous fraction (intracellular water) and three non-aqueous fractions (proteins, lipids mitochondria);
- The cell growth and division model that is based on the four cell cycle phases;
- The toxicity and effects model that simulates the direct effects of a chemical concentration on cell dynamics;
- Experimental set up model that takes into account the surface, area, size and shape of the well.

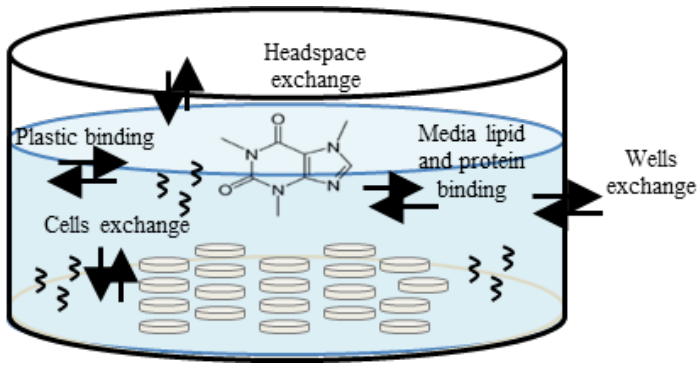

Figure 4.55 *Virtual Cell Based Assay (VCBA).*

Biokinetics / Toxicodynamics Models for Mixtures

The **COSMOS** Project aimed to develop biokinetics / toxicodynamics models to predict the HepaRG viability over time resulting from repeated exposures to the cosmetic mixtures. Long-term cell viability was studied through impedance measurements of HepaRG cells exposed repeatedly every 2 to 3 days for 4 weeks to mixtures of three hepatotoxic cosmetic ingredients: coumarin, isoeugenol and benzophenone-2 (BP2). Based on preliminary analyses on the cell viability following exposure to the single compounds, the mixture biokinetics / toxicodynamics model to predict the real-time cell viability following repeated exposures was built.

Physiologically Based Kinetic (PBK) Model

In order to support *in vitro* to *in vivo* and route-to-route (oral to dermal and inhalation to dermal) extrapolations, several PBK models were developed and calibrated for cosmetic ingredients and drugs (*Gajewska et al., 2014; 2015*). Those PBK models take into account of uptake in different tissues (gastrointestinal tract, skin, lungs) and a methodology to calibrate them without animal testing has been developed. PBK models have been coupled with the VCBA models to enable realistic estimates of *in vivo* effects from *in vitro* data. In a further step toward integrated multi-scale modelling, a 2D liver model including mechanisms for cell necrosis and cell proliferation has been coupled to the PBK models. This allowed for the analysis of the effect of the accumulation of compounds on hepatocyte viability and detoxification capacity after long-term repeated exposure.

Development of a Human Bioaccumulation Model

Traditionally, bioaccumulation potential has been assessed in aquatic or terrestrial organisms, but not directly in humans. To address this shortcoming, an approach for predicting human bioaccumulation potential based on the use of a simple PBK model was developed. This

generic PBK is able to predict the bioaccumulation potential of a chemical, expressed as the human bioconcentration factor (hBCF), based on selected physicochemical parameters, *in vitro* human liver metabolism and plasma-protein binding data, minimal renal excretion and a constant exposure scenario.

The PBK model was coded in R, and implemented as an open source KNIME workflow. This model was designed to incorporate not only the chemical properties of the compounds, but also the processes that tend to decrease the concentration of the compound, such as metabolism.

IVIVE Extrapolation to Target Organ Level

Two independent approaches for extrapolating *in vitro* toxicity effects to the target organ level have been developed.

The first approach models long-term toxicity data using impedance metrics. Due to the cost of the long-term toxicity testing, the acute toxicity data to extrapolate to chronic toxicity data were also used. The model couples dynamic descriptions of the major *in vitro* kinetic processes involved with a simple model of viability loss. The model was applied to describe HepaRG cell viability loss following exposure to three cosmetic related substances: coumarin (*Figure 4.56),* isoeugenol and benzophenone-2.

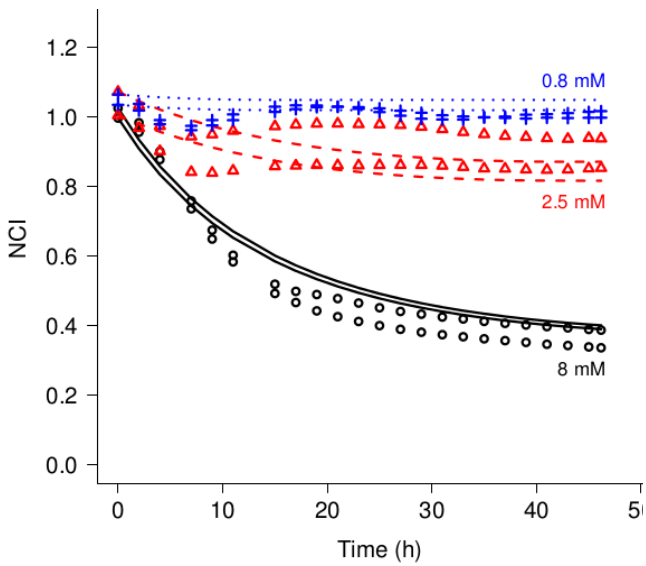

Figure 4.56 *Normalised Cell Index (NCI) of HepaRG cells exposed once to coumarin at various concentrations. The data and the model predictions are represented by points and lines, respectively.*

In the second approach the PBK model was coupled with a cell compartment by using dynamic outputs from the Virtual Cell Based Assay and simulating cell viability and mitochondrial membrane potential. This approach was used to perform *in vitro* to *in vivo* extrapolation (IVIVE) with respect to two case study compounds, caffeine and coumarin. The time profile curves as well as dose response curves were stimulated in order to be able to perform forward as well as backward extrapolation. The combined models have been automated and implemented into a KNIME workflow which is able to predict internal concentrations. By applying real case scenarios it was showed how the tool can be used to calculate the so called Margin of Internal Exposure, which forms the point of departue of a risk characterisation.

4.5.3.5 Integration of Computational Models, Tools and Data Sources into an *in silico* Workflow Environment

Improvements of KNIME Server and KNIME Analytics Platform

KNIME is the modular integration platform for the database and computational toxicity prediction methods which are being developed in the **COSMOS** Project. By means of graphical workflows, data are read from various data sources and subsequently transformed into suitable formats for model building and/or visual analysis. The KNIME technology integrates access to databases, data processing and analysis, as well as modelling approaches into flexible computational workflows that will be adaptable and form a set of building blocks allowing users to incorporate their own data and search existing data compilations.

KNIME provides a simple extension application programming interface which allows for easy integration of new methods which are usually represented by so-called nodes. Since KNIME is open source it is a suitable platform for implementing and deploying the computational methods that were developed in the different COSMOS working areas.

The KNIME Analytics Platform is a desktop program which runs locally on a computer and uses a directory on that computer to store the workflows. In context of the **COSMOS** Project it was desirable that workflows can easily be shared by all groups during development and once they are usable for the public they should be easily accessible and usable even for non-experts in KNIME. The KNIME Server offers a central storage for workflows that can be accessed directly from within the KNIME Analytics Platform client. The following extensions and improvements were triggered by the **COSMOS** Project:

➠ In order to support the implementation and dissemination of the computational models developed in the **COSMOS** Project, the KNIME Server as well as the KNIME Analytics Platform desktop application have been extended and improved in many ways in order to support the implementation and dissemination of the computational models developed in the **COSMOS** Project.

➠ An interactive integration of the R programming language: In some workflows R scripts are used and the interactive R nodes make it easy to develop them directly inside the workflow.

➠ A set of nodes for calculating common statistics and hypothesis testing, such as t-tests, Cronbach correlation, or Kruskal-Wallis test.

➠ The ability to select columns in node dialogs based on exact names, on patterns, or based on the type. Especially if user-supplied data are used column names often cannot be determined in advance or are spelt incorrectly. In this case the new column selection components that are used in most nodes by now offer more flexibility.

COSMOS KNIME Workflows

Computational models developed in the **COSMOS** Project have been coded using the open access KNIME workflow technology. KNIME workflows were developed in the local desktop application (which is completely Open Source).

The public area of the COMSOS KNIME Server contains the following workflows:

➠ Absorption
- PAMPA permeability estimation;
- Skin permeability estimation.

➠ Biokinetics
- Bioaccumulation;
- *In vitro* to *in vivo* extrapolation for: caffeine, estragol, styrene;
- Physiologically based kinetics for: caffeine, coumarin, estragol, ethanol, hydroquinone, isopropanol, methyliodide, nicotine, styrene;
- Virtual Cell-Based Assay.

➠ Chemical Space Analysis

➠ Molecular Initiating Events
- Covalent DNA Binding Alerts;
- Covalent Protein Binding Alerts;
- Hepatoxicity Alerts;
- Mitochrondrial Toxicity Alerts.

➠ Nuclear Receptor Binding
- LXR binding potential (based on PaDEL or RDKit);
- Nuclear receptor ligand binding.

➠ Ranking

COSMOS KNIME WebPortal

The COSMOS workflows were also transferred into user-friendly WebPortal versions. The COSMOS KNIME WebPortal is freely accessible at *http://knimewebportal.cosmostox.eu*, and is supported by COSMOS Space *(http://cosmosspace.cosmostox.eu)*. The KNIME WebPortal allows for access the KNIME Server and execution of workflows through a web interface from any recent web browser without installation of the software locally and without knowledge of the KNIME workflows as such. Thus, as opposed to access from the KNIME desktop application mainly for workflow developers, end users that only want to run a workflow with custom data can access the server via the COSMOS KNIME WebPortal. The WebPortal allows for a step-by-step execution. Each step asks for user input, such as files or model parameters, potentially also dependent on previous inputs. After all inputs have been provided, the workflow is executed and the results can be downloaded as files and/or graphical reports are generated as summaries.

Documentation of Computational Models

Documentation has been developed within the **COSMOS** Project to provide key information on the workflows, including information on workflows development, model background, encoded algorithms, required parameters and user guidance. Specifically, the user guidance explains in an easy to follow, step by step manner, how to execute the workflow. Such documentation is provided for the execution of both desktop and WebPortal version of the KNIME workflows and is available through COSMOS Space *(http://cosmosspace.cosmostox.eu)*.

Additionally, for most workflows short tutorial videos were created and are available on YouTube via *http://www.cosmostox.eu/what/webtutorials/*.

These web tutorials are also accessible directly from the following links:

- Tutorial for 'Physiologically Based Kinetic Models':
  https://www.youtube.com/watch?v=Ihn4I_g981Q
- Tutorial for 'Virtual Cell Based Assays':
  https://www.youtube.com/watch?v=BeV9NJd4E4c
- Tutorial for 'Skin Permeability Estimation':
  https://www.youtube.com/watch?v=DtNdwtKuY74
- Tutorial for 'PAMPA logPm Predictor':
  https://www.youtube.com/watch?v=WJLAz9GnvcM
- Tutorial for 'LXR Binding Prediction':
  https://www.youtube.com/watch?v=EkB1i9a4TWI
- Tutorial for 'Potential Nuclear Receptor Ligands and Alerts towards Hepatosteatosis':
  https://www.youtube.com/watch?v=ggkU6lZfDfY

Moreover, the COSMOS models translated into KNIME workflows were also documented in the EURL ECVAM DataBase service on Alternative Methods to animal experimentation (DB-ALM) available at *http://ecvam-dbalm.jrc.ec.europa.eu/beta/index.cfm/home/index*. The DB-ALM is a public, factual database service that provides evaluated information on development and applications of advanced and alternative methods to animal experimentation in biomedical sciences and toxicology, both in research and for regulatory purposes.

4.5.4 Contributions to SEURAT-1 Case Studies

Molecular modelling studies performed within **COSMOS** for the prediction of LXR and PPARγ binding (and activation) were implemented as a level 2 **SEURAT-1** Proof-of-Concept Case Study. Molecular modelling methodologies were used to address specific molecular initiating events (MIEs) involved in the development of liver steatosis, i.e. LXR binding and PPARγ binding (and activation). To predict LXR binding potential, several of these methodologies were used, including both ligand- and structure based methods (i.e., ensemble docking, e-Pharmacophore and fingerprints-based similarity). The molecular modelling approaches developed were then integrated by means of data fusion/consensus modelling. To predict PPARγ binding, a virtual screening protocol was assembled by first applying molecular docking, and then by filtering the generated poses with a pharmacophore model which was generated based on the X-ray complexes of the three most active PPARγ full agonists. 3D QSAR (CoMSIA) models were also developed to predict PPARγ activation.

The usage of molecular modelling approaches to predict receptor binding/activation provided hints for the characterisation of molecular mechanisms that trigger further downstream events and promote the development of liver toxicity. In the AOP framework, molecular modelling may thus play an important role by working in synergy with other *in silico* (QSAR, chemotypes, alerts) and *in vitro* approaches.

Moreover, the **COSMOS** *in silico* tools developed, which integrate a variety of *in silico* approaches (e.g., molecular modelling, QSAR and structural alerts) and chemometric tools in an innovative way, was also employed for the screening and ranking of chemicals to support the Read-Across and *Ab Initio* **SEURAT-1** case studies.

Furthermore, **COSMOS** evaluated the extension of the TTC approach to cosmetics-related chemicals in view of its use for safety assessment for cosmetics (see section 3.4). The new non-cancer TTC dataset of NOAEL values was enriched with appropriate data in order to increase their suitability for the evaluation of cosmetics ingredients. Furthermore, the difference in exposure scenarios and bioavailability issues, such as dermal absorption and skin metabolism, was addressed for the oral-to-dermal extrapolation of the TTC.

4.5.5 Potential Impact

The **COSMOS** Project has provided a high quality database of toxicological information and computational models which will assist in the prediction and assessment of the safety of cosmetics related chemicals. These outputs will directly support the safety/risk assessment of the use of existing, and potentially novel, ingredients in cosmetics. This research undertaken in the **COSMOS** Project supports the area of computational modelling as it is being implemented in the vision of 21st Century Toxicology. Overall, this enables more relevant and reliable information relating to human safety to be obtained; it contributes to the reduction of animals for toxicological assessment; and it assists in the development of cheaper and greener products.

Provision of data is crucial for the assessment of toxicity. The **COSMOS** Project has provided a database which will greatly improve data availability for repeated-dose toxicity assessment of cosmetics-related chemicals. This has been achieved by **COSMOS** partners working with regulatory authorities, industry and other trade organisations to obtain donations of data. These have been supplemented by direct data harvesting within the **COSMOS** Project in order to extract data from publicly available sources such as the Opinions of the SCCS. Further, these data were supplemented by those for other toxicities. There is the potential for a considerable impact of COSMOS DB on regulatory agencies, cosmetics industry, research institutes, universities, and small/medium enterprises who may use this information to make safety decisions regarding new and future ingredients and impurities. The data held within such a database are also vital for efforts to develop novel computational models and to support read-across predictions of toxicity to fill data gaps. The **COSMOS** Project has also provided strategies for the curation and harvesting of chemical structures and toxicity data. This is vital to ensure the reliability of data held within such databases. **COSMOS** supports information exchange and feedback enabling use of, and confidence in, project outcomes through access to the freely available public resources: COSMOS DB, COSMOS Space and COSMOS KNIME WebPortal. As a legacy of the COSMOS DB, the integrity of the data has been assured by the availability of a 'flat' file that may be read into a spreadsheet, as well as the establishment of the COSMOS DataShare Point to maintain the database and provide a means of collecting further data in an openly available format.

The Threshold of Toxicological Concern (TTC) approach has had a considerable impact on risk assessment of chemicals in many sectors e.g. pharmaceuticals, foods. Specifically TTC has the potential to have an enormous impact on the safety/risk assessment of cosmetics-related substances for which chemical-specific toxicity data are lacking. Provided that reliable exposure data are available, the TTC approach provides a relative assessment of the safety of exposure to a substance without recourse to any testing. As such, it provides a potential solution to the safety/risk assessment of many cosmetics ingredients and impurities, or any material that cannot be tested. Traditional approaches to TTC have been derived from data and chemicals not necessarily representative of cosmetics ingredients. The **COSMOS** Project

has provided a new database for the application and extension of the TTC approach. The database has enriched, and greatly expanded, the existing (Munro) dataset by the inclusion of No Observed (Adverse) Effect Levels (NO(A)ELs) for cosmetics-related chemicals. Importantly, the new non-cancer TTC database is transparent and open (available via COSMOS DB) and incorporates recommendations for how the data should be used. As part of the development of the new COSMOS TTC database, considerable effort was placed into quality assurance, essentially providing a blueprint for new studies in this area with regard to assessing the quality, or otherwise, of published toxicity studies. Since TTC is a pragmatic method recommended by the European Food Safety Authority (EFSA) as well as SCCS for the safety/risk assessment of chemicals found in food, cosmetics or consumer products, this new TTC database will have broad economic impact on the cosmetics industry and be a well applied tool. In order to apply the TTC values derived from oral data to use cases relevant to cosmetics, the **COSMOS** Project has extended the current TTC approach to cosmetic-related chemicals. Among the strategies to address this issue, a tiered approach has been developed for the assessment of the chemicals' bioavailability, which takes into account the absorption/permeability via dermal or oral routes. This provides novel models indicating possible systemic bioavailability following dermal or oral absorption. The findings of the TTC approach have been presented to the SCCS (November 2015) and have been commented upon in the Opinions of the SCCS (*SCCS, 2012; 2015*).

Novel models for the prediction of toxicity of cosmetics ingredients have been developed within the **COSMOS** Project. These models are openly and freely available and allow the user to make a prediction from the chemical structure. The models make predictions for the following properties that are important for risk / safety assessment of cosmetics ingredients: absorption of a compound following oral and dermal administration; binding to nuclear receptors (important receptors, interaction with which can be a pre-cursor to toxicity) that may be indicative of harmful effects, e.g. steatosis, to the liver; ranking of chemicals in terms of toxic potential; and structural alerts (i.e. specific fragments of molecules) associated with binding to DNA and protein, general hepatotoxicity and specific effects relating to mitochondrial toxicity. These models require the input of chemical structure only; as such they are increasingly seen as viable alternatives to animal testing. Results from the **COSMOS** Project, along with efficient use of data and information from COSMOS DB will support the increasing trend for using 'read-across' following the formation of categories of similar compounds for organ level toxicity prediction. These techniques will be supplemented by the optimisation of molecular modelling methodologies for their application in predictive toxicology, particularly assisting with the implementation of the mechanistic (Adverse Outcome Pathway - AOP) framework for toxicity prediction. Studies within the **COSMOS** Project have been performed to generate knowledge and set the foundation for future development of models and applications. The impact of these models will be through the area of computational toxicity prediction with particular significance to industry (cosmetics and beyond) as well as international efforts to improve this area of science.

The findings and models from the **COSMOS** Project will also support the overall area of *in vitro* to *in vivo* extrapolation (IVIVE). The project has provided models and methodologies for route-to-route extrapolations with an emphasis on oral-to-dermal extrapolation. This is important for safety/risk assessment when only oral data are available. The extension of models for IVIVE has improved the relevance of *in vitro* testing. Further, the calibration of Physiologically-Based Kinetic (PBK) models based on *in vitro* and *in silico* data has provided tools to assist in the prediction of target organ concentrations following a specific topical exposure. These models have been supported by a 2D liver model that can be coupled to PBK models to refine kinetics and hepatotoxic effects assessment as well QSAR models to predict metabolites and rate of disappearance of the parent compound (total and hepatic clearance). Overall, these models will have a significant impact on safety/risk assessment of the potential organ level toxicity to humans of cosmetics and related ingredients.

The data compilation and modelling efforts in the **COSMOS** Project have been supported by appropriate open access informatics and workflow approaches through the KNIME technology. This has enabled data and models to be accessed and utilised by all stakeholders and has provided a platform, through the COSMOS KNIME Web Portal, for the development of further tools beyond the lifespan of **COSMOS**. The impact of this approach has been to make the models publicly available ensuring their uptake by industry, regulators and academia. The models are transparent and can be developed further by the user e.g. incorporating new data or knowledge.

The **COSMOS** Project had considerable impact with cross-(**SEURAT-1**) cluster activities. For instance, the **COSMOS** Project contributed data curation and organisation to a number of high level case studies demonstrating the use of read-across as a technique to predict toxicity and fill data gaps, as well as input to an '*ab initio*' case study devising a workflow for safety assessment without relying on animal testing, particularly considering exposure. Further, **COSMOS** partners were instrumental in developing read-across arguments and understanding. The impact of these activities has been to realise a strategy and framework to document and support read-across which is relevant to industry users when making regulatory submissions.

COSMOS successfully implemented a dissemination plan targeting key stakeholders. The public face of the **COSMOS** Project was the web-site *(www.cosmostox.eu)* which was updated regularly with news items, project results, publications and links to COSMOS DB and the models. Newsletters were distributed electronically and a series of project leaflets (general information, COSMOS DB, computational models) were created and distributed widely at over twenty international key conferences and symposia. General information on the **COSMOS** Project (and on the TTC approach) were presented at meetings of the SCCS. A large number of conference presentations were made including the organisation of specific sessions (for example at the Society of Toxicology annual meeting in 2014); the **COSMOS** legacy also benefitted from a dedicated COSMOS dissemination day *(http://www.cosmostox.eu/output/newsletter/)*,

contributions to training events and the final **SEURAT-1** symposium in Brussels (December 2014, see section 4.10). In addition to the resources (COSMOS DB and KNIME workflows) made available on the internet, a large number of training videos and tutorials have been developed. The project also provided a permanent legacy of its work through publication in a number of key journals.

In summary the **COSMOS** Project will provide a legacy of data compilation, strategies for the further use of informatics to support computational toxicology and models for toxicity prediction. There will be a very broad impact to all stakeholders involved in the safety/risk assessment of cosmetics related substances as well as in other sectors. The public will ultimately benefit in the development of safer and cheaper products, with reduced reliance on information from animal studies.

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Awards

The QSAR and Molecular Modelling Group, Liverpool John Moores University, England received the 2013 Lush Science Prize

The group received the Lush Science Price for developing computational alternatives to animal testing to predict the effects of chemicals. The Lush prize is an annual prize fund for researchers working in the alternatives to animal testing field, focusing on consumer products and ingredients, and is funded by Lush cosmetics in the UK, a company committed to the non-animal test methods for all of their products, and Ethical Consumer magazine.

Dr. Steve Enoch was jointly awarded the 2013 Lush prize for science for the work of the QSAR and Molecular Modelling group at Liverpool John Moores University to develop *in silico* methods for the non-animal risk assessment of skin and respiratory sensitisation. The award was made for notable contributions to the field of predictive toxicology focussing around efforts on the development of computational methods applicable to cosmetics ingredients.

Poster Awards

Bucher, J., Diaz Ochoa, J.G., Mauch, K., Terfloth, L. (2015): Coupled modelling of PBPK and toxic mechanisms of action of valproic acid in liver. Poster award at the SEURAT-1 5th Annual Meeting 2015, 21–22 January 2015, Barcelona, Spain.

Hristozov, D., Jeliazkova, N., Kleinoeder, T., Lan, Y., Meinl, T., Miller, S., Neagu, D., Schwab, C.H., Richarz, A.-N., Hardy, B., Cronin, M.T.D., Yang, C. (2014): COSMOS Database: Public availability of repeated dose toxicity data and collaborative interoperability with the ToxBank data warehouse supporting integrated data analysis. Poster award at the SEURAT-1 4th Annual Meeting 2014, 5–6 February 2014, Barcelona, Spain.

Jereva, D., Al Sharif, M., Diukendjieva, A., Alov, P., Pencheva, T., Tsakovska, I., Pajeva, I. (2014): Nuclear ERα and PPARγ: receptor- and ligand-based analysis. Poster award at the 16th Congress of the European Neuroendocrine Association, 10-13 September 2014, Sofia, Bulgaria.

Kovarich, S., Bassan, A., Cronin, M.T.D., Fioravanzo, E., Manelfi, C., Worth, A.P., Yang, C. (2013): Molecular Modelling to Predict and Understand Chemical Toxicity in the AOP framework – Case Study: MoA from LXR Activation to Liver Steatosis. Poster award at the SEURAT-1 3rd Annual Meeting 2013, 6–7 March 2013, Lisbon, Portugal.

Nelms, M.D., Enoch, S.J., Fioravanzo, E., Madden, J.C., Meinl, T., Richarz, A.-N., Schwab, C.H., Worth, A.P., Yang, C., Cronin, M.T.D. (2012): Strategies to Form Chemical Categories from Adverse Outcome Pathways. Poster award at the SEURAT-1 2nd Annual Meeting 2013, 8–9 February 2012, Lisbon.

Paini, A., Benito, J.V.S., Gajewska, M., Worth, A.P., Zaldivar Comenges, J.M. (2013): Human Bioaccumulation Potential Simulated in R and Implemented in KNIME. Poster award at the SEURAT-1 3rd Annual Meeting 2013, 6–7 March 2013, Lisbon, Portugal.

Richarz, A.-N., Neagu, D., Yang, C., Fioravanzo, E., Péry, A.R.R., Berthold, M.R., Cronin, M.T.D. (2012): COSMOS: An International Cooperative Project Developing Computational Models for Repeated Dose Toxicity. Poster Award 2012 at the European Partnership for Alternative Approaches to Animal Testing (EPAA) Annual Conference “Global Cooperation on alternatives (3Rs) to animal testing”, 16 November 2012, Brussels, Belgium.

Richarz, A.-N., Enoch, S.J., Hewitt, M., Madden, J.C., Nelms, M.D., Przybylak, K.R., Yang, C., Berthold, M.R., Meinl, T., Ohl, P., Cronin, M.T.D. (2013): Flexible computational workflows to predict toxicity. Poster Award at the UK-QSAR and ChemoInformatics Group Autumn Meeting, AstraZeneca, 15 October 2013, Alderley Park, England.

Teng, S., Barcellini, S., Beaudouin, R., Rahmani, R., Péry, A. (2014): TK/TD modelling to analyse real time hepatotoxicity data for cosmetics. Poster award at the SEURAT-1 4th Annual Meeting 2014, 5–6 February 2014, Barcelona, Spain.

Teng, S., Péry, A., Kovarich, S., Bois, F. (2015): SEURAT-1 Level 3 *ab initio* case study: PBPK predictions of methotrexate and valproic acid tissue concentration in humans. Poster award at the SEURAT-1 5th Annual Meeting 2015, 21–22 January 2015, Barcelona, Spain.

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4.6 NOTOX: Predicting Long-Term Toxic Effects Using Computer Models based on Systems Characterisation of Organotypic Cultures

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4.6.1 Executive Summary

NOTOX used a systems biology approach based on data rich '-omics' technologies and modelling. Traditional cell culture is usually carried out in the presence of foetal bovine serum which leaves a xenogenic signature in the various '-omics' readouts. **NOTOX** developed serum free long-term cultivation of its cell systems which are compatible with the systems toxicology methods. In long-term repeated-dose experiments the long term-effects of a series of compounds including valproic acid, bosentan and chlorpromazine were characterised. In parallel, **NOTOX** developed organotypic cultivation of its human cell systems in order to mimic as close as possible the *in vivo* situation. 3D HepaRG spheroid cultures were characterised in detail. These 3D HepaRG cultures were used in acute but also repeated-dose toxicity studies. HepaRG spheroid cultures were successfully used for the identification and study of compounds with cholestatic liability. 3D cultivation techniques developed with HepaRG spheroid cultures were further extended to primary human hepatocytes (PHH) as well as to co-cultures with non-parenchymal cells for fibrotic and inflammatory responses. Additionally, 3D cardiomyocyte cultures derived from stem cells were also applied for long-term studies.

Applying new methods of two-photon microscopy, dynamics of transport of fluorescent

compounds into hepatocytes and secretion into biliary structures *in vivo* and *in vitro* provided data for modelling of liver tissue. A simple work-flow in cryo-electron tomography and sub-volume averaging was introduced that allows to obtain improved high-resolution, reliable results with little expert-supervision. This technology can be used as a tool to link cell biology to structural biology aiming towards a more complete understanding of physiological processes particularly related to the interaction of compounds with macromolecular and supramolecular structures in the cell.

Long-term toxicity was characterised with '-omics' analyses. A major **NOTOX** case study focused on valproic acid long-term repeated-dose toxicity (14 days) in 2D in a joint large-scale experiment. A similar setup was used on a smaller scale with 3D HepaRG spheroids. Various '-omics' methods were improved (e.g., epigenetics using ChiP-Seq, expression using Affymetrix GeneChip HTA, proteomics with improved sensitivity for spheroids). A reference proteome of HepaRG was created (4000 extracellular and 240 extracellular proteins) to support studies.

For simulations aiming at *in vitro* to *in vivo* extrapolation (IVIVE), models of different levels of complexity were developed and applied for long-term toxicity. In all cases, biokinetics were considered for modelling. A simple model based on PBPK principles was used to estimate oral equivalent dose (OED). Drug degradation and time-dependent response was measured *in vitro* and a virtual population was applied to capture potential variability. Dynamic EC_{10} determinations over two weeks permitted safety predictions very close to *in vivo* data.

Detailed mode of action based kinetic models were developed in the context of two joint large-scale case studies using acetaminophen and valproic acid. The acetaminophen mode of action model focuses on processes relate to oxidative stress, e.g. formation of radicals (NAPQI) and glutathione metabolism. Expression analyses of primary human hepatocytes in sandwich culture exposed to acetaminophen showed time dependent exposure effects clearly distinguished from culture effects. The acetaminophen mode of action model was incorporated into a liver lobule module resulting in a multi-scale model describing damage, including apoptosis pathways, cell death, regeneration, flow and transport. The model of valproic acid metabolism includes glucuronidation, beta-oxidation and CYP oxidation. The valproic acid mode of action model couples kinetics in the signalling and gene regulation of fatty acid synthesis and metabolic enzymes. These models were incorporated into PBPK models and used for IVIVE. These support the identification and validation of key events in the adverse outcome pathways (AOP).

A whole toolbox for agent-based modelling of toxicological responses was developed: (i) TiQuant for image processing and analysis, (ii) TiSim for simulation of a liver lobule including cell death, regeneration, flow and transport and (iii) CellSys II for toxicity testing simulations. This systems biology toolbox developed in **NOTOX** will have a significant impact in the scientific and industrial communities.

4.6.2 Project Context and Objectives

Validated alternative assessment methods for long-term systemic toxicity are urgently required to cope with the complete ban (enforced from 11 March 2013) on animal testing of cosmetic products in Europe. Animal models do not adequately predict human toxicity and at the same time incur huge costs to the industry in the safety assessment of their products.

It is expected that a combination of *in vitro* and *in silico* approaches will pave the way towards animal free human relevant and reliable safety predictions (*Figure 4.57*). *In vitro* test systems based on human cells could provide dose response data for the estimation of toxicity threshold. *In silico* tools and models taking into account population stratification could be used for the prediction of concentration-time profiles and the safe exposure limits. In addition, various '-omics' data is set to provide mechanistic details on the adverse effects for a better understanding of the pathways involved in the biological response.

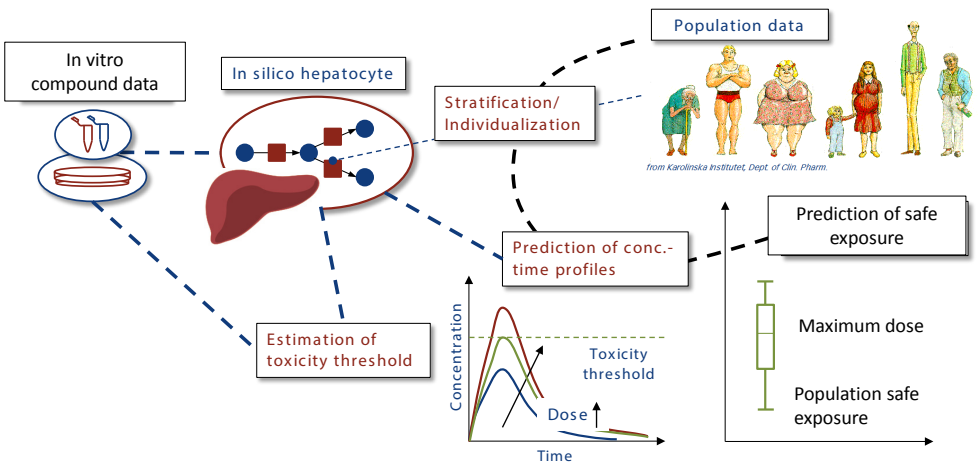

Figure 4.57 *Replacement of cost- and time-intensive animal or preclinical studies by in vitro and computational approaches. Detailed in silico hepatocyte models which are validated by experiments permit the prediction of dose-dependent concentration-time profiles of compounds. The knowledge of appropriate toxicity thresholds and the implementation of population data into predictive models enable an individualised or stratified estimation of maximum serum concentrations of test compounds and their metabolites as well as an individualised risk assessment.*

The **NOTOX** Project assembled experts for *in vitro* test systems together with scientists from the field of systems biology in order to establish these new systems-based models for the prediction of long-term toxicity. **NOTOX** developed and established a spectrum of systems biology tools including experimental and computational methods for: (i) organotypic human

cell and tissue cultures suitable for long-term toxicity testing with focus on the mode-of-action (MoA); and (ii) the identification and analysis of adverse outcome pathways (AOP). The overall goal was to predict long-term toxicity (repeated dose) on the basis of these models and well-designed experiments using an iterative systems approach.

Following this strategy, **NOTOX** established an integrated multifaceted experimental and computational platform. The experimental work focused on the application of cellular systems that come closest to the human *in vivo* situation while at the same time allowing their transfer into applicable and easy to handle test systems. Since testing on the target organisms (humans) is not possible, human organotypic cultures were applied to permit reproducible and transferrable testing of the highest possible relevance. As the liver plays a central role in metabolism, in both its inherent and xenobiotic conversion functions, we selected hepatic cultures for the **NOTOX** Project and used HepaRG, a hepatocarcinoma cell line, and primary human hepatocytes (PHH) in our experimental work. The HepaRG cell line has been shown to be closest to primary human cells in terms of the metabolism of xenobiotics, expressing important CYPs at high levels (*Kanebratt & Andersson, 2008a; 2008b*). For validation purposes, and for the development of new techniques, we also used primary human hepatocytes. In these test systems viability and physiological toxicity response parameters ('-omics') were monitored together with epigenetic and structural characteristics in parallel. Large-scale network models of regulatory and metabolic pathways and cellular systems together with bioinformatics integration of human and across species literature data were used for toxicity prediction (*Figure 4.58*).

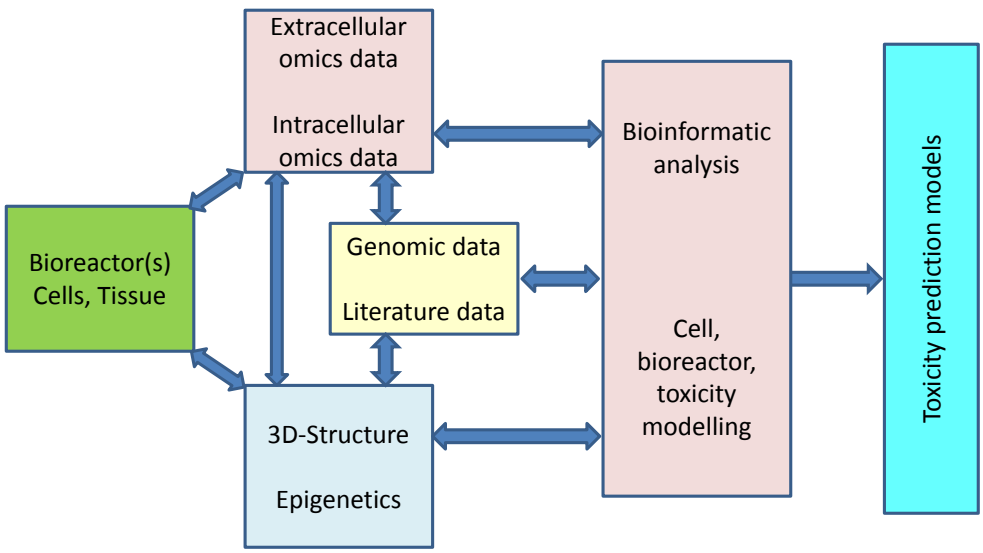

Figure 4.58 *The* ***NOTOX*** *approach combining human based cell systems including 3D culture with computational modelling for the prediction of toxicity.*

The major objectives of **NOTOX** were to

- supply a versatile methodology for systems-based analysis and prediction of long-term toxicity of test compounds on organotypic 3D cultures;
- develop and apply experimental and computational methods for continuous, non-invasive and comprehensive physiological monitoring (respiration, metabolomics, fluxomics, proteomics and peptidomics, epigenomics, transcriptomics, viability and toxicity reporters, cellular toxicity models) of organotypic test systems upon exposure to selected test compounds;
- develop and apply experimental and computational methods for the comprehensive characterisation of 3D organotypic cultures after long-term repeated dose exposure to selected test compounds (individual epigenetic chromosomal profiling, 3D electron tomography, 3D-topographic analysis and modelling, bioinformatics characterisation);
- develop causal and predictive large-scale computer models based on the integration of the experimental data with available data (from various databases) and high-performance grid computing for identification of predictive endpoints;
- provide cheaper, more ethical, scientifically based testing strategies for repeated dose toxicity in order to meet the European legislative demands. For this purpose we illustrate how computer models calibrated with *in vitro* experiments could be used in combination with human parameters to predict the possible toxicity in humans.

4.6.3 Main Achievements

4.6.3.1 Cell Cultivation Systems

Long-term *in vitro* Cultivation of Human Cells for Biological Studies

A system for long-term cultivation of liver cells has to maintain high liver specific functions as well as viability over time. In an effort to find ideal conditions for long-term repeated-dose exposure, serum-free conditions for long-term cultivation of HepaRG cells were established in 2D (*Klein et al., 2014*) as well as 3D (spheroids) environment. HepaRG cells were maintained for 30 days without the loss of viability and only minor loss of liver specific functions. Four different conditions without foetal bovine serum (FBS) were compared with standard long-term medium with FBS. SFM1, 2 and 3 were supplemented with growth factors (GF) making up for withdrawal of FBS. SFM4 was used for cultivation without FBS and GF. SFM1, 2 and 3 differed in respect to their DMSO concentrations (no DMSO, 0.5% DMSO and 1.8% DMSO

respectively). Investigation of viability of 2D HepaRG cultures over time showed that HepaRG cells can be cultivated without addition of FBS for at least 30 days, while maintaining cells viable (SFM1 and SFM2).

At the same time, the cytochrome P450 (CYP) activity of HepaRG cells during long-term cultivation in different media was monitored (*Figure 4.59*). While activities for CYP1A2, CYP2C9 and CYP2D6 only differ slightly between the tested conditions (SFM2 and serum supplemented medium; SSM), we found significantly higher activities for CYP2B6 and CYP2D6 when cells were cultured in SSM with 1.8% DMSO. For both, SFM2 and SSM, CYP activities generally decreased from day 0 to day 30, however were still on a high level (around 65% on day 30 for both conditions relative to original activity on day 0). This finding is very important since CYPs are key enzymes for the metabolism of xenobiotics.

Under these conditions, we analysed the extracellular metabolome (amino acids, glucose, pyruvate and lactate) of HepaRG cells. The uptake/production of various amino acids is depicted in *Figure 4.60*. The lactate/glucose ratios on days 6 and 30 were identical for SSM, SFM1 and SFM2.

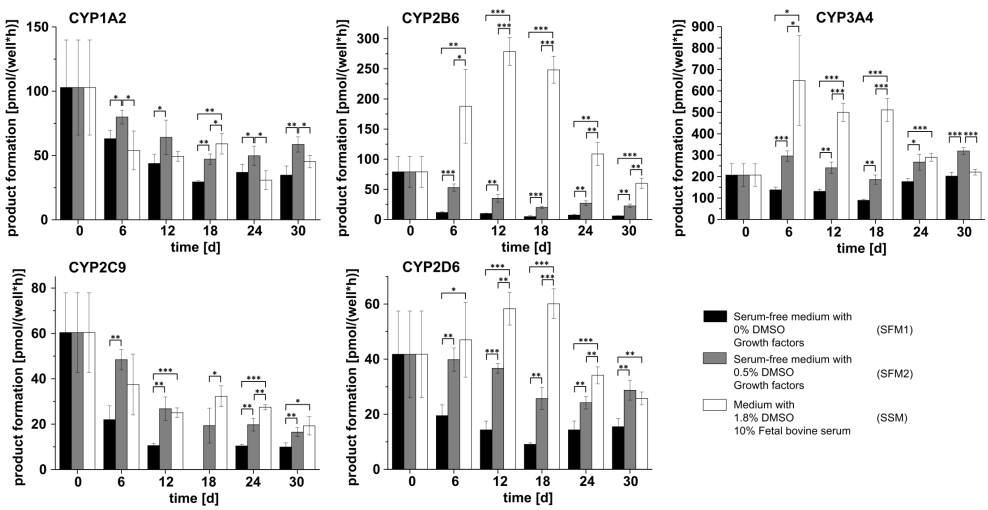

Figure 4.59. *Activities of CYP1A2, CYP2B6, CYP3A4, CYP2C9 and CYP2D6 enzymes in HepaRG cells during long-term cultivation with daily medium renewal. Error bars indicate standard deviation (n = 3). *, **, *** indicate p < 0.05, p < 0.01 and p < 0.001 respectively (Klein et al., 2014).*

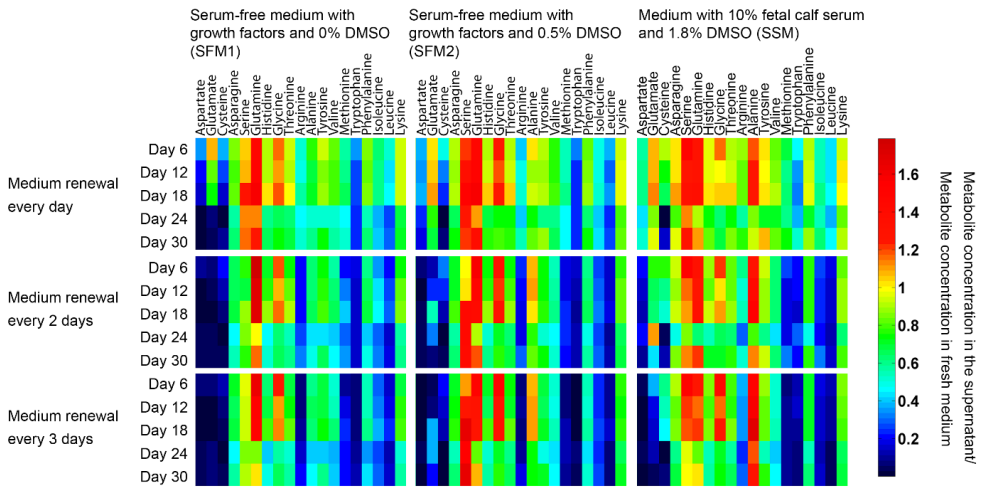

Figure 4.60 *A heat map showing the ratio of amino acid concentrations in the supernatants upon medium renewal every day, second or third day versus amino acid concentrations in the fresh medium, given for each investigated time point. An orange to red colour indicates production of amino acids, a yellow colour indicates that the amino acid was neither consumed nor produced. Green to blue colour indicates increasing consumption (Data source: Klein et al., 2014).*

In addition, metabolic flux analysis was applied to analyse metabolic changes over time and to investigate differences in metabolism between the different cultivation conditions. Metabolic flux analysis was shown to be a valuable tool for understanding the effects of experimental conditions and substrates as well as toxic compounds on cells. Several reaction and transportation rates are depicted in a flux distribution map (*Figure 4.61*).

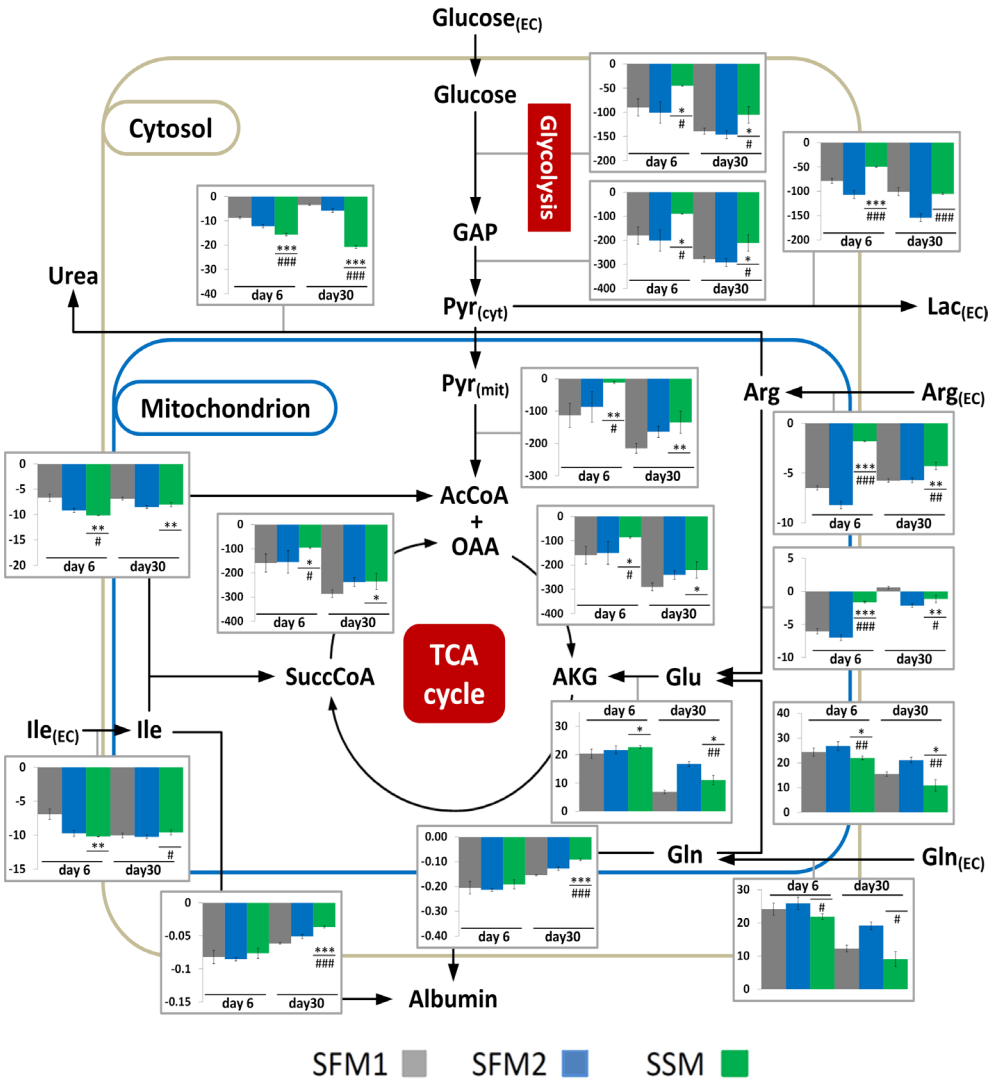

Figure 4.61. *A flux distribution map of HepaRG cells upon long-term cultivation for days 6 and 30 for SFM1 / 2 (serum-free medium with growth factors and 0% or 0.5% DMSO, respectively) and SSM (serum-supplemented medium with 1.8% DMSO). Negative values indicate fluxes into the direction of the arrow and positive values in reversed direction. Error bars indicate standard deviations (n = 3). *, **, *** (comparison of SSM to SFM1) / #, ##, ### (comparison of SSM to SFM2) indicate significance at p<0.05, p<0.01 and p<0.001, respectively. GAP, glyceraldehyde 3-phosphate; Pyr, pyruvate; AcCoA, acetyl coenzyme A; OAA, oxaloacetate; AKG, α-ketoglutarate; SuccCoA, succinyl coenzyme A; Lac, lactate; Glu, glutamate; Gln, glutamine; Arg, arginine; Ile, isoleucine; cyt, cytosolic; mit, mitochondrial; EC, extracellular; TCA, tricarboxylic acid.*

Reaction rates in the glycolytic pathway of HepaRG cells were significantly lower in serum-supplemented medium (SSM) as compared to serum-free medium (SFM). In accordance, lactate secretion was lower for cells maintained in SSM. Generally, glycolytic activity increased for all conditions over time. For cells cultivated in medium without DMSO (SFM1), approximately 40% of the glycolytic pyruvate was converted to lactate; for cells kept in 0.5% and 1.8% DMSO respectively (SFM2 and SSM), about 50% to 55% of glycolytic pyruvate was metabolised to lactate. Increased uptake of nutrients and consequential increased TCA cycle fluxes at later time points of cultivation for all conditions may be associated with increased energy demand for general maintenance, e.g. repair of DNA damages. An increased uptake of the branched-chain amino acids (BCAAs) isoleucine and leucine in SSM cultivation was observed. These amino acids are mostly metabolised to acetyl-CoA.

3D-Hepatic Model Using HepaRG Cells for Acute and Repeated Dose Toxicity Assessment

Liver cultures lose their functions rapidly in 2D cultures and cannot be maintained viable and functional longer than a few days. Additionally, many transport reactions depend on cell-cell contants and transporter activities often not sufficiently present in 2D cultures. Therefore, **NOTOX** established 3D cultures of liver cells (HepG2, primary human hepatocytes and HepaRG cells) and maintained them in culture for several weeks. The viability was constant for 4 weeks and longer. These hepatic cultures were functional during the tested period of 3 weeks as was shown by constant albumin production, transporter and CYP 450 activity.

The established system allows accurate adjustment of spheroid size, medium refreshment and spheroid harvesting and can therefore be used for the analysis of intra- and extracellular parameters. HepaRG cells formed compact spheroids between days 2 – 3 after initial seeding which did not grow in size since differentiated HepaRG cells do not further proliferate under used conditions (*Figure 4.62*). Cells containing up to 2000 cells per spheroid were found not exhibiting any visible transport limitations of e.g. oxygen, amino acids and other nutrients.

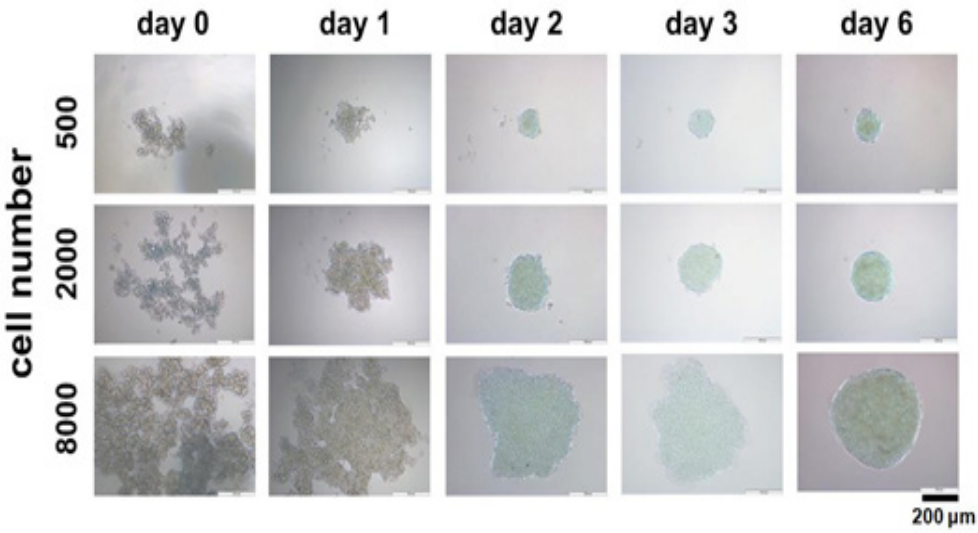

Figure 4.62 *Formation of HepaRG spheroids (initial cell numbers 500 – 8000) during 6 days of cultivation (d0 = seeding). Scale bars = 200 µm.*

Morphological analysis was performed using light microscopy and transmission electron microscopy. In *Figure 4.63* electron microscopy pictures of HepaRG spheroid cross sections are depicted. Typical structures found *in vivo* were consistently found in 3D HepaRG spheroids (*Figure 4.63A*). This includes typical liver structures such as bile canaliculi and microvilli (*Figure 4.63B* and *4.63C*) as well as transport vesicles found inside the bile canaliculi, indicating that HepaRG cells have an intact transport within these channels. The spheroid cultures were characterised for transporter activities and show e.g. functional MRP-2 transporters (e.g. *Figure 4.68* in the following section).

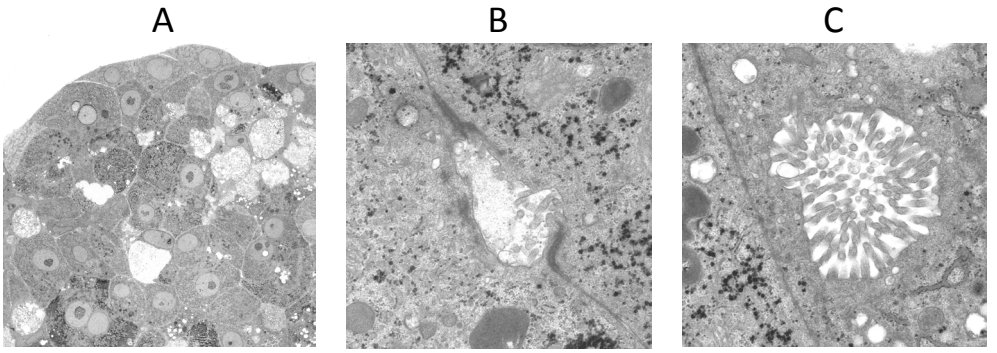

Figure 4.63 *TEM picture showing a HepaRG spheroid. A) Healthy HepaRG cells are showing the same characteristics as hepatocytes in normal liver, such as the variable quantity of glycogen (the dark stained material). B) A bile canaliculus is formed as an extracellular space, limited by two plasma membranes, sealed by tight junctions. In the cytoplasm next to the canaliculus, small irregular tubulo-vesicular structures of the smooth endoplasmic reticulum (SER) are seen. Dark granules are glycogen particles. C) Bile canaliculi with microvilli.*

Several compounds were tested in repeated dose toxicity studies applying 3D HepaRG cultures (*Gunness et al., 2013*). Compared to the 2D cultures, the 3D cultures were significantly more sensitive to acetaminophen exposure (*Figure 4.64a* and *4.64b*). The CYP2E1 enzyme activity assay supported these results (consistently higher enzyme activity was observed in the 3D versus the 2D cultures over the experimental period). The toxic metabolite of acetaminophen, N-acetyl-p-benzoquinone imine (NAPQI) is produced *via* metabolism by CYP2E1. In contrast to the 2D cultures, troglitazone was not toxic to the 3D cultures at both assessment time points (*Figure 4.64c* and *4.64d*). The mechanism of troglitazone-induced hepatotoxicity is not fully understood and it is uncertain whether the compound causes direct or idiosyncratic toxicity. The 3D cultures were more sensitive to rosiglitazone exposure at both exposure time points (*Figure 4.64e* and *4.64f*). Rosiglitazone is not hepatotoxic *in vivo*, however, it was found to be toxic *in vitro* in some donors of primary human hepatocytes.

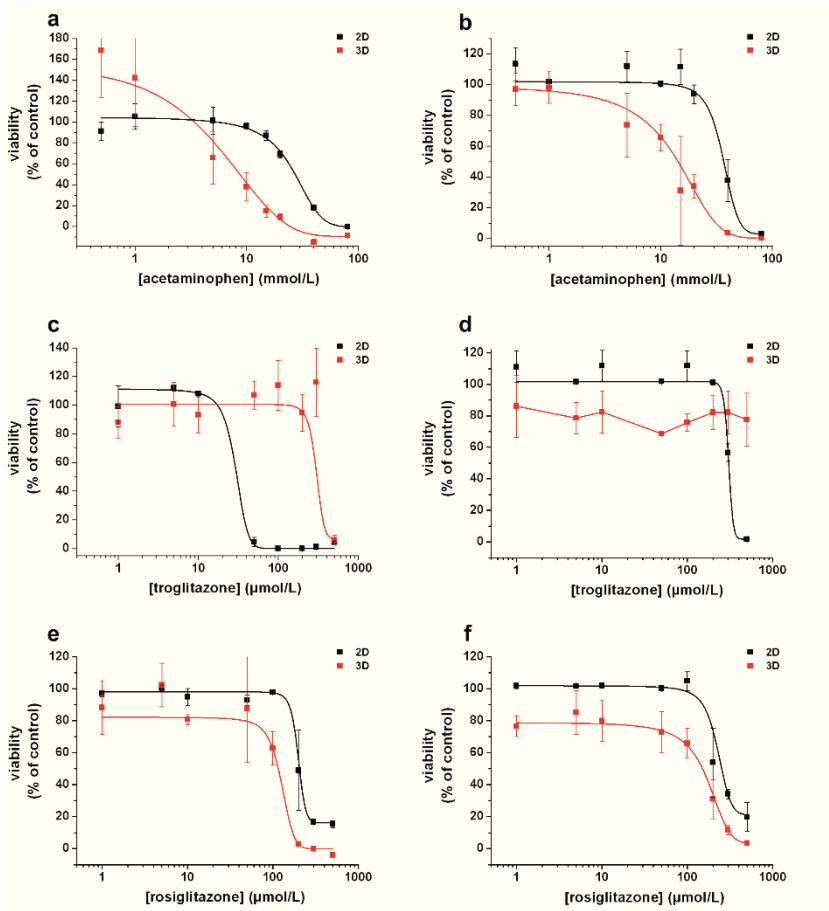

Figure 4.64 *Acute toxicity in 2D and 3D HepaRG cultures. The 2D and 3D HepaRG cultures were exposed to acetaminophen (a and b), troglitazone (c and d) and rosiglitazone (e and f) for 24 hours on culture days 4 or 21, respectively. Cell viability was assessed by measuring the intracellular ATP content.*

In another study, 2D and 3D HepaRG cultures were tested upon repeated exposures. 3D cultures of HepaRG are more sensitive to toxic response upon long term repeated exposure as seen by the shift in the dose-response curve (*Figure 4.65b*).

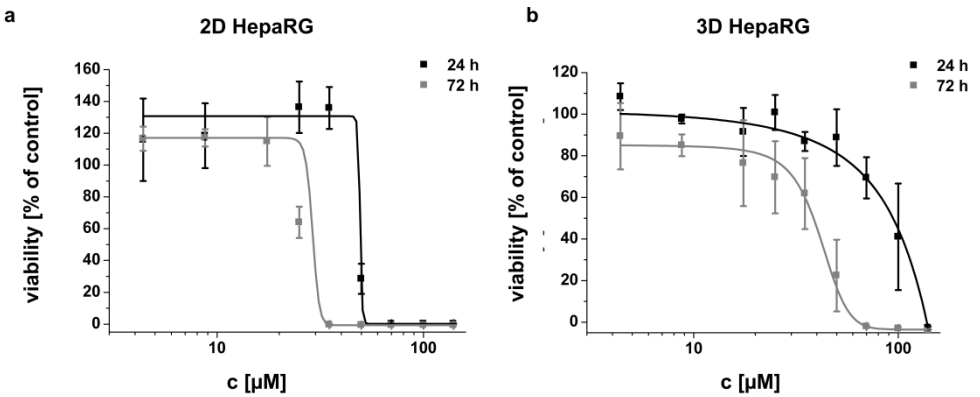

Figure 4.65 *Repeated dose toxicity of chlorpromazine in 2D and 3D HepaRG cultures.*

Taken all together, the results suggest that the 3D HepaRG cultures are suitable for metabolism mediated toxicity and can be extensively applied since these are amenable to high throughput screening of compounds. In conclusion, 3D organotypic HepaRG cultures are a promising preclinical tool in the study of human relevant long-term repeated effects and in the assessment of chronic drug-induced hepatotoxicity.

A 3D *in vitro* HepaRG Model for the Identification and Study of Compounds with Cholestatic Liability

Drug-induced cholestasis (DIC) is one of the leading causes of drug-induced liver injury and often only manifests weeks/months after the start of drug treatment. Preclinical detection of DIC is still often limited to measuring the compound's potential to inhibit the bile salt export pump (BSEP). Yet, recent studies emphasise the importance to consider other mechanisms by which drugs can induce cholestasis and it is clear that there is a need for novel *in vitro* models which allow for a comprehensive analysis of the cholestatic risk of compounds. HepaRG cells and spheroids have previously been described as appropriate for long-term toxicity testing and here we show that the spheroids accurately express two main bile acid (BA) transporters, bile salt export pump (BSEP) and multidrug resistance-associated protein 2 (MRP2) at the site of the bile canaliculi (*Figure 4.66A*); this makes them suitable for long-term toxicity testing of cholestatic drugs. By co-exposing the HepaRG spheroids to a non-toxic dose of a bile acid mix and compounds known to cause cholestasis (bosentan, chlorpromazine and troglitazone) we show that the bile acid co-exposure led to enhanced toxicity after 14 days of repeated dosing (*Figure 4.66B*). Importantly, this effect was not observed with the hepatocellular toxicant paracetamol or steatosis-inducing tetracycline (*Figure 4.66B*). Furthermore, in accordance with the definition of cholestasis, chlorpromazine induced accumulation of bile acids after 8

days of repeated dosing in the spheroids, which is accompanied by decreased BSEP mRNA expression (*Figure 4.66C*). Finally, when further investigating the mechanism behind the synergistic toxicity between bile acids and chlorpromazine, we found a selective increase in oxidative stress dependent sulfiredoxin 1 (SRXN1) expression as well as an increase in death receptor 5 (DR5) RNA (*Figure 4.66D*). In summary the use of HepaRG spheroids allows for distinction of compounds with cholestatic liability, as well as in depth mechanistic studies of cholestatic liver injury.

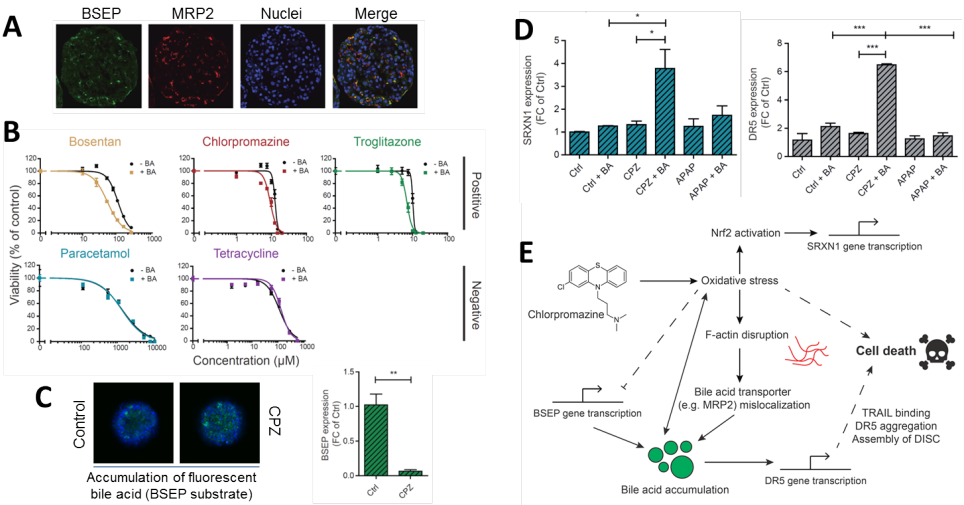

Figure 4.66 *HepaRG spheroids as a model for the detection of compounds with cholestatic liability and for mechanistic studies. A: Immunofluorescent staining of fixated and cryosectioned HepaRG spheroids; B: The viability (ATP levels) after repeated dosing of compounds for 14 days with and without the presence of bile acids (BA); C: Live confocal imaging of fluorescent bile acid and RNA expression of BSEP in spheroids exposed to chlorpromazine (CPZ) for 8 days; D: CPZ+BA selectively induces the oxidative stress Nrf2 target gene SRXN1 as well as the death receptor 5 (DR5) RNA; E: Proposed mechanism of chlorpromazine induced bile acid accumulation (cholestasis) and their combined toxicity.*

4.6.3.2 High Resolution Imaging to Monitor Structural Changes

Functional Imaging on Hepatocyte *in vitro* Systems for Toxicity Testing

The spheroid cultivation system displays a distinct cellular differentiation compared to classical hepatocytes monolayer cultures. The formation of bile canalicular structures at hepatocyte interfaces is a sign of marked differentiation (*Figure 4.67*). In collaboration with other projects

an automated quantification system for bile canalicular dynamics was developed. This system offers the possibility to systematically study disturbed bile canalicular dynamics which is a major reason for intrahepatic cholestasis (*Reif et al., 2015*).

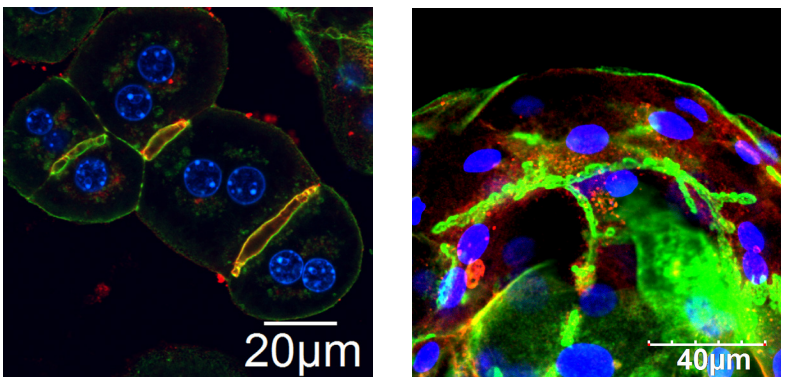

Figure 4.67 *Immunostaining for bile canaliculi in sandwich culture (cells embedded in a collagen matrix) and spheroids: Green fluorescence shows the bile canalicular structures visualised with an antibody against the marker protein DPPIV. Both cultivation systems, left sandwich culture - right spheroid, display distinct cell polarity with bile canaliculi formed at the apical cell membrane. Nuclei in blue and plasma membrane in red.*

The compound uptake efficiency in different hepatocyte cultivations systems was compared in order to estimate the applicability for compound testing in toxicological studies. For this purpose, passively diffusing Hoechst and actively taken up CMFDA were chosen as model compounds. Live cell imaging of these compounds into the spheroids revealed a distinct delay for the inner hepatocytes compared to the outer cell layer. The functionality of the formed bile canalicular system was shown by the biliary elimination of fluorescent metabolite of CMFDA (*Figure 4.68*).

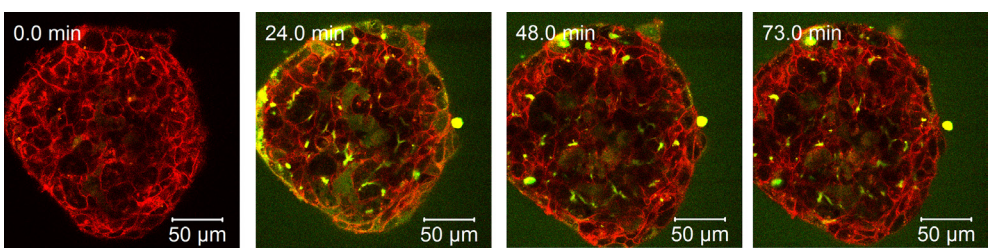

Figure 4.68 *Uptake of CMFDA into spheroids: Spheroids of primary mouse hepatocytes (td-tomato) were fixed after four days in cultivation on a collagen layer and were exposed to 3.2 µM CMFDA (metabolite in green). Compound activation and elimination was measured in a time dependent manner using two-photon microscopy.*

In order to compare the uptake efficiency between different culture systems, live cell imaging was also carried out for hepatocytes in sandwich cultures. In sandwich cultures the uptake of the model compounds was fast and homogeneous at the tested concentrations (data not shown). To compare uptake efficiency, kinetic parameters were determined for both cultivation systems using various concentrations of the fluorescent compounds. Finally these kinetic parameters were compared to the uptake measurements *in vivo*. This method contributes to transport related toxic effects in liver. In conclusion, both *in vitro* systems, hepatocyte sandwich cultures and spheroids can be used to study bile canalicular transport. CMFDA is taken up by hepatocytes, converted into the corresponding acid that exhibits green fluorescence and is transported to bile channels *via* the MRP2 transporter. This has a great potential for monitoring of cholestatic compounds.

Supramocular EM-Imaging for Compound Interaction

New methods of high resolution EM tomography allows near *in vivo* adequate imaging of macromolecules and supramolecular complexes. To detect and interpret biologically and toxicologically relevant drug-induced macromolecular changes, high-resolution structures with sufficient details have to be extracted from the tomograms. One of the issues that limit obtaining such structures with resolution sufficient for detection of drug-induced changes is modulation of the measured projections by so-called contrast transfer function (CTF), which is caused by microscope aberrations and defocusing. CTF causes a periodic inversion of contrast in high frequencies of the measured projections, making their direct interpretation impossible. The correction of CTF is performed by first creating a mathematical model of the effective CTF, i.e. the CTF that has actually distorted the acquired projections and then correcting the projections by de-convoluting them with this model CTF. To model the CTF, its key parameter - the microscope defocus for each projection - has to be estimated directly from those projections.

NOTOX developed a technique to estimate this defocus for each projection in the tilt series, drawing on *a priori* information about the series acquisition process and noise properties of the estimates. This estimation is incorporated into the whole pipeline as follows. Tilt-series is acquired and then aligned using the IMOD software package. A final result of this procedure is an electron scattering potential model of a representative of each structurally unique class. *Figure 4.69* details the structure of these representatives (70S ribosome locked by EF-G, *Figure 4.69A*; 70S without the EF-G, *Figure 4.69B*; the EF-G alone, *Figure 4.69C*). We were able to identify also standalone 50S subunits (*Figure 4.69D*), which are expected to be in the sample, since not all of the ribosomes in a cell are fully assembled. What should be noted is that neither of the classes depicted in *Figure 4.69* has been specifically searched for. They have been revealed automatically by the classification procedures. This shows the potential of the method to reveal unexpected structural events on the macromolecules upon exposure

to a drug. It allows to semi-quantitatively characterise the biochemical processes, such as effectiveness of the antibiotics binding, since we could count the total number of EF-G bound and EF-G free ribosomes, giving an approximation of the drug binding efficiency.

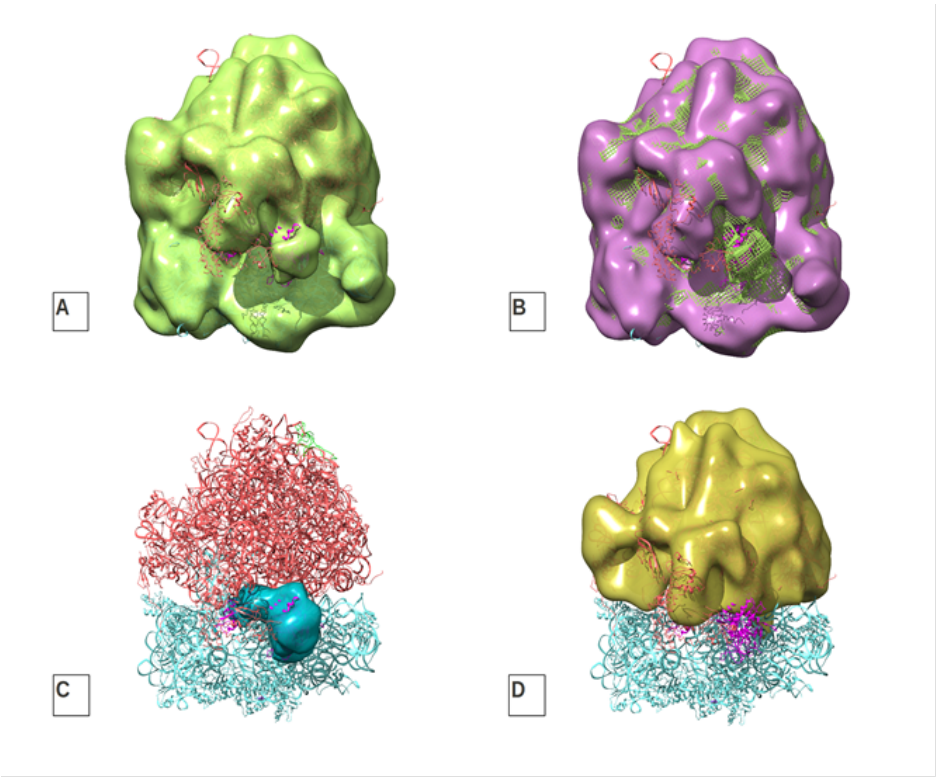

Figure 4.69 cryo-ET Structures of *ribosomes. Structures of E. coli ribosomes with docked inside the X-ray* structures *of Thermus thermophilis 70S. In green is shown the EM-map of the 70S+EF-G (surface panel A and green mesh panel B), in magenta the density relative to the 70S without EF (surface panel B), where we have coloured in yellow (panel D) the density of the 50S only. The small density in turquoise (panel C) corresponds to the EF-G only. The X-ray structures docked in the EM map shows ribbons of the 50S in green, the 30S in cyan, and the EF-G in pink. A) 70S+EF-G. Most of the density of the cryo-ET map, including the one relative to the EF-G, is occupied by the pdb model, as expected. B) Overlap of the 70S+EF-G and 70S only. The density relative to the EF-G is present only in the map 70S+EF-G (mesh). C) Subtraction, made with Chimera in between the 70S+EF-G (green map) and 70Sonly (magenta map). D) Map of the 50S only.*

4.6.3.3 '-Omics' Data for Toxicity Assessment

In vitro Based Prediction of Human Hepatotoxicity Using Transcriptomic Data of Human Hepatocytes

In vitro based prediction of hepatotoxicity is challenging, because it requires an *in vitro* system, which reflects critical mechanisms of *in vivo* toxicity. In this project we evaluated transcriptomic readouts to identify predictive biomarkers and established an *in vitro* based model to predict human hepatotoxic blood concentrations. We used publically available, genome wide expression data from 150 compounds tested in primary human hepatocytes (PHH) at a slightly cytotoxic concentration. The following strategy was applied to identify potential biomarker candidate genes: (i) identification of genes that are altered by many compounds; (ii) identification of genes, which are as well altered in human liver diseases such as cirrhosis, hepatocellular carcinoma and non-alcoholic steatohepatitis; (iii) exclusion of unstable baseline genes, which are altered just by the hepatocyte isolation and cultivation procedure; (iv) selection of genes belonging to various biological motifs to cover the most relevant toxic mechanisms. From the top genes with the highest fold changes among all compounds, 7 genes were selected as biomarkers: CYP 1B1, CYP 3A7, SULT 1C2, G6PD, TUBB2B, RGCC and FBXO32 (*Table 4.3*). These genes cover the biological motifs metabolism of xenobiotics, energy and lipid metabolism, cell cycle and cytoskeleton as well as protein degradation.

Table 4.3 *Identified biomarkers for further evaluation.*

Symbol	Liver disease	SV Up (FC3)	Gene Function	Category
CYP1B1	UP	18	metabolic enzyme in the ER (phase I enzyme)	Metabolism xenobiotics
CYP3A7	UP	39		
SULT1C2	UP	22	cytosolic enzye; catalyzes sulfonation (phase II enzyme)	
G6PD	UP	7	Enzyme in pentose phosphate pathway → fatty acid synthesis	Energy and lipid metabolism
TUBB2B	UP	5	major constituent of microtubules; functions in mitosis and intracellular transport	Cytoskeleton Cell cycle
RGCC	UP	25	cytosolic protein; induced by p53 modulates the activity o fcell cycle specific kinase in response to DNA damage	
FBXO32	UP	29	cytosolic protein; ubiquitination and proteasomal degradation	Protein degradation

A set of hepatotoxic as well as non-hepatotoxic compounds was defined and literature search was performed to identify plasma peak concentrations at therapeutic doses. HepG2 cells as well as PHH were exposed for 24 h and each compound was tested in a concentration range covering the plasma peak concentration but also ranging up to slightly cytotoxic concentrations. Two readouts were used to evaluate the hepatotoxic potential of the compounds: (i) the expression of the selected biomarker genes was analysed, that is the *in vitro* alert concentration was defined as the lowest concentration that causes a significant increase of at least 2.5 fold induction of at least one biomarker; (ii) cytotoxicity tests were performed to identify the lowest cytotoxic concentration, corresponding to 20% loss of viability. Both readouts were considered to identify the lowest observed effect concentration *in vitro*, which was finally compared to the plasma peak concentration of a therapeutic dose *in vivo*. Already in HepG2 cells, the prediction model separates hepatotoxic from non-hepatotoxic compounds. The majority of hepatotoxic compounds show alerts at concentrations *in vitro* which correspond to therapeutic doses *in vivo*. In PHH, the prediction sensitivity improves and hepatotoxic effects are observed at even lower concentrations. Preliminary results indicate that both systems are suitable to predict human hepatotoxic blood concentrations, at least within a certain error range.

Multi-Omics Analyses for Long-Term Toxicity Characterisation

In an early step towards '-omics' analyses on **NOTOX** cell systems, the expression profiles in different cell types, namely HepG2, HepaRG and PHH were compared (*Figure 4.70*). Clusters of coherent gene expression kinetics were identified and the potential regulatory circuitry underlying them was studied. This coarse-grained description of the transcriptional network in HepaRG serves as the basis for characterising more subtle responses during long-terms exposure and low dose experiments.

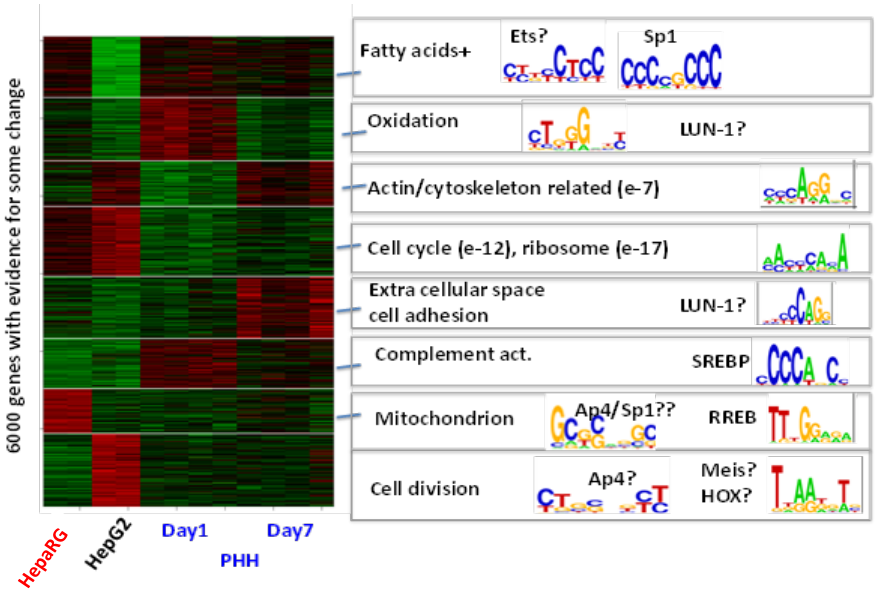

Figure 4.70 *Gene* expression *of HepaRG, HepG2 and exposed PHH cells was clustered using a variety of methods (K-means is shown here), as depicted by the heat map on the left (green – low, red – high). Encode-derived enhancer and promoter functional sequences were then associated with genes according to spatial proximity, generating a set of potentially regulatory sequences associated with each responding gene. Functional enrichment using GO annotation was associating putative function with the gene in each cluster (Fisher exact p-value is shown). De-novo motif finding was performed using the inferno algorithm developed at 'Weizmann Institute', which is capable of fully controlling for background distribution heterogeneity in enhancer sequences. PWM logos of identified motifs are shown.*

The multi-omics studies were extended in a joint experiment for the assessment of long-term effects of valproic acid (VPA). VPA is among the standard reference compounds selected by the ToxBank Project (see sections 4.7 and 4.9.4) and is used in the treatment of several diseases like epilepsy, bipolar disorders and migraine headaches. Molecular mechanisms of VPA effects, however, are still poorly understood. Within **NOTOX** effects of VPA were studied using HepaRG cells in 2D and 3D cultures. Viability of HepaRG cells during two-week VPA treatment decreased in a dose- and time dependent manner. Treatment with concentrations below the c_{max} (0.29 mM), resulted in a decreased viability after 8 and 14 days.

Treatment of HepaRG cells with VPA resulted in decreased uptake of glucose, followed by a decrease in secretion of lactate (*Figure 4.71*). Likewise, rates in the tricarboxylic acid (TCA) cycle and those of branched-chain amino acid (BCAA) degradation decreased. These observations are consistent with the literature *in vivo* effects of VPA.

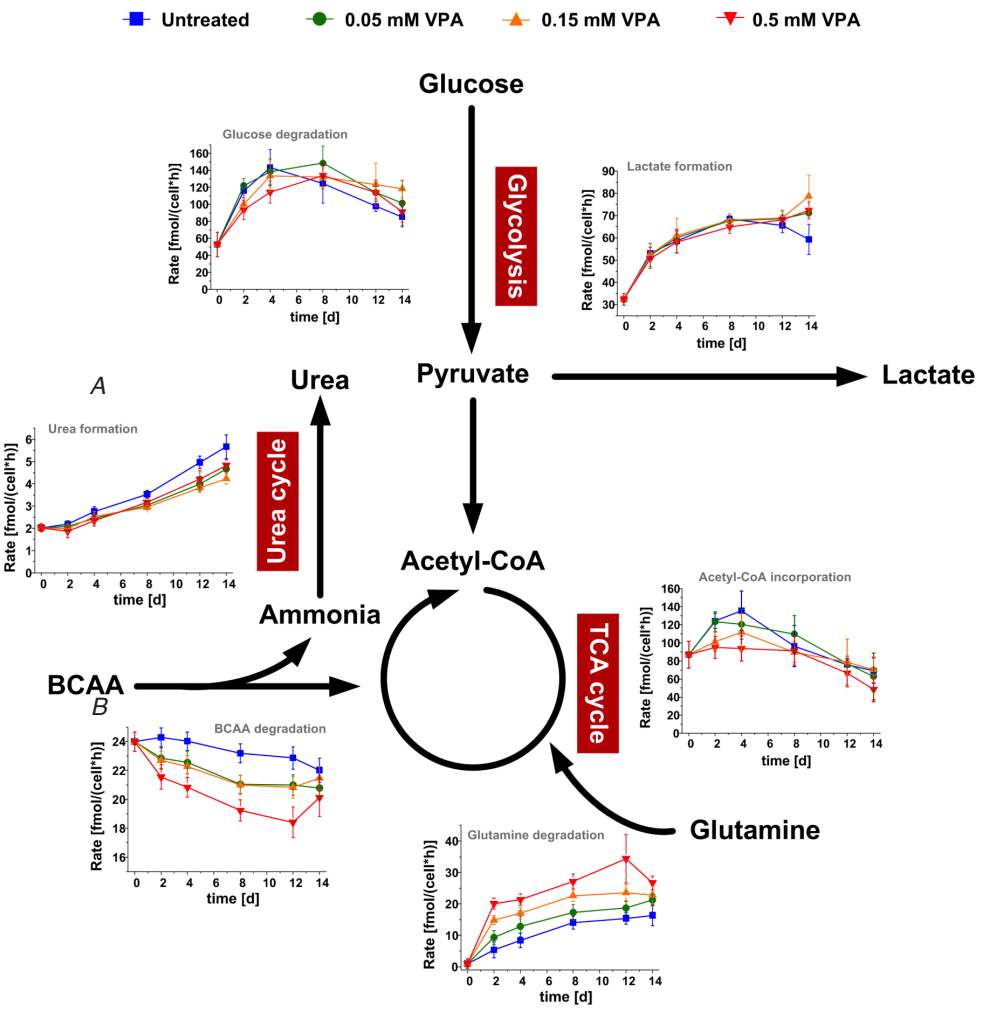

Figure 4.71 *Selected conversion rates (metabolic fluxes) in the central carbon metabolism of HepaRG cells upon repeated dose treatment with VPA. A reaction occurring in the direction of the arrow is indicated by a positive sign. Error bars indicate standard deviation (n=3). BCAA = branched-chain amino acids.*

Observed changes in the glucose metabolism and urea production may have a pronounced impact in susceptible patients such as those with compromised liver function and urea cycle deficiency leading to idiosyncratic toxicity. The combination of modelling based on long-term *in vitro* repeated-dose data and metabolic changes allows the prediction of human relevant *in vivo* toxicity with mechanistic insights.

On the gene expression level, approximately 300 genes were found to be up- or down-

regulated more than 1.5 folds (*Figure 4.72*). Based on a cluster analysis, the genes can be divided into 4 different groups. In one of the groups of genes in *Figure 4.72*, one can observe a considerable induction as a late response after 14 days of VPA treatment. Among these genes we identified are several stress response genes, e.g. FOS, JUN, ATF3, which indicate a more severe toxic response after long-term VPA treatment of the HepaRG cells.

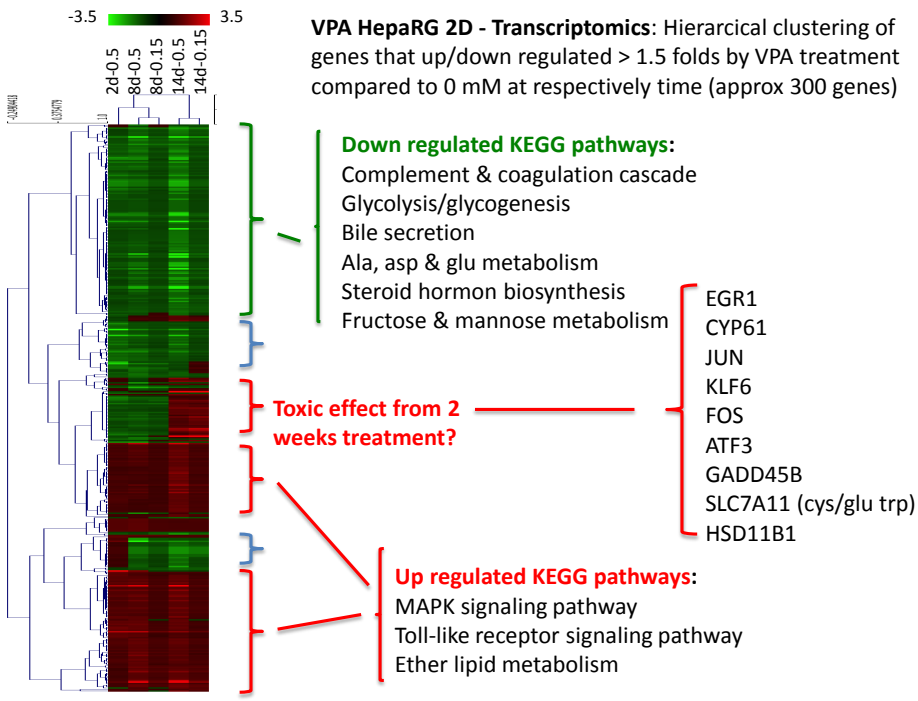

Figure 4.72 *Hierarchical clustering of fold change at each time points compared to the corresponding control.*

Among the down-regulated genes the bile secretion pathway can be exemplified with the BSEP transporter, which is down regulated already after 2 days treatment with 0.15 mM VPA *(Figure 4.73 left)*.

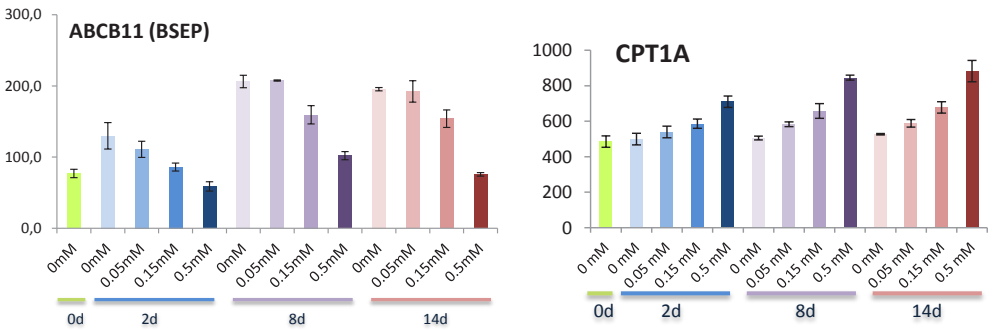

Figure 4.73 *Signal intensity of gene expression analysis of BSEP mRNA (left) carnitine palmitoyl transferase 1 mRNA (right).*

VPA enters the liver mitochondria with the help of carnitine and carnitine palmitoyltransferase I (CPT1A). Long-term treatment with VPA will deplete carnitine and inhibit the CPT1A enzyme, resulting in a compensatory up-regulation of the CPT1A gene expression (*Figure 4.73 right*).

On the proteomics level, using a label-free proteomics approach including manual validation of more than 4000 peptide signals, 1300 proteins could be quantified across all groups analysed with an intragroup coefficient of variation of less than 20%. Abundance of around 200 proteins changed significantly between all samples in a dose- and time-dependent manner, i.e. the longer and the more VPA was applied, the more proteins turned out to be significantly altered. Pathway analysis of the differential proteins indicated that especially lipid metabolism was affected by VPA-treatment, which is in accordance with the expected steatotic effects of VPA. Furthermore, the effects on the proteome were visible already when commonly applied endpoint assays like e.g. cell viability or ATP content did not yet indicate toxicity.

VPA is known to induce epigenetic changes. Therefore, genome-wide DNA methylation (Illumina 450K BeadChip array) and histone modification analysis (ChIP-seq) at different time points were applied. Epigenetic data were also correlated with global gene expression data (Affymetrix). As VPA is known to inhibit histone deacetylases, HepaRG cells cultivated for 14 and 21 days were analysed for the histone modifications H3K9ac and H3K4me3 using ChIP-seq. Preliminary bioinformatic analysis showed an overall decrease of normalised H3K9ac peaks during cultivation, while H3K4me3 appears to be more stable. This points to VPA blocking partially the loss of H3K9ac. Interestingly, also new H3K9ac and H3K4me3 peaks were detected at d14 and d21, among them sites that coincide with changes in gene expression (data not shown).

In brief, clear dose and time dependent trends were observed at all '-omics' levels. These '-omics' endpoints are now assessed in detail to develop a pathway of VPA effects.

Quantitative Proteomics for Toxicity Assessment in 2D and 3D Cultures of Hepatocytes

The emerging field of toxicoproteomics has been boosted by recent advances in proteomic technologies and its increasing applications in toxicology testing/research. In the context of toxicants, studying cellular protein dynamics on a global scale is indispensable for identifying molecular initiating events as well as adverse outcome pathways, especially in connection with other systems biology approaches like transcriptomics and metabolomics. Therefore, in an attempt to develop *in vitro* toxicological approaches, *in vitro* screening tests for toxicity evaluation nowadays should take advantage of the wide diversity of proteomic platforms and '-omics' approaches in general. **NOTOX** developed quantitative proteomics workflows for investigating both intracellular and extracellular proteomes of hepatocytes in both holistic and targeted approaches as well as bioinformatic methods for '-omics' data integration. The developed analytical methods provide good coverage of the different cellular compartments including the extracellular space (up to 4.000 proteins analysed; *Figure 4.74*), bearing in mind that secreted proteins are a valuable source for biomarkers of drug-induced toxicity. Moreover, the methods allowed accurate and reproducible relative quantification of more than 1.200 intracellular and 200 extracellular proteins in large-scale toxicological studies comprised of up to 13 groups including time series and different doses, a common experimental setup in toxicity studies. Using the quantitative data we differentiated treatment- as well as cultivation-dependent changes in the intracellular proteome upon treatment with acetaminophen and valproic acid (*Figure 4.74B-C*). Proteomics results were integrated with other '-omics' data using in-house developed bioinformatics tools (*Carapito et al., 2014*), as exemplified in *Figure 4.74D*.

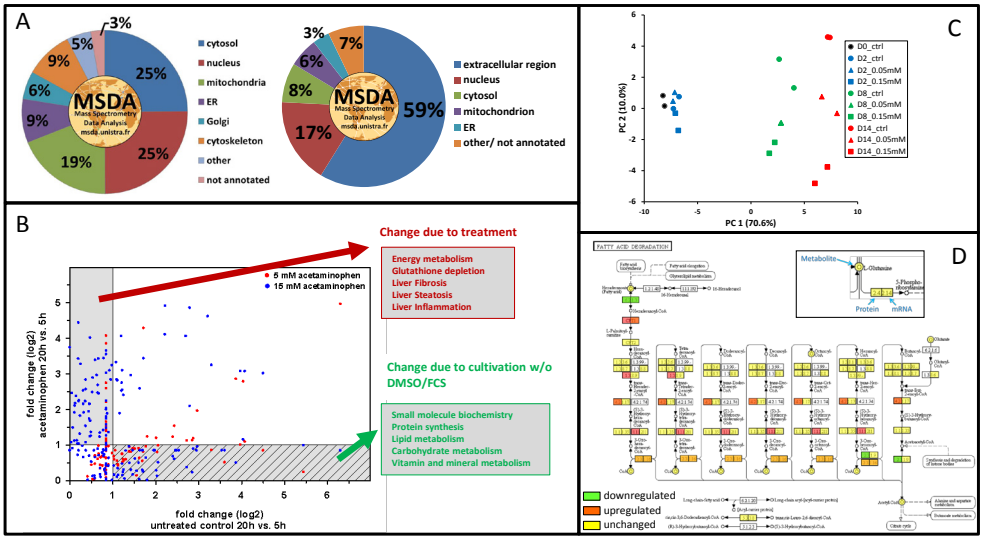

Figure 4.74 *Global toxicoproteomics within* ***NOTOX****. Subcellular locations of identified intracellular (A, left) and extracellular/secreted (A, right) proteins were determined, as well as changes in protein abundances upon treatment with acetaminophen (B) and valproic acid (C). Simultaneous visualisation of significantly changing transcripts, proteins and metabolites in HepaRG cells data was created using in-house developed bioinformatics tools (msda.unistra.fr), as exemplified here for long-term effects of valproic acid exposure on fatty acid degradation (D).*

Toxicity Assessment by Combining Multi-Omics Analysis and Database Search

In order to develop computational models of biological pathways for long-term toxicity assessment, a comprehensive NOTOX Knowledge Base (a database for long-term toxicity data) was established. This enabled the experimental partners to get access to information on all selected standard reference compounds together with their therapeutically relevant targets (proteins/genes). In the knowledge base, each compound was connected to tables of all metabolising enzymes, nuclear hormone receptors, other affected proteins and deregulated genes in the context of long-term toxicity. In addition, each relation in the knowledge base was underpinned with literature references. We performed systematic knowledge extraction, i.e. text mining and expert curation of the relevant scientific literature and publicly available knowledge bases (*Figure 4.75A*). The knowledge base contributed significantly to the interpretation of the experimental data, particularly with respect to the vast amounts of '-omics' data. A module for importing different '-omics' data (proteins and genes) was developed to analyse and visualise the data in the context of their mode of action on the molecular level. Pathway visualisation provides relevant information about molecular interactions and enables biologically informed

decisions on further actions. Moreover, by using pre-processed experimental data from partners, we designed and completed the module for analysing '-omics' data in the context of most strongly indicated toxicological pathways related to exposure to test compounds and associated with genes/proteins from the '-omics' data (*Figure 4.75B*). Toxicity assessment of compounds used for **NOTOX** pilot studies (acetaminophen and valproic acid) considers curated existing knowledge, experimental '-omics' data and data derived from bioinformatics analysis in order to enable super-imposition of characterised '-omics' data onto maps of affected biological pathways, in order to create hypothesis-based models of mechanisms of action.

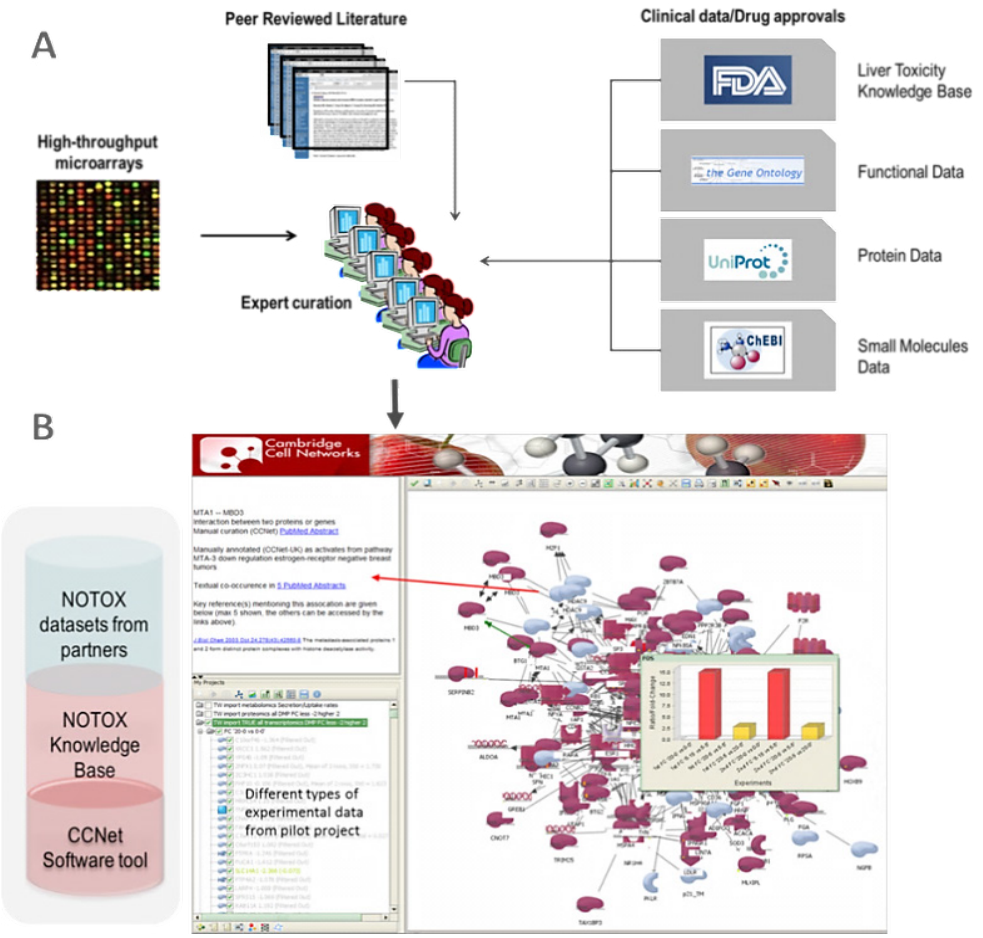

Figure 4.75 *The manually curated database for liver toxicity. The database was developed by manual annotation of 30.000 scientific publications related to drug-induced hepatotoxicity. It contains 5.000 compounds, nearly 10.000 individual expression values and more than 2.500 literature articles (A), as well as proteomics, transcriptomics and metabolomics data from* ***NOTOX*** *partners. These data are accessible for analysis and visualisation via project workspace in NOTOX Knowledge Base (B).*

In silico Modelling for the Prediction of Dose and Pathway Related Adverse Effects in Humans from *in vitro* Repeated-Dose Studies

For the reduction and ultimately replacement of animal studies in preclinical long-term toxicity assessment, a combined strategy of using advanced *in vitro* cell culture methods based on functional human cell cultures and computational modelling is expected to play an essential role. HepaRG cultures remain viable and functional for long periods and have been applied in toxicity studies. **NOTOX** established conditions for HepaRG cells in which they retain viability, transporter and metabolic activity (see section 4.6.3.1 above). These cultures were used to assess long-term toxicity of valproic acid and bosentan over a period of 28 days. *In vitro* biokinetics were assessed for the consideration of plastic binding and compound degradation (*Figure 4.76A*). The long-term dose response data was used to calculate oral equivalent doses for both valproic acid (*Figure 4.76C*) and bosentan. Using a simple PBPK model (*Figure 4.76B*) with a virtual population of 100 individuals, *in vitro* to *in vivo* extrapolation (IVIVE) is possible as shown for valproic acid (*Figure 4.76D*). The model predicts that valproic acid is hepatotoxic in 4 and 47% of the virtual population at the maximum daily recommended dose after 3 and 4 weeks of exposure, respectively (*Klein et al., 2016*).

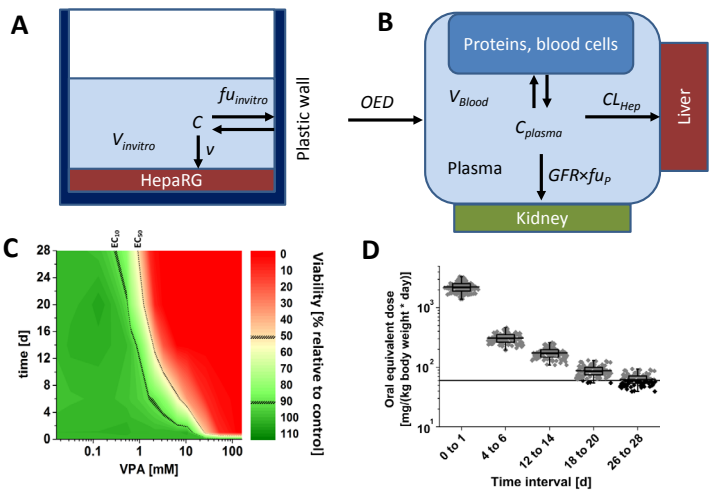

Figure 4.76 *Estimation of oral equivalent doses (OEDs) from in vitro data using a physiological model. A: model scheme for the in vitro test system; B: human physiological model; C: Viability data from 2D in vitro HepaRG culture during VPA exposure; D: estimated OEDs using a population 100 individuals.*

Combined PBPK and Metabolic Models for Toxicity Prediction

Cellular metabolic and toxicity-related mechanism of action models coupled to pharmacokinetic or physiologically-based pharmacokinetic (PBPK) models provide insight into the relation of

route and dose of administration to local effective concentrations. **NOTOX** provides a multi-scale whole body model describing the molecular toxic mechanisms of valproic acid (VPA). For this purpose, a PBPK model of valproic acid (*Figure 4.77A*) was combined with a model of the hepatic VPA metabolism (*Figure 4.77B*), based on a set of coupled ordinary differential equations, as well as toxic mechanisms/mode of action (MoA) steps, which attenuate cell viability due to toxic metabolites and disturbance of lipid metabolism. The coupled PBPK-VPA-MoA-model was pre-set with *a priori* parameters, to fulfil following demands: (i) the model was adapted to reflect *in vivo* plasma concentration profiles (*Figure 4.77C*); (ii) the model was enabled to reflect potential VPA-triggered dynamic response in key players in fatty acid synthesis and oxidation (*Figure 4.77D*). The hepatic VPA-model was further identified with experimental metabolite, transcript and protein data from the VPA experiment on HepaRG culture. The combined modelling approach introduced supports the dose-dependent simulation of cellular VPA dynamics and toxic effects and can be adapted and extended to a large range of compounds.

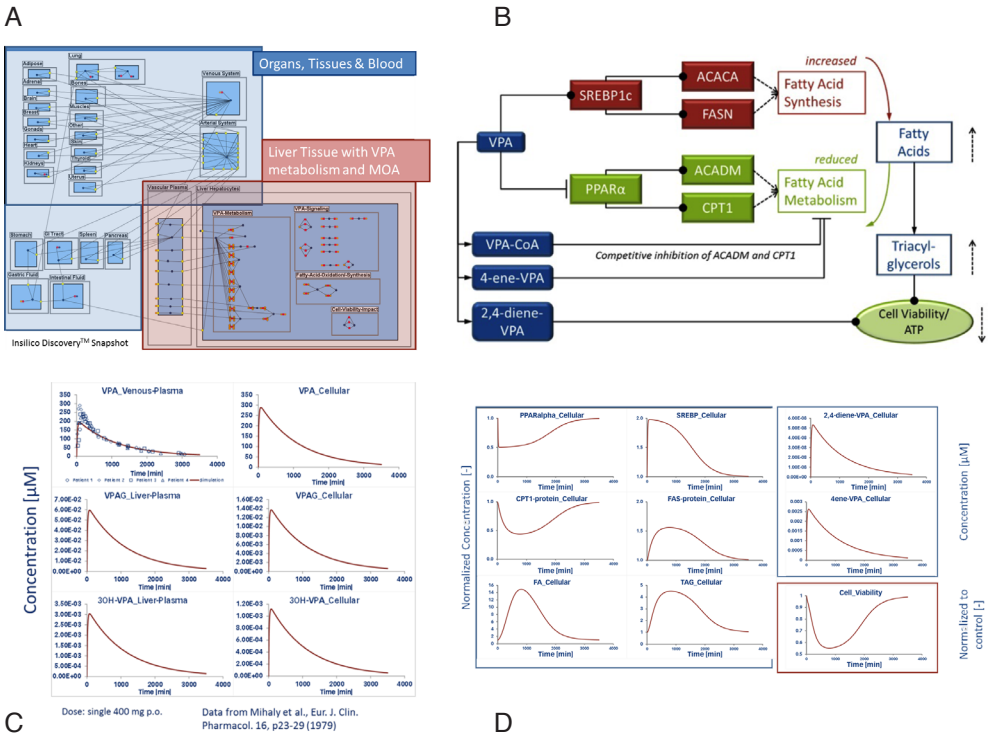

Figure 4.77 *(A) The VPA-MoA-model is integrated in the liver compartment of Insilico's PBPK model. (B) VPA toxic mechanism/mode of action model reflecting FA (Fatty Acid) metabolism disturbance and cell viability impact. (C) Plasma and Cellular VPA and –metabolites' profiles (D) Potential mode of action response profiles in fatty acid regulation and metabolism and in cell viability.*

Agent-Based Modelling for *in vitro* to in vivo Toxicity Extrapolation

Agent-based models display every individual cell and hence permit representing the precise experimental setting of *in vitro* experiments as well as the precise architecture of liver lobules which constitute the smallest anatomical and functional unit of the liver. *Figure 4.78* shows how the detailed mechanistic model on the molecular level is incorporated into an agent-based representation of a liver lobule that is then incorporated into a whole body PBPK model.

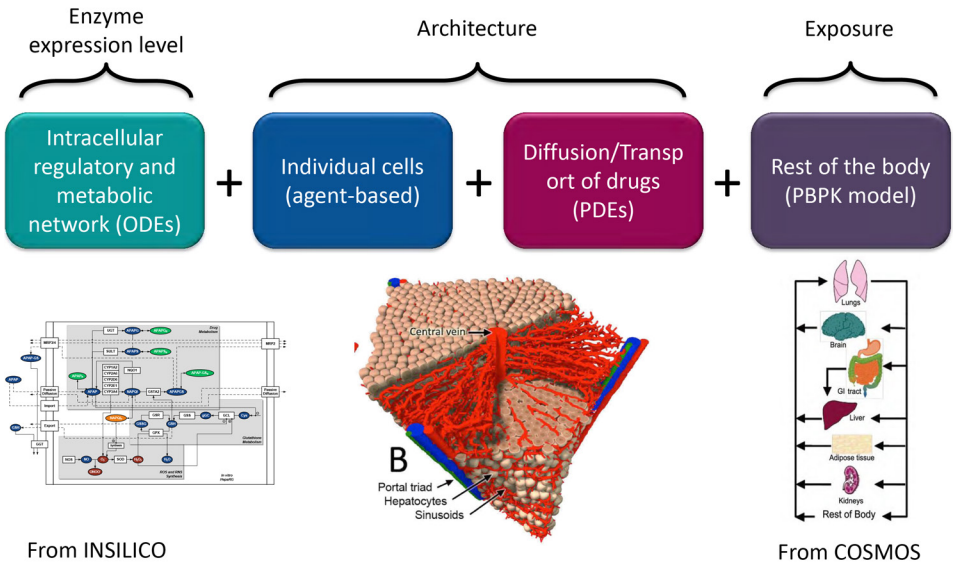

Figure 4.78 *Concept of application of agent-based multi-scale modelling for IVIVE. A detailed regulatory and metabolic model is plugged into individual cell constituting an organ or the elementary unit of an organ, e.g. a liver lobule. This organ model is then part of a classical PBPK model primarily describing the distribution of compounds in the body as well as their removal, e.g. by the kidney.*

Zonated ammonia detoxification. We investigated how liver function and in particular ammonia detoxification is impaired during drug-induced liver damage and regeneration. We showed that the classical reaction scheme for ammonia detoxification in a compartment model was sufficient to reproduce the measured concentrations of ammonia and glutamine at the liver outlet in the healthy case but not in the case of a drug-damaged liver (*Schliess et al., 2014*). In an integrated metabolic spatio-temporal model, it was shown that adding an *ad hoc* ammonia sink during the damage could resolve the mismatch. The reversible reaction catalysed by GDH is a good candidate that could act as an ammonia sink during the damage, whereas producing ammonia in the healthy situation. We developed and implemented an

alternative model taking into account the GDH reaction. This model was now able to describe cell damage by ammonia as well as the recovery of the cells (*Figure 4.79*). As could be shown in an animal model, the identified mechanism can be used therapeutically to prevent hyperammonemia.

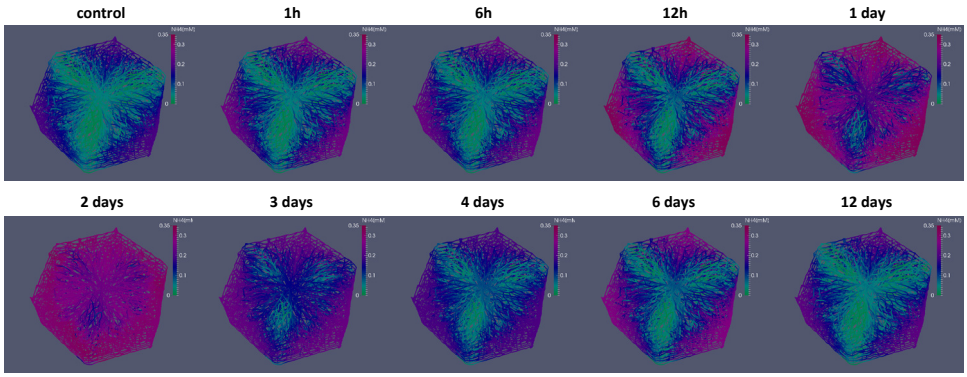

Figure 4.79 *Simulation snapshots of ammonia concentration in lobule (green: low, violet: high). 3 portal veins per lobule were implemented in the model together with a septum-like connection of the sinusoidal network in the portal field with the portal veins. Ammonia detoxification is better along the porto-central axis.*

Acetaminophen (APAP) overdosing causes necrosis in hepatocytes expressing CYP2E1 and CYP1A2 (about 50% of all hepatocytes) resulting in a necrotic lesion in the central region of liver, similar to ammonia. Necrosis most severely affects hepatocytes expressing the enzyme glutamine synthetase, localised in the lobule centre. These most efficiently detoxify the blood from ammonia. The integrated mathematical model described above recently predicted the existence of a hypothetical ammonia sink (*Schliess et al., 2014*) that could later be identified experimentally (*Ghallab et al., 2015*). **NOTOX** used a similar modelling strategy to infer the *in vivo* APAP toxicity from *in vitro* experiments by stepwise extending a mathematical model calibrated with *in vitro* data to extrapolate *in vivo* toxicity. In its final version, the model is multi-scale and spatial temporal, representing liver lobule architecture, including APAP detoxifying pathway in each hepatocyte, and the flow of blood and transport of APAP with the blood. First, a population of hepatocytes exposed to different concentrations of APAP was modelled to reproduce the measured *in vitro* toxicity curves. Cell-to-cell variability has been taken into account by permitting 30% variability for enzymes involved. Next, differences of the *in vivo* to *in vitro* situation were systematically explored: (i) the difference in the temporal profile of exposure; (ii) the higher expression of metabolic enzymes *in vivo* compared with *in vitro*; (iii) the lobule architecture. While including (i) generates dose-independent cell survival (*Figure 4.80a*), including in addition (ii), all CYP positive cells died (*Figure 4.80b*). Integrating the

APAP toxicity pathway into each CYP2E1/CYP1A2 positive hepatocyte of a spatial-temporal model resolving each hepatocyte as a single agent within realistic lobule architecture, first simulations indicate that still all cells are dying (*Figure 4.80c*). We conclude that our modelling tool permits systematic *in silico* testing of death mechanisms, which can be used as a guiding tool for planning of experimental designs.

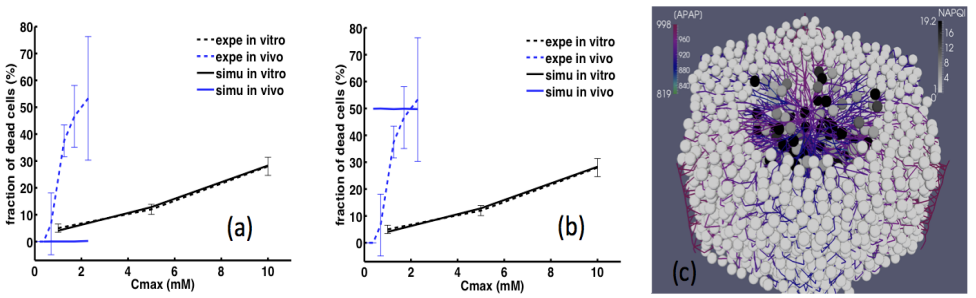

Figure 4.80 *(a)-(c) Modelling steps to infer in vivo from in vitro toxicity (full lines: model, dashed: experiments). (a) temporal exposure profile included, (b) higher CYP levels in vivo are included in addition, (c) both in spatial temporal model (black: dead cells), snapshot before all cells have died.*

Various simulations of a series models showed that a detailed agent-based model is especially important when tissue regeneration or tissue formation plays an important role as was shown for ammonia, CCl_4 and also acetaminophen. A simplified model involving several compartments, e.g. mimicking the different cellular characteristics along the sinusoid, may be necessary and sufficient for compounds that are metabolised with a relatively high rate and for taking into account the different sensitivities of these cells to the toxic compound they are exposed to. In those cases where the first pass through the liver would not create significant gradients, a simple one-compartment model may be sufficient.

4.6.4 Contributions to the SEURAT-1 Case Studies

The **NOTOX** approach with its multi-'omics' and high content imaging characterisation combined with informatics analysis of the data permits the identification and characterisation of pathways leading to toxic effects and contributed significantly to the cross-cluster case studies defined at the cluster-level of the **SEURAT-1** Research Initiative. Specific data, e.g. expression of certain genes, changes in the proteome, metabolic changes or changes related to the generation of reactive oxygen species, were used as markers for the quantification of effects. Specific contributions from the **NOTOX** Project were

- the identification of changes in expression of CPT1 and BSEP, changes in metabolic fluxes, changes in histone modification and various protein levels in the context of the steatosis case study;
- functional imaging in sandwich and spheroid cultures allowed determination of bile flux in the context of the cholestasis case study. Furthermore, modified susceptibility of HepaRG detected compounds with cholestatic liability;
- the quantification of the formation of reactive oxygen species in response to treatment with paracetamol;
- the establishment of high resolution EM-tomography for exploration of molecular initiating events in the context of the adverse outcome pathway approach.

Furthermore, the two detailed case studies using paracetamol (acetaminophen, APAP) and valproic acid (VPA) that were conducted under the umbrella of the **NOTOX** Project supported the **SEURAT-1** case studies. The data from these case studies permitted the generation of detailed gene regulatory and metabolic models that could be incorporated into agent-based models and further into PBPK models that allow a quantitative description of the metabolism and distribution of compounds in the human body. Using such models various scenarios of exposure can now be studied in detail, e.g. uptake via different routes, oral or via the skin.

4.6.5 Potential Impact

Strategic Impact of NOTOX

The **NOTOX** Project delivered new methods based on a systems biology approach. The **NOTOX** approach created examples of *in silico* predictive models at various levels of details that will be beneficial to all stakeholders, consumers, regulatory bodies and producers and paves the way how new alternative testing methods may be developed in the future. Particularly the development of alternative methods in long term repeated dose toxicities will have a tremendous impact on all these communities and will also bring about harmonisation, transparency and better understanding on various levels. The potential impact of **NOTOX** may be seen in different directions: strategically, with respect to the application in safety assessment, to innovation and to the support of European industries.

Adoption of the results from the **NOTOX** Project by the concerned European industries will have an impact in

- ensuring safety of consumer products as defined by the European Scientific Committee on Consumer Products (SCCP) directives;

- providing *in vitro* alternative methods for long term toxicity testing based on predictive computer models that have potential to enter pre-validation and finally validation;
- decreasing animal testing;
- reducing the workload during product development and testing;
- improving predictability of safety of substances of importance for cosmetics and other industries (chemical and pharmaceutical);
- allowing new systems-oriented approach for novel cost-efficient and time-efficient product testing;
- strengthening competitive edge of cosmetic and pharmaceutical industries by providing knowledge-based solutions;
- accelerating systems-oriented toxicity testing in product development.

Impact on Safety Assessment

The potential impact can be best explained by briefly looking at some major results of **NOTOX** that promise integration into safety assessment procedures in the foreseeable future. We are fully convinced that future testing will very much rely on methods based on systems biology approach that is characterised by a broad understanding of mechanisms of toxicity often called mode of action. Mode of action of a compound can be most thoroughly studied using '-omics' methods, comprising genomics, epigenomics, proteomics, metabolomics and fluxomics combined with powerful new imaging methods using fluorescence techniques and high resolution 2D/3D electron microscopy. Indispensably, this requires reliable cellular systems mimicking the human *in vivo* situation as closely as possible.

NOTOX paved the way to using only human cells, particularly cell lines (HepaRG, stem cell derived hepatocytes and cardiomyocytes) for long-term toxicity assessment instead of animals. At the beginning of **NOTOX**, HepaRG cells were occasionally used for toxicity testing, but not for long-term effects. However, during the course of time, the **NOTOX** results have shown the suitability of HepaRG cells in long-term applications. Other projects of the **SEURAT-1** Research Initiative started using the HepaRG model. Moreover, **NOTOX** realised early the value of emerging new methods for 3D cultures as was indicated already in the proposal. **NOTOX** started to extensively use such techniques in toxicity studies which were mainly based on 2D cultures or animals in the near past. In addition, **NOTOX** extended high content screening methods to the 3D cultures, i.e. spheroids and sandwich cultures. More recent developments are further stimulating the use of human cells, particularly the increasing availability thanks to the breath taking progress in the isolation, propagation and differentiation of embryonic and induced pluripotent stem cells (hESC and hiPSC). **NOTOX**

presents examples for the cultivation of such cells including micro tissues of multi-organ mimicking systems (hepatocytes and cardiomyocytes). Activities of cellular systems were characterised with a range of '-omics' methods and with optical and electron microscopy methods. Bioinformatic methods for the evaluation of the huge amount of data created were applied and further developed. Computational modelling is still a tedious and challenging effort, however, in **NOTOX**, mechanistic and predictive mathematical models with much higher resolution and degree of detailing were created in its last phase.

Application of **NOTOX** Methods in Safety Assessment

A crucial question is how such new developments can contribute to the safety assessments of compounds in the cosmetics, pharmaceutical and other industries. *Figure 4.81* shows schematically some important levels of testing that contribute to safety assessment. A first and nowadays quick computational method uses chemical knowledge combined with *a priori* knowledge on structurally related chemicals for read across, as has been successfully further developed within the **SEURAT-1** Research Initiative (see section 3.2.2).

NOTOX contributes significantly to methods in the other three areas indicated in *Figure 4.81* by providing a whole toolbox of methods for testing compounds using human cell *in vitro* and *in silico* methods. This first method capitalises on the developed organotypic cultures of the HepaRG cell line that has been extensively studied in **NOTOX** and combines it with simple PBPK type of modelling (*Klein et al., 2016*). It was stimulated by American activities like ToxCast. Our newly developed 2D and 3D cultivation methods are not only applicable for short-term toxicity testing but allow also long-term and repeated-dose testing.

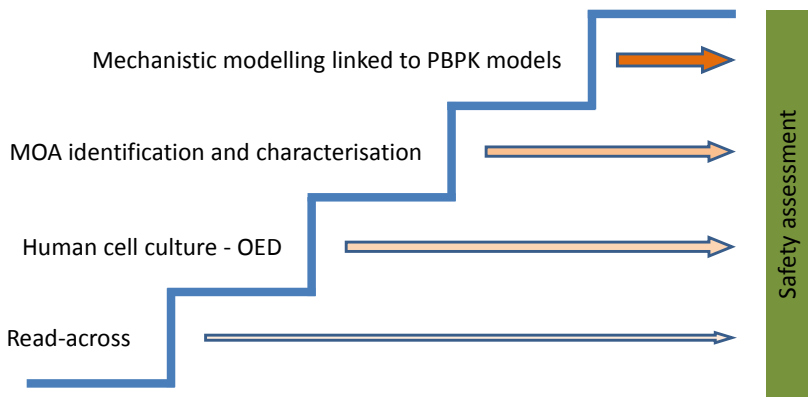

Figure 4.81 *The* ***NOTOX*** *approach combining human based cell systems including 3D culture with computational modelling for the prediction of toxicity. OED: oral equivalent dose, PBPK: physiologically based pharmacokinetic modelling.*

The principle of oral equivalent dose (OED) determination was highlighted above (see *Figure 4.76* in the previous section). Generally, a simple model of the *in vitro* systems such as shown in *Figure 4.76* allows the estimation of parameters needed for the *in vivo* estimations. The pharmacokinetic parameters liver clearance and plastic binding are determined using modern analytical techniques, usually involving mass spectrometry. For reliable determination of liver clearance, cell cultures with *in vivo* like activities of liver specific enzymes and transporters are essential that are now available by the 2D and 3D culture methods using HepaRG (*Gunness et al., 2013; Mueller et al., 2014*) or other human cells. The serum free media (*Klein et al., 2014*) developed in **NOTOX** improves such studies by practically eliminating protein binding effects *in vitro*. Protein binding is determined in separate *in vitro* experiments or estimated by computational methods.

Dose-response curves for liver cells can be determined reliably using our HepaRG cultures. All these studies can be made in a high-throughput manner using e.g. 96-well plates. Having the glomerular filtration rate (GFR) hepatic clearance can be estimated (*Figure 4.76B*). Taking known data of liver weight, serum protein concentration and blood volume allows a direct estimate of OED. In combination with a useful exposure scenario the assessment of the risk of liver toxicity is possible also for a population. This method is also straight forward applicable for new liver cell lines that will be developed, e.g. derived from human embryonic or human of induced pluripotent stem cells (hESC or hiPSC). It has potential to be applied for liver co-culture systems, e.g. with Kupffer or stellate cells. Such systems have been explored within **NOTOX** with Kupffer cells and with stellate cells in collaboration with *HeMiBio* (*Leite et al., 2016*).

Additionally HepaRG cells can be combined with cells of another organ of interest for the determination of metabolism mediated toxicity. We recently used cardiomyocytes derived from hESC and hiPSC cells (obtained from the *SCR&Tox* project, see section 4.2.3.1, *Figure 4.4*) for long-term toxicity testing. These cultures can be kept in a common medium to test for toxic compounds created by liver metabolism. This shows the collaboration between the different projects of the **SEURAT-1** Research Initiative as well as the huge future potential of methods developed within **NOTOX**.

Aspects of the identification and characterisation of mode of action for safety assessment were highlighted in the previous section in the context of the contribution of the **NOTOX** Project to the **SEURAT-1** case studies.

Strengthening the Community Industry

The **NOTOX** Project by its diverse activities focusing on improved prediction of long-term toxicity has potential impact on the community industry:

- The SMEs which were partners in the project namely; Biopredic International, Insilico Biotechnology and Cambridge Cell Networks, have access to the results and prototypes for own business development;
- The dissemination of results such as the developed predictive models for long-term toxicity according to the exploitation plan of the project can be used by new or existing SMEs in the bioinformatic and bioinstrumentation business sector;
- New methods for cosmetics, pharmaceutical and chemical industries were developed and disseminated.

A number of software packages were developed in the **NOTOX** Project (some available freely) and are ready for exploitation. Examples of these software packages are

- Cell Sys and TiQuant for agent based modelling;
- Ettention software framework for tomographic.

The company EYEN SE was founded by the former **NOTOX** researcher Lukas Marsalek as a Spin-Off from DFKI GmbH. The company aims to provide electron tomography, sub-tomogram averaging and macromolecular structural analysis as a commercially available service and thus is a large step to transfer some part of the techniques and pipelines developed during **NOTOX** to a persistent state usable after the end of the project.

Integration in Community Research

The importance of the area addressed in the proposal call required actively engaging the scientific community. Especially a systems biology approach necessitated a joint effort by a multidisciplinary team composed of biologists, bioinformatics, biophysicists, toxicologists, engineers, mathematicians and modellers. The **NOTOX** Project integrated the expertise of these diverse community research groups

- by a cross disciplinary research effort between frontline researchers in the bioinformatics and biomedicine sciences, in particular with the German ‘Virtual Liver Initiative’;
- by setting up collaborations with the other projects of the **SEURAT-1** Research Initiative through exchange of people, methods and material. These collaborations resulted in joint publications;
- by mobilising front researchers and users by thorough dissemination activities in the scientific community. **NOTOX** organised satellite meetings/workshops at the ESTIV and EUROTOX meetings. In addition, the general public was also reached by media communications and press releases;

- by the active involvement of **NOTOX** in the **SEURAT-1** case studies;
- by the active contributions from **NOTOX** to the **SEURAT-1** workshops.

Animal Welfare Policy and 3R Principle

The complete ban on animal testing for cosmetics and cosmetic ingredients from March, 2013 (Cosmetics directive 76/768/EEC) necessitates the development of non-animal test systems. To date no alternative validated long term repeated dose toxicity methods exist. The **NOTOX** Project considered the situation of experimental animal testing as stated in the Community Directive 86/609/EEC (OJ L 358, 18.12.1986, p.1) and is in agreement with the protocol annexed to the Treaty of Amsterdam on Animal Protection and Welfare regarding the formulation and implementation of Community Policies including research. In addition, this project is in accordance with the 3Rs concept (reduce, refine or replace the use of laboratory animals) of Russell & Burch (Russell, W.M.S. & Burch, R.L., 1959, The principles of humane Experimental Technique, 238 p.p., Methuen, London).

NOTOX supported this policy

- by reducing the need for animals during compound screening by using organotypic human cell cultures in 2D and 3D (HepaRG cells, primary human hepatocytes, human stem cell derived hepatocytes and cardiomyocytes) for long-term repeated dose toxicity studies;
- by replacing animal tissue material with human cell and tissues systems in addition to replacement of foetal bovine serum with other components allowing serum free cultivation;
- by providing powerful *in vitro* bioinformatic and mathematical models of human cell systems. Such causal computer models can be used for long-term toxicity studies that are also more predictive for responses in humans than in animals. An example close to application is the OED calculation using *in vitro* long term data and PBPK modelling.

Project-related Publications from the NOTOX Consortium

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Awards, Prizes and other Achievements

Daniel Müller and Sebastian Klein received bursaries for young scientists at the Systems Biology of Liver conference held in Luxembourg 21–23 February 2013.

A figure from a publication (*Gunness et al., 2013*; see above) was used on the cover of *Toxicological Sciences* (Vol. 133, Is. 1).

The **NOTOX** publication *Klein et al., 2013* (see above) has been selected as a highlight in the Journal of Chemical Research in Toxicology, in the special issue on Systems Toxicology, March 2014 (Dahlmann, H.A. (2014): Spotlight. *Chem. Res. Toxicol.*, 26: 312-313).

Lukas Marselek, a former postdoc of **NOTOX** at the German Research Centre for Artificial Intelligence, has established an SME (Eyeon) in Prague in 2014.

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4.7 ToxBank: Supporting Integrated Data Analysis and Servicing of Alternative Testing Methods in Toxicology

Emilio Benfenati and Barry Hardy on behalf of the ToxBank Consortium

4.7.1 Executive Summary

ToxBank established a dedicated web-based warehouse for toxicity data management and modelling, a 'gold standards' compound database and repository of selected test compounds, and a reference resource for cells, cell lines and tissues of relevance for *in vitro* systemic toxicity research carried out across the **SEURAT-1** Research Initiative. **ToxBank** developed infrastructure and service functions to create a sustainable predictive toxicology support resource going beyond the lifetime of the reearch programme. Specifically, based on an extensive requirements gathering from all **SEURAT-1** projects and an analysis of this data, a production version of the ToxBank Data Warehouse has been implemented that provides access to all experimental, processed data and protocols alongside relevant public information *(https://services.toxbank.net/toxbank-ui/login)*. This includes the development of web-based interfaces for linking and uploading data, including raw and processed data, and model results. All steps of any experiments are linked to protocols describing the procedures. A web-based user interface for searching, browsing, and filtering the results has been implemented to provide access to all protocols and data across the cluster in a way that is sensitive of any intellectual property restrictions on access. Public access to the ToxBank Data Warehouse was established in Autumn 2014. Public data on reference compounds was incorporated into the warehouse supporting meta analysis and risk assessment being carried out on the **SEURAT-1** case studies. A collaborative ToxBank Gold Compound database was established using the MediaWiki platform *(http://wiki.toxbank.net/)*. It has been populated with a set of approximately 50 reference compounds, including information about chemical identities, adverse effects, toxicity mechanisms and therapeutic targets. These compounds were used as reference compounds by the **SEURAT-1** Research Initiative.

ToxBank Tutorial and Workshop proceedings have been captured and integrated into

the ToxBank website *(http://www.toxbank.net/)*. In 2015 ToxBank partners agreed to the continuation of ToxBank as a sustained OpenTox resource *(http://www.opentox.net/)* beyond the end of the project, including its extension and support of the EU-ToxRisk programme *(http://www.eu-toxrisk.eu/).*

4.7.2 Project Context and Objectives

The primary goal of the **SEURAT-1** Research Initiative was the development of human safety assessment strategies which may be used to replace, reduce or refine (3Rs principle) repeated-dose systemic toxicity testing historically carried out in animals. The target of **ToxBank** within the **SEURAT-1** Research Initiative was the establishment of a dedicated web-based Data Warehouse (DW) for toxicity data management and modelling, a 'gold standards' compound database and repository of selected model compounds, and a bank for cells and cell lines, including stem cells, and tissues of relevance for *in vitro* toxicity testing.

The objectives were the following:

- Establishment of a dedicated web-based data warehouse: The ToxBank Data Warehouse (TBDW) has established a centralised compilation of information and data for systemic toxicity. Links to relevant public databases are provided for data import. All projects of the **SEURAT-1** Research Initiative uploaded their raw and processed data into the TBDW as soon as these become available. Data generated were analysed and the outcome integrated whenever possible into computer models capable of predicting repeated-dose toxicity. The TBDW has been organised so that it provides a sustainable source of information for toxicological research going beyond the life-time of the research projects, through sustainability and business planning carried on throughout the project, including discussions with industry groups such as Cosmetics Europe to meet their requirements.

- Establishment of a database of selected model compounds: The ToxBank Gold Compounds were selected to meet the highest quality standards. Chemicals in the database have been selected based on the scientific literature, including review of mechanistic publications, high-quality repeated dose toxicity *in vivo* data from animal studies, and adverse event and epidemiological data from humans. The database covers cosmetic ingredients, industrial chemicals, and pharmaceuticals that meet high-quality selection criteria for reference compounds. In addition, a list of selected model compounds, standard operating procedures for data quality control, processing and analyses have been provided. Whenever compounds are needed for training or validation purposes, they can be selected from the database in correspondence with targeted mode

of actions in systemic toxicity. An extensive information resource on reference compounds was created for public dissemination through the ToxBank wiki *(http://wiki.toxbank.net/)*.

➠ Establishment of a repository for the selected model compounds: The ToxBank Chemical Repository ensures the availability of test chemicals accompanied by analytical quality control procedures to the research projects of the **SEURAT-1** Research Initiative. We have investigated service models so that the chemical repository could be maintained beyond the end of the programme to provide ongoing services to further toxicology research and validation programmes.

➠ Setting up of a cell and tissue bank for *in vitro* toxicity testing: An important service to European scientists is the formation of a bank of cells, cell lines (including stem cells and stem cell lines) and tissues to be used in the projects of the **SEURAT-1** Research Initiative and beyond the end of the programme. A biomaterials information resource was developed and incorporated into the ToxBank wiki.

4.7.3 Main Achievements

4.7.3.1 Data Warehouse

Approach to Collecting Requirements

The **ToxBank** consortium used a methodology referred to as contextual inquiry/design for the collection of user information, from visits and direct interviews with many **SEURAT-1** partners, to use in developing the system requirements for the TBDW. An analysis of this data resulted in the user requirements for the TBDW. This included the need to develop a solution to manage, register, assign a status, comment, peer review, and sensitively share the diverse protocols being generated throughout the cluster as a high priority. Handling data presented a number of complex problems as a result of the diversity of the experiments conducted within the **SEURAT-1** Research Initiative as well as the different workflows that were in place across the cluster. It was seen as essential that each step of an investigation be documented with a protocol and annotated with the resulting data, both the original results and any subsequent processed data. Providing information on the cells, reagents, and compounds was also highlighted as an important activity. It should be possible to search and download any protocols or investigation data.

To support the second phase development (integrated data analysis) **ToxBank** partners visited a further 10 **SEURAT-1** partner sites and conducted interviews with ca. 20 investigators. The new requirements included the ability to precisely search for significant up or down regulated genes or proteins. In addition, chemical structure searching (exact, substructure and

similarity) was added to support use cases such as read across. To understand how individual investigations, from a list of search results, were performed and what sort of data is available, a dashboard was implemented to summarise multiple investigations. This information allows users of the ToxBank Data Warehouse to understand both the experimental factors that were the basis for the investigations, but also the parameters and technologies used in producing the data. Data from the selected investigations can then be exported in a standardised way to enable combining data from different experiments. Since many tools were being used to perform data analysis and visualisation across the cluster, it was decided not to replicate or build any of these specialised tools, but to enable their use through a variety of data export options. Specifically, linking the information to pathway tools is important to help understand the biological context.

Design of System Architecture

The TBDW architecture consists of a set of web services, providing access to protocols and data, a search service, and a Web GUI application, offering user-friendly access to the above functionality. ToxBank currently adopts the OpenTox framework design and incorporates the REpresentational State Transfer (REST) software architecture style, a formally defined common information model, based on the W3C Resource Description Framework (RDF) and authentication and authorisation based on OpenAM. All TBDW component and web service developments were based on these previously selected technologies.

Incorporation of Systemic Toxicity Ontology

The **ToxBank** consortium created a keyword hierarchy that is used in the TBDW. In addition to its use in facilitating collaborations, the keyword hierarchy is used to support searching, browsing and linking of resources within the warehouse. When information is uploaded into the TBDW, terms are selected from this hierarchy and linked to protocols and investigation datasets in the warehouse. The keyword hierarchy is currently organised into six main branches: biomaterials, investigative techniques, data and readouts, adverse events, modes-of-action, and gold compound standards. In addition, the TBDW incorporated existing life-science related ontologies through the use of ISAcreator data entry tools that create ISA-tab compatible archives, where concepts (e.g. cells, experimental unit, and so on) in the data are linked to existing ontology terms.

Implementation of the Data Warehouse through a Suite of Distributed Data Marts

To handle all of the data generated in the diverse investigations being performed across the cluster, as well as relevant public data, **ToxBank** adopted the ISA-TAB universal data

format to represent the experiments, including the toxicity studies, any chemical analysis, and '-omics' experiments. Data access and upload procedures are defined by the Investigation API. Data was uploaded in ISA-TAB format; data queries are performed with the SPARQL query language. REST operations are available for accessing individual investigations, studies, assays and data files. Work has been ongoing throughout the project on extending the data warehouse framework to support data uploading and processing.

Implementation of Data Warehouse Operations Using Web Services

The warehouse has a series of operations for managing protocols, data, and searching. These operations have been implemented as a set of distributed web services that make use of existing OpenTox APIs. The specific services implemented were defined according to the needs of the cluster, as defined in the requirements described earlier. **ToxBank**'s REST resources are instances of the relevant RDF classes. **ToxBank** put special emphasis on data confidentiality. The Authentication & Authorisation infrastructure (AAI), in particular, builds upon what was already developed and well tested in the OpenTox project and strived to further enhance it. Searching within the **ToxBank** system is provided as a separate web service that is deployable within an existing web container or as a stand-alone application. It was developed using Java and various open source technologies including Restlet *(http://www.restlet.org/)* and elasticsearch *(http://www.elasticsearch.org/)*. The Search service is primarily accessed by the Protocol, Investigation, UI services and desktop applications such as ISAcreator *(https://github.com/ISA-tools/ISAcreator)*. When protocol or data resources are uploaded, the corresponding service notifies the Search service that a new resource is available. The search service then retrieves the resource and makes it available for indexing.

To support the precise searching of '-omics' and other data, the isa2rdf tool has been extended to support conversion of microarray, mass spectrometry and protein assignment data files to RDF/XML in addition to converting the ISA-Tab metadata. The RDF representation of the data files is based on an extension of the OpenTox Dataset RDF representation and each data file item is linked to the relevant sample, described by the ISA-Tab metadata. The conversion to RDF is performed transparently for the user, who uploads an ISA-Tab archive. The server preprocesses the archive, using ISA-Tab validation and isa2rdf and imports the triples generated into a triple store *(http://4store.org/)*. Once all the information is available in the triple store, the relevant queries are defined as a set of predefined SPARQL queries and exposed as REST services in the general form of /investigation/sparql/{template_name}. The approach described allows a common data model for both metadata and data files, which is independent of a database technology.

To support searching by chemical structures, a dedicated **ToxBank** instance of OpenTox compliant web services from IdeaConsult *(http://ambit.sf.net)* was installed on a ToxBank server. The content is updated on demand through the OpenTox dataset API.

An ISAcreator plugin allowing to query the chemical structure services was developed *(https://github.com/vedina/opentox-isa-plugin)*. It allows searching for chemical compounds (by identifier, similarity or substructure search) within ISAcreator and links the experiment metadata with the chemical structures in TBDW.

Development of Graphical User Interface

Web-based graphical user interfaces have been designed, customised and implemented for loading the data into the TBDW as well as accessing the information and model results generated.

Data Entry

A series of forms-based user interfaces have been developed and/or customised for loading experimental data and related descriptions of experimental protocols. To collect investigation data in a consistent manner across the cluster, the **ToxBank** consortium selected to use ISAcreator, an open access tool *(http://isatab.sourceforge.net/isahelp/ch03.html)*. ISAcreator provides a graphical user interface to create a consistently recorded series of data files that include the experimental design and information concerning the overall investigation, information on the experimental steps linked to both protocols as well as raw or processed data files.

Data Access and Analysis

The protocols and investigation housed in the TBDW are available from the web-based user interface. This GUI is a front-end user interface for the repository services defined by the **ToxBank** API. It is a standalone web application allowing users to log in, search and review existing protocols and investigations, and to upload new protocols and investigations. The interaction between the users' web browsers and the **ToxBank** UI server relies on standard HTML/CSS/Javascript content, generated dynamically within a Java-based web application framework (the Play Framework).

A series of user interfaces to support chemical searching as well as searches on genes and proteins have been developed. The chemical structure search user interface allows structure queries to be specified in order to perform an exact, substructure or similarity search. The query molecule is defined as either a SMILES string, a MOL file or is drawn within an integrated structure-drawing editor. The search queries all investigations with chemical structures that are defined as experimental factors in ISA-Tab. A biomaterials search window was developed that allows a **ToxBank** user to define a precise query for different biological materials. An additional option to display a dashboard for a selected set of investigations is also provided. The new dashboard was developed to help understand a specific list of investigations in terms of what experimental factors were considered, what was the source of the biological material,

as well as what protocols, technologies, and endpoints were used. From these investigations, it is possible to download and combine specific data to use in external bioinformatics, chemoinformatics, advanced data analysis or visualisation software as well as data mining applications.

Integration of Tools for Data Analysis, Mining and Model Building

To support an integrated view of the derived or processed data generated from experiments across the **SEURAT-1** Research Initiative as well as outside the cluster, **ToxBank** uses preconfigured templates for assay metadata (as part of the **ToxBank** customised ISAcreator distribution) and proposed a standard file format for processed data. In this proposed standard, each type of experiment (e.g., transcriptomics, proteomics, and so on) has a different file format. The file containing this processed data is uploaded as part of an ISA-Tab archive (containing the experimental design, raw data, and links to the protocols) and can be used in **ToxBank** to support precise searching (e.g. identify all investigations where a specific gene has a fold change greater than 1.5) as well as a consistent integrated analysis of the data over the entire cluster. This standardisation also supports effective integration with data analysis, data mining and model building applications.

Data Warehouse Support Facilities

ToxBank has been supporting the preparation and upload of protocols and data into the data warehouse. To support the upload and use of the ToxBank Data Warehouse, a series of on-line tutorials have been generated. These include lectures on background material such as bioinformatics and tutorials to support the formatting of data, the upload of protocols, reports, and data as well as how to search, analyse and download information from the data warehouse. These tutorials are available through the *toxbank.net* website.

Operation and Support of ToxBank Warehouse during the SEURAT-1 Research Initiative and Beyond

An interface to the COSMOS database via an API was developed and implemented in addition to approaches to integrate information on external resources with **SEURAT-1** generated information. One of these projects was to integrate the ToxCast and Tox21 data (see section 5.2.2) with the **SEURAT-1** data to support a meta-analysis of the combined information.

The TBDW has been organised to provide a sustainable service for toxicological research beyond the lifetime of the research projects and resources set-aside such that the public reference data will be available for at least five years after the completion of **SEURAT-1** as an OpenTox resource.

Scientific Coordination and Interaction with other Related Activities

A number of tools have been adopted to support this task: Mantis, ToxBank wiki web-pages, Google Docs and the Jenkins integration server. Weekly technology focused meetings were held to discuss the ToxBank technical progress and the development and supported through ToxBank wiki web-pages. The web-pages developed outlining the APIs are made available publicly for other developers to build interfaces using the OpenTox/ToxBank web services.

4.7.3.2 Compound Database

Selection of ToxBank Gold Compounds

ToxBank was tasked to create a quality-controlled curated cheminformatics database for standard reference compounds ('gold compounds') that can be used in the training and validation of *in vitro* assays and *in silico* models. Gold compound selection criteria and standardised curation and operating procedures were established in order to support the earliest possible creation of the database and its use in decision making on project and assay design across the **SEURAT-1** research programme. The **SEURAT-1** Research Initiative addressed hepatic, cardiac, renal, neuronal, muscle, and skin toxicities, with the largest effort directed towards hepatotoxicity. We therefore selected standards that are relevant to these pathologies, with the addition of specifically representing fibrosis with respect to cytotoxicity.

The **SEURAT-1** research strategy encompasses 'any substance' (see section 3.1). Thus, the reference compounds must cover a breadth of chemical classes that include cosmetic ingredients, agricultural and industrial chemicals, and pollutants, among others. Therefore the core criterion for acceptance was established to be a promiscuous mechanism of toxicity. While 'mechanism' may refer to several possible aspects of the overall mode of action of a toxicant, compound selection was based narrowly on the molecular initiating event for the adverse outcome pathway. Promiscuity refers to lack of structural specificity in ligand binding, where the specificity may relate either to ligand or receptor structure. The concept derives from the observation that small, hydrophobic ligands tend to have binding affinity for multiple different proteins, and conversely, receptors with large hydrophobic pockets tend to bind multiple diverse ligand structures. Promiscuity is clearly relevant to a strategy that must span a broad chemical space. The following were identified as basic promiscuous mechanisms of toxicity: alkylation, membrane disruption, and binding to (non-selective) nuclear hormone receptors. Additional standards were selected to represent especially well-characterised initiating events for the targeted pathologies.

General criteria such as stability, solubility, and availability were added to the mechanism-related criteria. The selection criteria are available at the ToxBank Gold Compounds Wiki site: *http://wiki.toxbank.net/w/index.php/Selection_Criteria*. Initial criteria also comprised non-idiosyncratic toxicity and relevance to repeated dose toxicity, but these criteria proved difficult

to relate to objective standards of selection. There is no agreed frequency of occurrence that defines 'non-idiosyncratic', for example; and these criteria had only secondary impact.

In the original **ToxBank** conception, identification of the gold compounds was to be accomplished via computational data mining. Once the **SEURAT-1** Research Initiative was constituted, however, it became apparent that only a small number of reference compounds was required and that manual selection and extensive curation were preferred. An initial set of 24 compounds was selected and published in 2012 along with information about chemical identities, adverse effects, toxicity mechanisms and therapeutic targets. This compound set completed the selection of hepatotoxin reference standards. The list was formally approved at the 2013 annual meeting. In 2013, the full compound set was completed by the addition of the full list of renal-, cardio- and neurotoxin standards. The complete list of compounds is available at *http://wiki.toxbank.net/wiki/CompoundSummaryTable*. Data associated with the Gold Compounds, especially the hepatotoxins, comprised in-depth textual analysis of the literature on mechanism of action (*Jennings et al., 2014*).

Selection criteria for the chemical standards included an evaluation of the highest purity available on the market, the reliability of information and traceability, suitability for cell culture application, ease of access and shipment availability in the different countries involved, and lot size and price. Standard compound suppliers and product numbers were provided to ensure that all labs were using a common compound source. Information on the stability of the chemicals was collected from the literature, and additional calculated biodegradation and metabolism properties were provided as a component of a data table that was constructed for each compound.

Standard Operating Procedures for Data Quality Control, Acceptance, Processing and Analyses of ToxBank Gold Compounds

The compound selection strategy evolved in consultation across the **SEURAT-1** Research Initiative. Each project team, the Scientific Expert Panel (SEP), and Colipa (now Cosmetics Europe) provided representatives at a kick-off meeting in Cascais, Portugal in February, 2011. An advisory Gold Compound Working Group with 18 members was assembled from the attendees at the Cascais meeting, and an evaluation team of 10 scientists was assembled from the SEP, industry, and academic labs to serve as the working Gold Compound Selection Team to evaluate specific compounds for acceptance (see also section 4.9.5). As a matter of process, it was agreed that compounds recommended as standards require unanimous agreement by the evaluation team and would be submitted to the working group for review and comment before being accepted as Gold Compound standards.

The selection strategy that emerged from the Cascais meeting was defined with respect to adverse events such as steatosis and cholestasis. The subsequent evolution to an MoA-based

approach was endorsed by the SEP and developed in a series of monthly teleconferences with the Gold Compound Working Group starting in early August 2011. Explicit consideration of repeated dose toxicity was initiated at a meeting of experts organised by COACH in Ispra and a subsequent meeting with the **SEURAT-1** cardiotoxicity team in Cologne in November of 2011.

The final compound list was formally submitted to the Scientific Expert Panel and accepted in February of 2013. Compound selection efforts culminated in 2014 with the publication of a peer-reviewed review of the hepatotoxin reference compounds (*Jennings et al., 2014*). This review assimilated information from the ToxBank Wiki data tables for individual compounds into an integrated discussion of mechanisms of toxicity.

Establishment of the Database, Procedures and Analytical Tools

Although it was originally envisaged that the Gold Compound Database would comprise a relational database, it was agreed with the Gold Compound Working Group in 2011 that a textual discussion of the rationale for selection of the compounds and their relevance to assay validation was preferred. Accordingly, a standard table of acceptance criteria was established and the data supporting compound acceptance was published as the ToxBank Gold Compound Wiki *(http://wiki.toxbank.net)*. The wiki format was selected because it supported incremental, rapid publication of compound data tables as they were developed and approved by the working group. This wiki ultimately became the primary repository for an extensively curated compilation of reference information on each Gold Compound. Data associated with the Gold Compounds that was generated internally by the **SEURAT-1** Research Initiative was then collected within the ToxBank Data Warehouse as described in the previous section 4.7.3.1.

The **ToxBank** data mining and analytical capabilities were directed to two outcomes. For the first outcome, efforts concentrated on supplementing the literature data in the Gold Compound Wiki with computed properties for the compounds. These properties were primarily physical properties such as solubility and predictions of elements of pharmacokinetics and can be accessed at the wiki. For the second outcome, efforts concentrated on integrating the ToxBank Gold Compound Warehouse with public databases and demonstrating proof of principle for mining the linked data sources. These capabilities were then applied to developing methods for computationally relating chemical structure to mechanism of action in order to support data mining efforts that address this demand.

In order to support development of data mining tools, the Gold Compounds were associated with external mineable database collections in 2013, including the well-known TG-Gates and assay data from PubChem as well as the COSMOS database of the **SEURAT-1** Research Initiative (see section 4.5.3.1). Application of analytical methods for read across, enriched meta-analysis of multiple '-omics' and functional data, background knowledge from GO

ontologies and Kegg pathways, and pathway visualisation for **SEURAT-1** Gold Compounds was demonstrated.

In 2014, links were extended to the Munro (2006) database of LO(A)EL and NO(A)EL data and HESS (Hazard Evaluation Support System) database from the OECD QSAR Toolbox and the EPA's Integrated Risk Information System (IRIS) database. Tools were then created to mine these databases to identify compounds chemically similar to the Gold Compounds, retrieve biological data for these compounds, and then predict biological targets for new compounds of interest, for example, in read across predictions and adverse outcome pathway predictions. These tools and proof-of-principle for their employment were exemplified by analysis of ToxCast/Tox21 Phase II data for potential hepatotoxicity and analysis of the databases mentioned above.

A summary of the ToxBank Gold Compounds and associated datasets is provided in *Table 4.4*. In addition to **SEURAT-1** datasets, discussions with the US EPA during the programme resulted in the additional generation of ToxCast and Tox21 assay datasets, which were incorporated into **ToxBank**.

Table 4.4 *Summary of* ***SEURAT-1*** *Gold Compounds and associated data.*

Adverse effect	CAS	SEURAT-1 Gold compounds	ToxBank	ToxCast	Tox21
Hepatotoxins	103-90-2	Acetaminophen	Yes	ToxCast - 1000 [ph1 + ph2], ToxCast - 1800 [ph1 + ph2 + e1k]	Yes
	144-48-9	Iodoacetamide	Yes	No	Yes
	107-18-6	Allyl alcohol	Yes	ToxCast - 1800 [ph1 + ph2 + e1k]	Yes
	6956-96-3	DMNQ	Yes	No	No
	56-23-5	CCl4	Yes	No	Yes
	1162-65-8	Aflatoxin B1	Yes	No	No
	642-15-9	Antimycin A	No	No	No
	1404-19-9	Oligomycin A	Yes	No	No
	83-79-4	Rotenone	Yes	ToxCast - 1000 [ph1 + ph2], ToxCast - 1800 [ph1 + ph2 + e1k], ToxCast - 300 [ph1]	Yes
	370-86-5	FCCP	Yes	No	No
	99-66-1	Valproic Acid	Yes	ToxCast - 1000 [ph1 + ph2], ToxCast - 1800 [ph1 + ph2 + e1k]	Yes
	50-53-3	Chlorpromazine	Yes	No	Yes
	1951-25-3	Amiodarone	Yes	No	Yes
	59-05-2	Methotrexate	Yes	ToxCast - 1000 [ph1 + ph2], ToxCast - 1800 [ph1 + ph2 + e1k]	Yes
	147536-97-8	Bosentan	Yes	No	Yes
	481658-94-0	Dirlotapide	Yes	No	No
	54910-89-3	Fluoxetine	Yes	No	Yes
	31282-04-9	Hygromycin B	Yes	No	Yes
	293754-55-9	TO901317	Yes	No	No
	13292-46-1	Rifampicin	Yes	ToxCast - 1000 [ph1 + ph2], ToxCast - 1800 [ph1 + ph2 + e1k]	Yes
	50892-23-4	WY14643	No	ToxCast - 1000 [ph1 + ph2], ToxCast - 1800 [ph1 + ph2 + e1k]	Yes
	6051-87-2	β-Naphthoflavone	No	No	Yes
	10540-29-1	Tamoxifen	Yes	ToxCast - 1000 [ph1 + ph2], ToxCast - 1800 [ph1 + ph2 + e1k]	Yes
Nephrotoxins	7758-01-2	KBrO3	Yes	No	No
	303-47-9	Ochratoxin A	Yes	No	No
Cardiotoxins	23214-92-8	Doxorubicin	Yes	No	No
	642-15-9	Antimycin A	No	No	No
	113558-89-7	E4031	Yes	No	No
	51-83-2	Carbachol	Yes	No	Yes
	7683-59-2	Isoproterenol	No	No	Yes
	21829-25-4	Nifedipine	No	No	Yes
Neurotoxins	18228-17-6	Naphthol AS-E Phosphate	No	No	No
	66575-29-9	Forskolin	No	ToxCast - 1800 [ph1 + ph2 + e1k]	Yes
	208255-80-5	DAPT	No	No	No
	53123-88-9	Rapamycin	No	No	Yes
	1227911-45-6	GSK2334470	No	No	No
	?	Akt 1/2 inhibitor	No	?	?
	31430-18-9	Nocodazole	No	No	Yes
	109511-58-2	U0126	No	No	No
	79-06-1	Acrylamide	No	ToxCast - 1000 [ph1 + ph2], ToxCast - 1800 [ph1 + ph2 + e1k]	Yes
	2078-54-8	Propofol	No	ToxCast - 1800 [ph1 + ph2 + e1k]	Yes
	7758-95-4	Lead(II) Chloride	No	No	No
	2921-88-2	Chlorpyrifos	No	ToxCast - 1000 [ph1 + ph2], ToxCast - 1800 [ph1 + ph2 + e1k]	Yes
	333-41-5	Diazinon	No	ToxCast - 1000 [ph1 + ph2], ToxCast - 1800 [ph1 + ph2 + e1k], ToxCast - 300 [ph1]	Yes
	60-57-1	Dieldrin	No	ToxCast - 1000 [ph1 + ph2], ToxCast - 1800 [ph1 + ph2 + e1k]	Yes
	?	Ni^{2+}	No	No	Yes
	4342-36-3 | 1461-22-9 | 2155-70-6	Tributyltin (TBT)	No	ToxCast - 1000 [ph1 + ph2], ToxCast - 1800 [ph1 + ph2 + e1k]	Yes
	1066-45-1	Trimethyltin (TMT)	No	No	No
	35065-27-1	PCB 153	No	No	Yes
	52663-68-0	PCB 180	No	No	Yes
	1499-55-4	Glutamate	No	No	Yes
Negative Control	69-65-8	*D*-Mannitol	No	ToxCast - 1000 [ph1 + ph2], ToxCast - 1800 [ph1 + ph2 + e1k]	Yes
			ToxBank Wiki	ToxCast	Tox21
SUMMARY		YES	25	16	33
		NO	27	36	19

4.7.3.3 Compound Repository

Physical Collection of Test Chemicals

The main objectives were the establishment of a physical repository of the test chemicals used, and the detailed characterisation of the chemical structure and relevant physico-chemical properties (including predictions on stability and binding properties) of the chemicals used within the **SEURAT-1** Research Initiative. We developed analytical methods for critical unstable test compounds, and the stability measurement under experimental conditions of doxorubicin, tamoxifen, amiodarone, bosentan, chloropromazine, valproic acid (and its liver metabolites), methotrexate and piperonyl butoxide. Particular attention was given to the measurement of the actual concentration of the substances within the *in vitro* assays at different time points. The measurement was made by LC-MS/MS in order to determine their stability during *in vitro* testing, at different time points. Furthermore, activities aimed to direct the development of standard operating procedures (SOPs) for the test protocols and education on Good Chemical and Cell Culture Practice.

A physical repository for test chemicals used within the **SEURAT-1** Research Initiative was established as a dedicated freezer at Mario Negri Institute, Italy, who held the responsibility for its maintenance and the associated chemical analysis facilities. Protocol guidelines were developed and uploaded in the ToxBank Data Warehouse.

Quality Assurance Framework for Data Inclusion on Test Chemicals

A quality assurance framework for data inclusion on test chemicals to be used within the **SEURAT-1** Research Initiative was defined. This work was strictly related to the extensive assessment performed for the selection of Gold Compounds for testing (see previous section 4.7.3.2), since data on physico-chemical properties, purity and stability were gathered for the evaluation of the suitability of the standards in term of physico-chemical behaviour in test conditions.

The collected properties were those required for the characterisation of the Gold Compounds: (i) testing within the US EPA ToxCast/Tox21; (ii) structure and isomeric form; (iii) stability to storage, light, freeze thaw; (iv) buffers and water solubility; (v) dimethylsulphoxide solubility; (vi) binding to plastic; (vii) availability commercially with highest purity; (viii) volatility.

The sources which were considered reliable and used were: (i) scientific literature; (ii) official databases; (iii) material certification; (iv) information sheets; and (v) specifications provided by the standard's suppliers and US EPA. For data confirmation, multiple sources were considered and compared contemporaneously, whenever available. For particular properties, such as water solubility and volatility, if data were not available, they were calculated through commercially and freely available software for modelling. The used software programs were

ACD Labs (Advanced Chemistry Development, Inc., Canada) for the calculation of solubility and Episuite (EPA, USA) for the calculation of vapour pressure for volatility evaluation. Quality principles were established and followed in order to guarantee the reliability and completeness of the information collected.

All the data were reported in a standardised form (scientific unit, conditions, etc.) along with specifications on how the data were generated (testing parameters and conditions, calculation methods) and with references.

Chemical Handling and Data Storing

The procedures for chemical handling during *in vitro* testing are strictly related to the specific test conditions (e.g. solvent, pH, temperature, duration, reactive conditions, other factors) and then to standard operating procedures (SOPs) which describe how an experiment is performed. SOPs were developed within the research projects of the **SEURAT-1** Research Initiative and published and shared through the ToxBank Data Warehouse. The entry of the relevant physico-chemical information on already selected test chemicals in the Data Warehouse was achieved through the organisation of these in a wiki resource, which was linked to the ToxBank Data Warehouse *(http://wiki.toxbank.net)*. The data are reported in the 'Physical properties' section of each compound's table in the wiki. For each chemical all the properties collected for the evaluation of the criteria defined above are reported, including source, structure, purity and specification of the isomeric form. Instructions on the correct handling of chemicals was transmitted through the indications reported in the 'Stability' part of the 'Physical properties' and in the 'Storage' part of the 'Recommended product and source' section on the wiki, where there is available specific information on the stability to light, storage, freeze-thaw, pH sensibility, etc.

Analytical Methods

The development of analytical methods for critical, unstable test chemicals and the following measurement of stability properties of test chemicals were performed to determine the concentrations of tamoxifen, amiodarone, bosentan, chloropromazine, valproic acid and its liver metabolites, methotrexate and piperonyl butoxide present in cell culture medium after different treatment times and in controls. A specific analytical method based on acetonitrile extraction followed by centrifugation was developed and optimised for the extraction of each chemical from cell culture medium and the subsequent determination was performed by electrospray ionisation liquid chromatography tandem mass spectrometry. Additionally, a chromatographic based method for the quantification of doxorubicin was developed on tandem mass spectrometry.

Stability and Binding: Measurements and Calculations

The developed LC-MS/MS analytical methods mentioned above were applied upon request by the **SEURAT-1** research projects to determine concentrations of the chemicals listed above in cell medium samples from acute and long-term toxicity studies. Upon request from the NOTOX Project (section 4.6) a particular focus was on HepaRG cells. The analysis was performed both on medium incubated with cells and without cells to estimate the stability of the chemical in cell medium under experimental conditions and to evaluate cellular uptake. Furthermore the stock solutions of the chemical in cell medium maintained in the fridge were analysed. Valproic acid concentrations were also measured in samples from different cultivation conditions: 2D and 3D HepaRG cultures; methotrexate and piperonyl butoxide was measured in samples from 3D HepaRG cultures. The measured concentrations were compared with the nominal concentrations and the partial loss of the active compound was related to different processes such as adsorption to containers, degradation, evaporation and absorption into cells.

Cells were exposed to the chemicals dissolved in the culture medium for acute and long-term (28 days) toxicity experiments, in which the medium was renewed every 48 h. Cells were treated with concentration in the micromolar range except for valproic acid which was tested in millimolar concentrations. After 48 h of incubation at 37°C, supernatants were collected in triplicates into 0.5 ml plastic reaction tubes and centrifuged for 10 minutes at 13k rpm (4°C). They were then transferred into glass vials with micro inlets and frozen at -20°C for storage. Before shipment from the NOTOX partner to the **ToxBank** partner, the samples were further thawed and aliquots were prepared. The samples for time points for days 6, 14, 20 and 28 were analysed for chemical quantification, except for methotrexate and piperonyl butoxide where the experiment was a long-term (21 days) toxicity experiment on 3D HepaRG cultures. Supernatant and cells were transferred in ice-cold plastic tube and then the separation of supernatants from cells was obtained by sedimentation and consecutive relocation of supernatant. The calculated limits of detections for the developed analytical method were 0.001 ng/µl for tamoxifen, 1 nM for amiodarone and bosentan, 8 nM for chlorpromazine, 0.2 µM for valproic acid, 0.021 µM for piperonyl butoxide and 0.0042 µM for methotrexate.

Results showed that the analysed compounds had different stability in the experimental conditions examined. Amiodarone was detected only in the acute toxicity test samples, when the tested concentration was higher than 10 µM, the measured concentrations were 1000 times lower than expected. The critical stability of amiodarone is probably related to its low solubility and binding properties towards plastic materials. Chlorpromazine was measured 3 to 27 times lower than the expected concentrations in the cell medium incubated for the long-term toxicity experiments without cells. Bosentan measured in cell medium incubated for the long-term toxicity experiments without cells was about 50-70% of the expected concentration. Valproic acid was measured nearly at the expected concentrations. Methotrexate level was about 50-70% and the piperonyl butoxide was calculated 2-5% due to solubility problems and

binding properties toward plastics. The protocols for extraction and LC-MS/MS analysis of the chemicals were uploaded on the ToxBank Data Warehouse.

In summary, the following tasks were achieved:

- Development of LC-MS/MS methods for doxorubicin, tamoxifen, amiodarone, bosentan, chlorpromazine, valproic acid and its metabolites, methotrexate and piperonyl butoxide.
- Determination of the actual concentration of amiodarone, bosentan, chlorpromazine, tamoxifen, valproic acid and its metabolites, methotrexate and piperonyl butoxide in cell medium samples from acute and long-term toxicity studies on HepaRG cells (overall more than 1,000 samples measured).
- Upload of developed protocol guidelines developed in the ToxBank Data Warehouse.

In addition to the experimental approach, the calculation of biodegradation and metabolism properties was performed with the following software: Episuite (EPA, USA), Topkat (Accelrys, USA), MetabolExpert (CompuDrug, USA). These software tools allow the estimation of several properties related to stability: atmospheric oxidation, Henry's law constant, melting point, boiling point, aerobic and anaerobic biodegradability, aqueous hydrolysis rate constant and half-lives (only for particular classes of chemicals), metabolites which may be formed in humans, animals or through photodegradation. These results contributed to the complete characterisation of the Gold Compounds and to the evaluation of possible problems of stability in specific test conditions. The calculations on binding properties were performed as well for the selected Gold Compounds.

Sustainability Planning

The repository of the substances will be maintained for 5 years. This is not demanding and critical, since it simply requires keeping the freezer at the Mario Negri Institute on. The chemicals will be possibly available for other projects, such as, in particular, EU-ToxRisk. Similarly, the methodologies for the analyses of the chemicals will be available within others projects.

4.7.3.4 Cell and Tissue Bank

Materials Requirements

The Biomaterials requirements for **ToxBank** were gathered as a cross-partnership collaboration following an initial scoping meeting (Milan, 2012) and subsequently through a combination of e-mail, phone calls, web-based surveying and participation in the ToxBank Knowledge Café

laboratory visits on user requirements. The developing materials requirements were further updated and coordinated at **ToxBank** partner meetings organised around the annual meetings of the **SEURAT-1** Research Initiative. This included fundamental criteria for acceptable characteristics of hPSC lines, which were established in a collaboration with the **SEURAT-1** project *SCR&Tox* (see section 4.4; *Pistollato et al., 2012*). Initiatives coordinating output from relevant national, regional or European activities (e.g. UK Stem Cells for Safer Medicine, California CIRM, International Stem Cell Banking Initiative) were also engaged in this process.

Suppliers (Biomaterials) Virtual Network and Quality Assurance Framework

This goal was addressed by reviewing the general resources available in Europe and identifying those with special relevance to *in vitro* systemic toxicology and the detailed user requirements. Based on information provided by suppliers and users, an evaluation procedure was set up to produce a web-based registry of suppliers that builds on existing registries focused on the central EC-funded database of hPSC lines hESCreg *(http://www.hpscreg.eu/)*.

Key quality criteria on which to base evaluation of suppliers and in addition provide advice on best practice for **SEURAT-1** partners and ultimately to the broader toxicology field were identified. These were based on key user criteria established in the earlier process of establishing biomaterials requirements. An additional and important development was an active collaboration with the **SEURAT-1** project *SCR&Tox* in the context of coordinating the **SEURAT-1** 'Stem Cell Working Group' (see section 4.9.9) incorporating input from ECVAM. These interactions led to joint poster presentations at annual meetings of the **SEURAT-1** Research Initiative, peer reviewed publications (*Pistollato et al 2012; Stacey et al., 2016*) and reports and guidance documents now available on the public ToxBank wiki. These dealt with accessing suitable human tissues and cell lines and key quality criteria for establishment and use of hPSC lines under Good Cell Culture Practice. Critical issues to be addressed to enable compliance with legal, ethical and commercial requirements for use of stem cell lines in the **SEURAT-1** Research Initiative and ongoing industry utility were identified and involved the completion of an ethics review questionnaire developed in collaboration with partners in *SCR&Tox*.

Inventory of Suitable Cell Culture Passaging Facilities and Procedures

An evaluation process was created to invite feedback from suppliers of stem cell lines and other key reagents, indicating how they meet the quality criteria established for biomaterials. This was developed to provide researchers with the ability to evaluate suppliers based on a standardised assessment based on information gathered using a standard questionnaire.

Securing satisfactory information from suppliers was a significant challenge and less than

50% of those suppliers contacted provided a response, or were able to provide sufficient information to use in the evaluation. All suppliers providing satisfactory responses were listed in a registry with details from their websites and their authorised response to the evaluation process. These details included any quality standards or regulations under which they are inspected or to which they proposed to be compliant. A formal review and a registry of these suppliers was compiled and published. These criteria were then utilised in the final registry of suppliers and a map with hyperlinks has also been developed for international suppliers of stem cell lines. Whilst not identified as a formal commitment, this activity has been extended to address the availability of biomaterials from outside Europe to identify international players in the biomaterials field with a special focus on human pluripotent stem cell. The template questionnaire was published on the ToxBank wiki and researchers can use it for ongoing selection of biomaterials.

Operations Beyond the Period of the Project, Maintenance of **ToxBank** Services, Security Updates and Sustainability Planning

Ongoing supply of materials could be delivered through a number of business models. A business case and service model were developed based on an options appraisal which concluded that a sustainable online Cell and Tissue Bank for provision of access to research materials and reagents for *in vitro* systemic toxicology was possible. An estimate for the investment required to achieve this was also conducted.

A significant virtual network engaging a range of stakeholder groups was developed and an outline for a supply network for materials for European *in vitro* systemic toxicology is now available through the ToxBank wiki *(http://www.toxbank.net/)*. These resources can be used to coordinate procurement of suitable research biomaterials and reagents for *in vitro* systemic toxicology procedures using stem cell lines which has ready capacity for expansion as a primary access conduit for users via a directory of suppliers.

At project-end the security of the **ToxBank** services were brought into state of the art condition. Secure communication between **ToxBank** web services and web clients are handled by HTTPS technology. HTTPS is a communication over HyperText Transfer Protocol within an encrypted connection by SSL or TLS. SSL, and its successor TLS, are cryptographic protocols to provide secure communication over the internet. In the current server setup outdated and unsecure SSL protocols in version 1 to 3 are blocked and only newer TLS protocols 1.0, 1.1 and 1.2 are in use. TLS do support a large number of ‘cipher suites’. Cipher suites are collections of symmetric and asymmetric encryption algorithms used by hosts to establish secure communication. Some offer better level of security than others. At the start of the connection between a web client and a web service the client sends a list of possible ciphers to the webserver. The webserver replies with the cipher suite that it has selected from this list. The ToxBank Data Warehouse server allows the use of a collection of ‘latest’ cypher

suites that offers a good balance between security and the possiblity to connect with a variety of current web clients. The web server's configration was adapted and tested with the free online SSL test service from Qallys SSL Labs at *https://www.ssllabs.com*. The web services operating systems were upgraded to newest versions (Debian 8 with support until May 2018/ May 2020 LTS) to ensure secure and 'easy to update' operating system over the end of the project.

The current on-line facility is accessible at the **ToxBank** website *(http://www.toxbank.net/)* where the resources described above are located with other elements of **ToxBank**. This system has a direct link with hPSCreg *(http://www.hpscreg.eu)* for access to scientific and ethics data on stem cell lines. **ToxBank** partners are also partners in the IMI EBiSC project (European bank of iPSC lines), where they lead the development of the information management system, which in turn enabled ongoing coordination and interoperability with **ToxBank** features and resources.

4.7.4 Cross-Cluster Cooperation

Right at the start of **SEURAT-1** Research Initiative, the first two **SEURAT-1** cross-cluster working groups were established by **ToxBank**: the Data Analysis Working Group (DAWG) and the Gold Compound Working Group (GCWG). DAWG meetings and communications discussed the expected data analysis requirements of the cluster. The GCWG meetings were held to finalise the list of standard reference compounds to be used in the **SEURAT-1** case studies. Furthermore, **ToxBank** together with the *SCR&Tox* Project took the lead in the Stem Cell Working Group, which gathered **SEURAT-1** partners together to discuss key issues of quality control for stem cell lines, formulated suitability criteria for cell lines used in **SEURAT-1** workplans and published a consensus, with a number of **SEURAT-1** partners, on quality assurance of hPSC lines and the development of stem cell-based toxicology assays (*Pistollato et al., 2012; Stacey et al., 2016*). The cross-cluster working group approach proved particularly successful and was adopted by COACH and expanded into other areas during 2012 as a key organisational structure for cluster activities (see also section 4.9.4). The working group activities provided valuable background information and interactions that aided the development of the warehouse design.

ToxBank has continued to collaborate with DETECTIVE, NOTOX, *HeMiBio*, and *SCR&Tox* to create ISA-tab formatted investigations and protocols to upload into the ToxBank Data Warehouse as well on the analysis of the data. A close collaboration was set up with the NOTOX Project regarding the analyses of standard reference compounds in 2D and §D HepaRG cultures (see above). **ToxBank** has also collaborated with COSMOS to provide access to the COSMOS data. A single structure search from the ToxBank Data Warehouse will return matching chemicals with integrated records that have been uploaded to **ToxBank** alongside COSMOS database records.

4.7.5 Potential Impact

We have uploaded numerous protocols, reports and datasets from the activities of the **SEURAT-1** Research Initiative. We have agreed as a consortium to maintain all **ToxBank** reference information as a public OpenTox resource for a minimum of 5 years beyond the end of the project, providing the scientific community access to the results of **SEURAT-1** at no charge for access. OpenTox was initially an FP7 project, but importantly has developed as a community around open resources and standards, and in 2015 was formed as an international member-based non-profit organisation (http://www.opentox.net/the-opentox-association).

During 2016 we continued to develop and extend case studies around the **ToxBank** resources in collaboration with COACH (see section 4.8) and the European Commissions Joint Research Centre in Ispra, Italy. Part of the **ToxBank** consortium is also engaged in the new EU-ToxRisk programme forming a bridge between **SEURAT-1** results and their extensions (see section 5.4). We continue to communicate with **SEURAT-1** coordinators and data owners throughout the final reporting and review process, and beyond. Data quality has been assured by continuing to support the best practices on data preparation as reported extensively in previous **ToxBank** tutorials (http://www.toxbank.net).

To promote wider access, public registration and access to **ToxBank** is supported, and will continue to be supported throughout the next five years. We also plan to further develop the **ToxBank** resource as an important key reference resource interoperating with the growing set of OpenTox resources on data, algorithms, modelling, and analysis and visualisation components.

As data owners provide public access to their datasets and results, we will promote their availability to users registered on **ToxBank**, and more broadly to the scientific and general public.

Project-related Publications from the ToxBank Consortium

Gadaleta, D., Pizzo, F., Lombardo, A., Carotti, A., Escher, S.E., Nicolotti, O., Benfenati, E. (2014): A k-NN algorithm for predicting the oral sub-chronic toxicity in the rat. *ALTEX*, 31: 423-432.

Grafström, R.C., Nymark, P., Hongisto, V., Spjuth, O., Ceder, R., Willighagen, E., Hardy, B., Kaski, S., Kohonen, P. (2015): Toward the teplacement of animal experiments through the bioinformatics-driven analysis of 'omics' data from human cell cultures. *Altern. Lab. Anim.*, 43: 325-332.

Hardy, B., Apic, G., Carthew, P., Clark, D., Cook, D., Dix, I., Escher, S., Hastings, J., Heard, D.J., Jeliazkova, N., Judson, P., Matis-Mitchell, S., Mitic, D., Myatt, G., Shah, I., Spjuth, O., Tcheremenskaia, O., Toldo, L. (2012): Toxicology ontology perspectives. *ALTEX*, 29: 139-156.

Hardy, B., Apic, G., Carthew, P., Clark, D., Cook, D., Dix, I., Escher, S., Hastings, J., Heard, D.J., Jeliazkova, N., Judson, P., Matis-Mitchell, S., Mitic, D., Myatt, G., Shah, I., Spjuth, O., Tcheremenskaia, O., Toldo, L. (2012): Food for thought … A toxicology ontology roadmap. *ALTEX,* 29: 129-137.

Jennings, P., Schwarz, M., Landesmann, B., Maggioni, S., Goumenou, M., Bower, D., Leonard, M.O., Wiseman, J.S. (2014): SEURAT-1 liver gold reference compounds: a mechanism-based review. *Arch. Toxicol.*, 88: 2099-2133.

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Kohonen, P., Ceder, R., Smit, I., Hongisto, V., Myatt, G., Hardy, B., Spjuth, O., Grafström, R. (2014): Cancer biology, toxicology and alternative methods development go hand-in-hand. *Basic Clin. Pharmacol. Toxicol.*, 115: 50-58.

Maunz, A., Gütlein, M., Rautenberg, M., Vorgrimmler, D., Gebele, D., Helma, C. (2013): Lazar: A Modular Predictive Toxicology Framework. *Frontiers Pharmacol.,* 4: Article 38 (published 9 April 2013).

Pistollato, F., Bremer-Hoffmann, S., Healy, L., Young, L., Stacey, G. (2012): Standardization of pluripotent stem cell cultures for toxicity testing. *Expert Opin. Drug Metab. Toxicol.*, 8: 239-257.

Pizzo, F., Gadaleta, D., Lombardo, A., Nicolotti, O., Benfenati, E. (2015): Identification of structural alerts for liver and kidney toxicity using repeated dose toxicity data. *Chem. Cent. J.*, 9: 62.

Stacey, G.N., Coecke, S., Bal-Price, A., Healy, L., Jennings, P., Wilmes, A., Pinset, C., Ingelman-Sundberg, M., Louisse, J., Haupt, S., Kidd, D., Robitzki, A., Jahnke, H.-G., Lemaitre, G., Myatt, G. (2016): Ensuring the Quality of Stem Cell-Derived *in vitro* Models for Toxicity Testing. *Adv. Exp. Med. Biol.,* 856: 259-297.

Toropov, A.A., Toropova, A.P., Pizzo, F., Lombardo, A., Gadaleta, D., Benfenati, E. (2015): CORAL: model for no observed adverse effect level (NOAEL). *Mol. Divers.*, 19: 563-575.

Awards

Pekka Kohonen and Roland Grafström (Karolinska Institute, Stockholm) received the 2014 Lush Science Prize

The Lush prize is an annual prize fund for researchers working in the alternatives to animal testing field, focusing on consumer products and ingredients, and is funded by Lush cosmetics in the UK, a company committed to the non-animal test methods for all of their products, and Ethical Consumer magazine.

Roland Grafström and Pekka Kohonen work at the Institute for Environmental Medicine, which is one of the largest research departments at the Karolinksa Institute in Stockholm, Sweden. They received the Lush Science Price for combining *in vitro* and *in silico* analyses in the context of the **ToxBank** Project. The award was made for notable contributions to the field of predictive toxicology focussing around efforts on the application, analysis, interpretation and storage of '-omics'-derived data.

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Introduction
Future
Context
Projects
Strategy

4.8 COACH: Coordination of Projects on New Approaches to Replace Current Repeated Dose Systemic Toxicity Testing of Cosmetics and Chemicals

COACH

Sara Skogsater, Bruno Cucinelli, Pierre-Antoine Legrix

4.8.1 Introduction

COACH is a coordination and support action of the FP7 HEALTH programme, which started on 1 January 2011, together with the six research projects of the **SEURAT-1** Research Initiative (presented in the previous sections). **COACH**, which will last until the end of 2016, is the only **SEURAT-1** project still ongoing as the six research projects finished on 31 December 2015. During this period, **COACH** will focus on the dissemination of results and handing over to new initiatives.

The main roles of **COACH** were to:

- Facilitate cluster-wide internal cooperation;
- Provide strategic guidance with the help of the Scientific Expert Panel (SEP);
- Prepare and distribute the **SEURAT-1** Annual Reports;
- Organise the **SEURAT-1** Annual Meetings, meetings of the SEP and workshops supporting cross-cluster activities and collaborations with external partners;
- Coordinate cluster-level dissemination and outreach activities.

COACH provided centralised scientific administration to the **SEURAT-1** Research Initiative (the '**COACH** Office'), organised cluster-level interactions and activities and was the main cluster-level entry point for all participants, including funding organisations such as the European Commission and Cosmetics Europe, as well as any external organisation looking to liaise with the **SEURAT-1** Research Initiative (*Figure 4.82*).

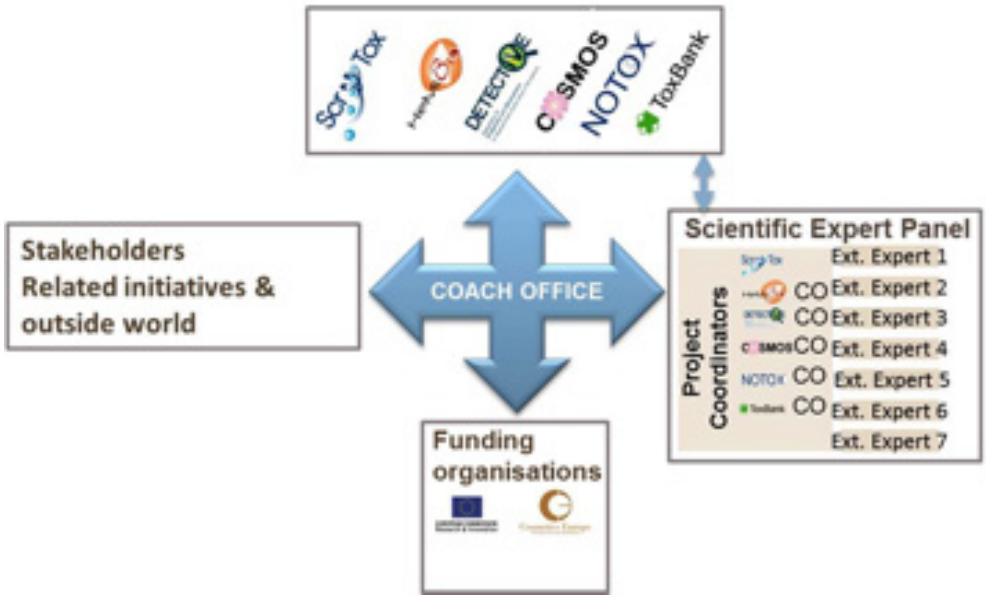

Figure 4.82 *The* ***COACH*** *Office as the central contact for cluster-level activities.*

The following sections highlight some important achievements of the first, second and early third periods (illustrated in *Figure 4.83* below).

Figure 4.83 *Main cluster-level achievements of the* ***SEURAT-1*** *Research Initiative since the launch of the initiative.*

4.8.2 Cluster-Level Coordination

As with any collaborative research initiative, the starting period for **SEURAT-1** was key to short- and long-term success. At the start of the collaboration, partners needed to establish the methods, means and common references that allowed them to organise the collaboration in the most efficient and productive manner. This was particularly important for **SEURAT-1** in the context of the simultaneous start of six individual research and development projects,

which formed a cluster of complementary research activities working towards a common aim. **COACH** played a key role in this specific context.

The scientific management and coordination of the **SEURAT-1** Research Initiative has been strongly supported by the Scientific Expert Panel (SEP), which has played a key role in providing scientific advice regarding the research and the future orientation of **SEURAT-1**. The SEP is comprised of the coordinators of the six cluster research projects plus six external experts. Details of the SEP members are given in *Table 1.1* in the Introduction of this Annual Report (see chapter 1).

Research Strategy, Strategic Review and Roadmap

The SEURAT vision and long-term research strategy were described in the first volume of the **SEURAT-1** Annual Report, issued in September 2011 (*Whelan & Schwarz, 2011*). The research strategy, adopted by the SEP in July 2011, was based on a discussion paper prepared by **COACH** partners at the University of Tübingen and Joint Research Centre. The strategy describes how the **SEURAT-1** Research Initiative aims to achieve the long-term target of replacing animal testing in human safety assessment, the overall research target of **SEURAT-1** and beyond.

In mid-2014, **COACH** partner the University of Tübingen encouraged creation of the so-called Strategic Group. Made up of six experts selected from SEP members, the Strategic Group aimed to prepare a strategy proposal for the next research activities following **SEURAT-1,** in line with the overall vision and long-term strategy elaborated by the initiative. In November 2014, the paper '*SEURAT: Safety Evaluation Ultimately Replacing Animal Testing – Recommendations for future research in the field of predictive toxicology*' was issued, published in *Archives of Toxicology* (*Daston et al., 2015*) and on the **SEURAT-1** website.

At the operational level, the SEP continued to monitor the cluster-level progress made by **SEURAT-1** towards its overall objectives via regular strategic reviews of **SEURAT-1**. The motivations for implementing this plan originally initiated by **COACH** were also to:

- Facilitate the engagement and advisory role of the SEP;
- Identify critical areas of project interaction;
- Establish a high-level roadmap indicating key milestones to serve as a basis for tracking progress;
- Provide analysis to aid strategic decision-making.

The strategic review process was prepared by **COACH** partner the Joint Research Centre (JRC) and consisted of two main components: (i) a SWOT analysis questionnaire as a practical tool to better understand how to benefit from strengths and opportunities and how to confront weaknesses and threats at the cluster level; and (ii) the development of a

roadmap for monitoring progress at the cluster level. The SWOT analysis was carried out as a brainstorming exercise by **COACH**, cluster coordinators, SEP members and Cosmetics Europe Advisory Board members. Feedback was collected and summarised, then further discussed by the SEP to identify actions that would improve cluster interactions and achieve a high-level outcome. This exercise was repeated annually and thus provided the SEP with a tool to understand whether improvement measures had been successful.

The cluster-level roadmap (as the second part of the strategic review) was prepared based on the following steps:

1. Identification of core topics of cross-cluster importance that are critical in achieving the **SEURAT-1** objectives;

2. Identification of the projects and project deliverables that are relevant for each topic;

3. Aggregation of the deliverables identified to determine high-level milestones that define the roadmap for each topic;

4. Assignment of the topics to dedicated working groups and a recommendation to organise workshops for formulating the cluster-level research questions.

The first strategic review carried out by **COACH,** with contributions by the project coordinators, was presented during the SEP meeting in June 2012. The presentation included a detailed description of the cluster-level objectives, the pooled results of the SWOT analysis, an analysis of cross-cluster interactions, and a preliminary outline of the **SEURAT-1** roadmap. The majority of SWOT analysis responses referred to 'strengths and weaknesses', while fewer responses referred to 'opportunities and threats'. Thus, in this first SWOT analysis, participants were apparently concerned with issues more of 'internal origin' than of 'external origin'. This inward-looking perspective is understandable considering that the questionnaire was circulated in the first years of **SEURAT-1**. The SEP identified and discussed areas within the cluster that needed more attention, and tried to find ways to benefit from strengths and tackle the problems arising from weaknesses. The SEP proposed possible solutions to these areas of concern and some additional activities were initiated. An update of the strategic review and the status of a more detailed roadmap based on the most recent contributions from the cluster coordinators were presented in a subsequent SEP meeting in November 2012, and the finalised second strategic review was presented at the SEP meeting in June 2013.

The third strategic review was presented and discussed at the SEP meeting in Paris in May 2014 and released in June 2014. Based on a comparison of the corresponding 2012 and 2013 results with the latest version, a clear change was observed in the SWOT exercise. It was agreed not to carry out a strategic review in 2015 but to focus on collecting highlights from the **SEURAT-1** projects for further dissemination, as the research project finalised their activities at the end of 2015.

Collaborations with Related Initiatives

Collaborating with related research initiatives and institutions in and outside Europe has been top of the **COACH** action lists since the start of **SEURAT-1**. Ties were established with many international actors, EPAA (The European Partnership for Alternative Approaches to Animal Testing), Tox21/ToxCast (research programmes of the U.S. Environmental Protection Agency) and ESTIV (European Society of Toxicology *In Vitro*). **COACH** also had interactions with the MIP-DILI initiative.

The second cluster training activity, called **SEURAT-1** & ESTIV Joint Summer School, also illustrated the role of **COACH** in supporting collaborations with related initiatives. The summer school in 2014, organised by **COACH** partner ARTTIC, took place in Amsterdam on 8-10 June. Thanks to the collaboration with ESTIV, it brought together *in vitro* and *in silico* toxicologists from many different countries, representing academia, industry and regulatory bodies and the young **SEURAT-1** scientists who showcased their work within **SEURAT-1**.

The **SEURAT-1** book (Annual Report) launch events represent an excellent opportunity to deepen the relationships with important initiatives and stakeholders and gain visibility for **SEURAT-1**. In 2012, the Annual Report was launched at EuroScience Open Forum (ESOF) 2012; in 2013, during the **SEURAT-1** & EPAA Stakeholders Event; and in 2014, during the 9th World Congress on Alternatives and Animal Use in the Life Sciences (WC9). The launch of the 5th Annual Report, built on this tradition, was organised at the EUROTOX 2015 conference in Porto, Portugal in September 2015. **COACH** coordinated the **SEURAT-1** corner, hosted at the Joint Research Centre booth, distributed **SEURAT-1** dissemination material (leaflets, posters, USB sticks, all volumes of the Annual Reports, etc.) and arranged associated activities such as interviews, short scientific sessions and demonstrations.

4.8.3 Facilitating Exchanges between SEURAT-1 Participants

The **SEURAT-1** Annual Meetings were the main face-to-face events gathering the cluster participants. The first two Annual Meetings (March 2011 and February 2012) were organised with a similar concept: (i) a plenary session involving a series of keynote speeches about important issues in alternative human safety testing international research, including progress made by the cluster projects; (ii) parallel working groups focusing on specific cross-cluster topics; and (iii) a panel discussion drawing conclusions from the discussions and providing a common view on future work orientations and priorities of the research initiative. The third and fourth Annual Meetings (March 2013 and February 2014) were organised differently, in order to adapt to the evolving cross-cluster cooperation needs of the initiative in the context of the **SEURAT-1** case studies. The fifth Annual Meeting (January 2015) showcased the impressive outcomes of the research project and initial results from the **SEURAT-1** proof-of-concept (PoC) case studies (see chapter 3). A non-exhaustive list of the tools and methods

for mechanism-based toxicology developed in the **SEURAT-1** projects was also presented in a dedicated session and it was agreed that all methods will be collected as a common outcome in the so called '**SEURAT-1** Tools & Methods Catalogue' (see section 4.9.3). The **SEURAT-1** Symposium, held in Brussels on 4 December 2015, aimed to present **SEURAT-1**'s main achievements. It gathered 120 researchers, regulators, EU officials and industry representatives in the alternative methods field. Section 4.10 provides further details on this public event.

Another important element of fostering collaborations between the scientists in the various research projects was the organisation and maintenance of cross-cluster working groups. Besides actively preparing workshops, **COACH** supported these working groups in organisational matters, organised teleconferences as required and set up mailing lists and dedicated workspaces for each working group on the private collaborative web platform, facilitating communication and collaboration among working group members. **COACH** partner the Joint Research Centre assisted in the coordination of the case studies for the **SEURAT-1** proof-of-concept exercises. In 2014, two workshops were organised in Ispra, setting the scene for the read-across case study (29-30 April) and the *ab initio* case study (9-10 October). At the read-across workshop, relevant experts, including from outside **SEURAT-1**, were invited to ensure a robust and well-informed basis for the project. The outcome of the workshop was also published in a review paper in *Environmental Health Perspectives* (*Berggren et al, 2015*). The activities of both case studies were followed up with workshops organised and chaired by the JRC in 2015. The read-across experts re-convened on 5-6 October, and the results discussed at that meeting are summarised in section 3.2. The Ab initio Working Group met to discuss how to further progress the case study 19 May 2015, and they agreed on the final *ab initio* workflow on 1-2 February 2016, again at the JRC facilities in Ispra. Both the read-across and *ab initio* case studies were presented at the final **SEURAT-1** Symposium in December 2015. In addition, they fed into the ECHA scientific topical workshop on New Approach Methodologies in Regulatory Science, held on 19-20 April 2016 in Helsinki, Finland (see section 2.3). In preparation for the ECHA workshop, two chemical read-across categories were worked out according to the assessment framework published by ECHA *(http://echa.europa.eu/documents/10162/13628/raaf_en.pdf)*, one by Cosmetics Europe and one by the JRC. This was done under the umbrella of the read-across case study and the results were discussed at the ECHA workshop. In both cases it was agreed that the data from alternative methods had increased confidence in the read-across.

Internal training encouraged contacts between younger scientists and facilitated the knowledge transfer developed within **SEURAT-1**. The training concept was homogenised by a special **SEURAT-1** training task force composed of representatives from each of the projects. In 2014, **COACH** organised the second cluster-level summer school (8-10 June), in close collaboration with the ESTIV2014 conference. The **SEURAT-1** part of the summer school covered mostly practical sessions (hands-on computer exercises, soft skills sessions,

discussions and workshops), while the ESTIV programme offered the participants scientific conferences and a career session.

4.8.4 Dissemination of Information

The **COACH** dissemination activities have evolved together with **SEURAT-1**, as each phase throughout has required specific attention. Since 2011, the activities have ranged from creating the **SEURAT-1** visual identity as a new player in the field of alternative testing and presenting the objectives of the Research Initiative to the entire scientific world, to showcasing the achievements for key stakeholders, the general public and other target groups via a number of channels. The following activities and tools were developed to support the dissemination as efficiently as possible:

- A consistent visual identity for **SEURAT-1** (logo, colours, layout of printed and electronic dissemination material, website appearance, etc.) was created at the outset of the initiative in collaboration with a professional design company;

- A variety of information dissemination support materials were created and distributed, including two leaflets, the second version containing an embedded USB stick; **SEURAT-1**, **COACH** and cluster roadmap posters; a 'who's who' booklet, which was distributed at each Annual Meeting (also available online); a standard PowerPoint presentation presenting the initiative and showcasing main results; unique roll-up banners illustrating the cluster composition and the conceptual framework, which were used during the **SEURAT-1** & ESTIV Joint Summer School, at the **SEURAT-1** corner at the 9th World Congress on Alternatives and Animal Use in the Life Sciences in Prague, Czech Republic, in August 2014, at the EUROTOX 2015 conference in September 2015 in Porto, Portugal and during the **SEURAT-1** Symposium on 4 December 2015; a booklet summarising a Guided Tour, which was developed for the final symposium of the **SEURAT-1** Research Initiative in Brussels on 4 December 2015. The tour was organised as a series of stations addressing the different steps required to answer a safety assessment question, and highlighted the use of non-animal testing tools and methods in this context;

- The public website *(www.seurat-1.eu)* is kept up to date. It presents the Research Initiative and its background and aims, the cluster projects and the involved partner organisations, and promotes research activities in the field of human safety assessment, particularly regarding alternatives to *in vivo* repeated dose systemic toxicity testing. Dedicated, regularly updated pages present related events, links, publications, job announcements, a press corner, etc.;

➠ The preparation of the **SEURAT-1** Annual Reports was coordinated by **COACH** partner the University of Tübingen, which proposed the content structure and specified the contributions required. For each report, the proposed structure and approach was reviewed and endorsed by the Scientific Expert Panel, which contributed actively to the writing and validation of the books' contents. The University of Tübingen collected, reviewed and edited the contributions, while ARTTIC took care of the book layout in collaboration with an appointed professional designer. Further to this well-established collaboration, the following Annual Reports were published and distributed in more than 1000 copies each:

- First Annual Report (*Schwarz & Gocht, 2011*) published in September 2011;
- Second Annual Report (*Gocht & Schwarz, 2012*) published in July 2012 and launched at the Euroscience Open Forum (ESOF) in July 2012;
- Third Annual Report (*Gocht & Schwarz, 2013*) published in July 2013 and launched at the **SEURAT-1** & EPAA Stakeholders Event in September 2013;
- Fourth Annual Report (*Gocht & Schwarz, 2014*) published in August 2014 and launched at the 9th World Congress in August 2014;
- The fifth Annual Report (*Gocht & Schwarz, 2015*), published in September 2015 and launched at the 51st Congress of the European Societies of Toxicology in September 2015 (EUROTOX 2015);
- This sixth and last Annual Report.

The Annual Reports were printed and distributed to individuals by post and at relevant conferences. The electronic versions of the Annual Reports are also made available for download from the **SEURAT-1** public website and distributed using USB sticks, thereby reaching even more of the target audience;

➠ A dedicated dissemination channel for the Annual Reports was created in the form of a mailing list, containing over 700 postal addresses of scientists, experts and stakeholders in **SEURAT-1** research results. It is regularly updated each year before the Annual Report's distribution;

➠ The **COACH** partners are aware of the importance of promoting the objectives, approach and progress of **SEURAT-1** at international conferences and workshops. Participation in such events with the aim of presenting the progress and main achievements has strongly contributed to increasing the visibility of the **SEURAT-1** Research Initiative in the scientific community. The

events with **SEURAT-1** contributions in the last year are listed in section 4.11.3;

➠ The excellent visibility of **SEURAT-1** and its recognition as the major European research initiative in the field of alternative human safety testing methods is the fruit of a dissemination plan prepared as a project internal working document by **COACH** in 2011. It defined the dissemination objectives and the appropriate means required to reach the targets, leading to a number of actions. In order to refocus and prioritise the dissemination strategy more towards the stakeholders of **SEURAT-1**, i.e. the industry, regulators, the public and policy- and opinion-makers, a paper describing an updated dissemination strategy was prepared by **COACH** and presented during the SEP meeting in 2013. Aiming to define dissemination objectives, means and channels to better target the stakeholder groups, and to establish a plan of appropriate concrete dissemination actions, this dissemination strategy was considered a living document that was reviewed in each SEP meeting;

➠ Associated with the above-mentioned dissemination strategy, a plan for communication in mass media was developed in 2014. The creation of the plan was initiated by the **COACH** partner ARTTIC and supported by the newly established Editorial Review Board containing representatives from the SEP, European Commission and Cosmetics Europe. Its objective was to reach out to public journals, radio, television and other media outlets to spread information about the **SEURAT-1** results (see section 4.11.6).

➠ A film highlighting the achievements of the **SEURAT-1** Research Initiative was produced as part of this dissemination strategy. This film was shown during the **SEURAT-1** Final Symposium (see section 4.10.4) and can be watched on YouTube *(https://www.youtube.com/watch?v=Ymzsh9p5pwM)*.

4.8.5 Final Steps

Preparation of the next phase towards the achievement of the SEURAT long-term goals:
In the last year of the project, **COACH** continued to stimulate and coordinate these tasks with the aim of handing over the **SEURAT-1** knowledge to future research initiative(s) in the most efficient way and thus bringing the achievement of the SEURAT long-term goals closer. EURL ECVAM, **SEURAT-1** and EU-ToxRisk co-organised a workshop on the regulatory use of alternative approaches in toxicology for the safety assessment of chemicals, which took place on 9-10 November 2016 at the Joint Research Centre in Ispra, Italy (see section 5.3).

Project-related Publications from the COACH Consortium

Bal-Price, A., Crofton, K.M., Sachana, M., Shafer, T.J., Behl, M., Forsby, A., Hargreaves, A., Landesmann, B., Lein, P.J., Louisse, J., Monnet-Tschudi, F., Paini, A., Rolaki, A., Schrattenholz, A., Suñol, C., van Thriel, C., Whelan, M., Fritsche, E. (2015): Putative adverse outcome pathways relevant to neurotoxicity. *Crit. Rev. Toxicol.,* 45: 83-91.

Berggren, E., Amcoff, P., Benigni, R., Blackburn, K., Carney, E., Cronin, M., Deluyker, H., Gautier, F., Judson, R.S., Kass, G.E.N., Keller, D., Knight, D., Lilienblum, W., Mahony, C., Rusyn, I., Schultz, T., Schwarz, M., Schüürmann, G., White, A., Burton, J., Lostia, A., Munn, S., Worth, A. (2015): Chemical safety assessment using read-across: assessing the use of novel testing methods to strengthen the evidence base for decision-making. *Environ. Health Perspect.,* 123: 1232-1240.

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Daston, G., Knight, D.J., Schwarz, M., Gocht, T., Thomas, R.S., Mahony, C., Whelan, M. (2015): SEURAT: Safety Evaluation Ultimately Replacing Animal Testing – Recommendations for future research in the field of predictive toxicology.- *Arch. Toxicol.*, 89: 15-23.

Gocht, T., Schwarz, M. [Eds.] (2012): Towards the Replacement of *in vivo* Repeated Dose Systemic Toxicity. Vol. 2: The Consolidation of the Research Strategy. 371 p., Paris (self-publishing).

Gocht, T., Schwarz, M. [Eds.] (2013): Towards the Replacement of *in vivo* Repeated Dose Systemic Toxicity. Vol. 3: Implementation of the Research Strategy. 397 p., Paris (self-publishing).

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4.9 Project and Cluster Activities

4.9.1 Project Meetings

Mark Cronin, Elmar Heinzle, Jürgen Hescheler, Marc Peschanski, Catherine Verfaillie

***SCR&Tox*:** The *SCR&Tox* Final Consortium Meeting took place in Paris, France on 4 December 2015.

HeMiBio: The final *HeMiBio* consortium meeting was combined with an International Symposium entitled 'Biology Meets Technology for Liver Toxicity Testing', which took place in Leuven, Belgium on 9 September 2015. The symposium highlighted the results obtained within the *HeMiBio* consortium, including the development and differentiation of iPSC reporter lines and hepatic differentiation protocols, microfluidic bioreactor design and construction and development of sensors capable of evaluating hepatocyte function in repeated dose toxicity settings; and the creation of an *in vitro* model hepatocyte-stellate cell co-culture model for assessment of liver fibrosis. In addition, sessions also highlight topics covered by other consortia of the **SEURAT-1** Research Initiative, aside from state of the art lectures by experts in these areas of research. The programme consisted of the following three sessions:

- Session 1 - Development of liver bioreactors for drug development and therapeutics:A state-of-the-art overview of liver-on-chip technology was given and clinical perspectives on bioartifical liver projects were highlighted.
- Session 2 - Implementation of the adverse outcome pathway concept in HeMiBio: A state-of-the-art overview of the AOP area with focus on liver toxicity was given and the *HeMiBio* contribution was presented.
- Session 3 - Combining pluripotent stem cell differentiation and genome engineering to create liver tissue *in vitro*: The cardinal features of the different liver cells, including hepatocytes, but also the non-parenchymal cells, including hepatic stellate cells, hepatic sinusoidal endothelial cells, kupffer cells and liver resident innate immune cells, were described in this session.

Overall about 90 attendees were registered for the meeting. All participants had been invited to submit original posters. 23 posters were presented of which seven had been invited for short oral presentations of 10 minutes. Finally, two poster prizes were awarded chosen for their content and clarity by the HeMiBio Advisory Board members Daniel Duche (L'Oreal, Cosmetics Europe) and Philip Hewitt (Merck).

DETECTIVE: The DETECTIVE Final General Assembly with reports about the project achievements took place in Brussels, Belgium on 24–25 November 2015. This meeting brought together data-producing and supporting consortium partners to discuss the final outcomes and achievements of the consortium. The presentations focused on the current state of ongoing and concluding projects and the strategies employed to identify new relevant biomarkers of toxicity. The outcomes and deliverables, including the dissemination of results in data-rich '-omics' databases, were discussed as were the future perspectives of the work completed under the DETECTIVE project.

The discussion during the meeting highlighted the following recurring themes that were critical to the success of the data output of DETECTIVE:

- The extension of studies to include more compounds so as to refine and strengthen the potential biomarker lists.
- The level of cross validation and correlation required between different '-omics' levels.
- How can newly identified biomarkers be included in the existing AOPs?
- The need for an agreement on the criteria that ideal biomarkers of toxicity should meet.
- The critical importance of statistics in the selections of the biomarkers was highlighted.

COSMOS: The final COSMOS Symposium on 'Computational Tools for Safety Assessment Focussing on Cosmetics Ingredients' took place in Liverpool, UK on 9 September 2015. The one day workshop event presented the findings of the COSMOS Project, within the context of **SEURAT-1**. Specific highlights included:

- Role of 21st Century Toxicology: Development of non-test methods to meet the demands of the Cosmetics legislation;
- COSMOS DB: A database solution to investigate toxicological data to support knowledge discovery and safety evaluation;
- Threshold of Toxicological Concern (TTC): The COSMOS TTC dataset enriched in cosmetics-related chemicals, available within COSMOS DB;
- Biokinetics Modelling: Freely available PBK models (for oral and dermal exposure), IVIVE, cell-based virtual assays;
- Toxicity Modelling: Structural alerts and molecular modelling profilers to support toxicity prediction;
- Computational Workflows: Freely available KNIME workflows, KNIME WebPortal, supporting documentation, user guidance and on-line tutorials;

- Read-Across: Presentation of a strategy and templates to define uncertainty and assist in data gap filling;
- Hands-On Demonstrations: The computational tools were used by interested participants under expert guidance;
- Interactive feedback opportunities: Applications could be discussed in direct contact with the developers.

The Symposium has been recorded, please find the recorded presentations at *http://www.cosmostox.eu/home/symposium*.

NOTOX: At the 51st Congress of the European Societies of Toxicology (EUROTOX), held on September 13-16 in Porto, Portugal – the NOTOX consortium presented the outcomes of its project. The Congress' agenda covered a variety of topics highlighting the latest research results and trends in the field of toxicology.

The NOTOX symposium took place on September 14th during the EUROTOX 2015 Congress. The topics presented focused on the application of cellular systems that come closest to human *in vivo* situation and their transfer into applicable and easy-to-handle test systems. Prof. Magnus Ingelman-Sundberg (Karolinska Institutet) presented *'Improved in vitro systems for prediction of hepatotoxicity'* while Fabrice Bertile (CRNS) discussed the newest research outcomes concerning *'Toxicoproteomics applied to in vitro investigation of liver toxicity using HepaRG cells'*. The topic of *'Model and in vitro based prediction of human hepatotoxicity'* was elaborated by Jan G. Hengstler (IfADo) and, finally, Lothar Terfloth (Insilico Biotechnology AG) provided participants with insights into the *'Prediction of long term toxic effects by genome based network models'*. Overall, the latest findings by the NOTOX consortium were well appreciated and the coordinators of the symposium, Elmar Heinzle and Fozia Noor, were pleased to welcome an audience of 200 attendees.

4.9.2 The SEURAT-1 Roadmap

Mark Cronin, Barry Hardy, Elmar Heinzle, Jürgen Hescheler, Marc Peschanski, Catherine Verfaillie and the COACH Team

All **SEURAT-1** projects contributed individually or collaboratively to the cluster-level objectives, which were: the formulation of a mode-of-action-based research strategy; the development of innovative testing methods; and the demonstration of proofs-of-concept (PoCs), thus providing a blueprint for expanding the applicability of the research strategy. **SEURAT-1** projects fed directly into these objectives, either through working groups (see below) or other coordinated cluster activities, and contributed to demonstrating the PoC at multiple levels.

The three levels for PoC studies were intensively discussed in the previous volumes of this book series and are also discussed in chapter three of this final volume, with a strong focus on the application level. Cross-cluster working groups were established (see section 4.9.4) in order to support the design of case studies in relation to the three PoC levels. The PoCs identified were regarded as cluster milestones, into which projects and working groups fed. They were the backbone of the **SEURAT-1** roadmap, which was developed by COACH to provide a tool for monitoring project deliverables contributing to **SEURAT-1** cluster objectives. Altogether, this roadmap provides an overview of cross-cluster interactions and cluster-level milestones, which were formulated to achieve the cluster-level objectives.

The **SEURAT-1** timeline (illustrated in *Figure 4.84*) maps out the milestones of the cluster. It illustrates the timing of PoCs at three conceptual levels and further milestones as the backbone for interactions between the **SEURAT-1** projects. In the last year, the 'Tools and Methodology catalogue' milestone was organised and comprises now the collection of tools and methodologies developed within **SEURAT-1** (see below). This collection fulfils the second cluster-level objective (i.e., the development of highly innovative tools and methodologies that can ultimately support regulatory safety assessment). The third cluster-level objective was achieved by the PoC case studies as described above.

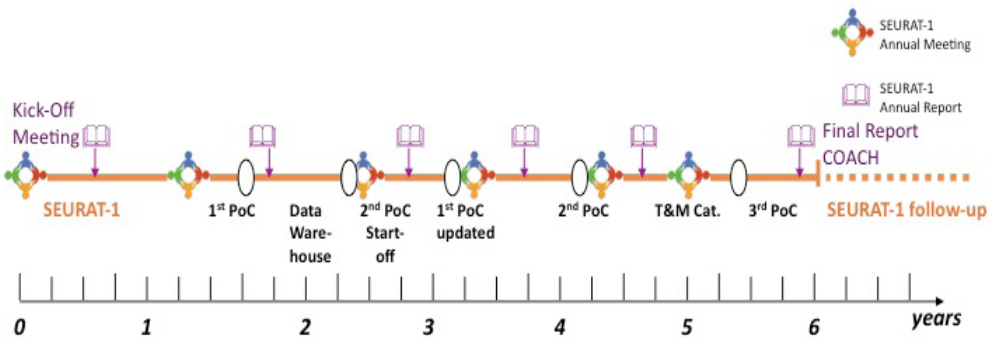

Figure 4.84 *The* ***SEURAT-1*** *timeline.*

At the beginning of **SEURAT-1** the deliverables from all projects were collected and compiled in a Gantt chart. This tool proved difficult to use as the deliverables were too numerous and detailed to give any useful overview. In addition, the Description of Work (DoW) of each project had not been developed in close collaboration with the other projects. It was therefore suggested to take a more top-down approach using the cluster-level objectives to identify and work towards the key deliverables, which are the essential project deliverables for achieving cluster objectives or triggering cross-cluster interactions.

SEURAT-1 project coordinators were first asked to identify major project milestones contributing to the **SEURAT-1** objectives (presented in the second **SEURAT-1** Annual Report). They then identified the key deliverables from the project DoW that contribute to these milestones.

Based on this, the projects were incorporated into the roadmap and the key deliverables became the basis for the **SEURAT-1** monitoring table and roadmap. The roadmap has been created in such a way that it is possible to follow the timescale for the **SEURAT-1** cluster-level milestones in the main roadmap, while the timescale for each separate project or working group is elucidated in segmented maps (not shown). The development of this roadmap was thoroughly discussed in the third Volume of this Annual Report, outlining the contributions from the projects and the working groups to the cluster-level milestones separately. The overall result is summarised in *Figure 4.85* and demonstrates the progress of the **SEURAT-1** Research Initiative along its own roadmap.

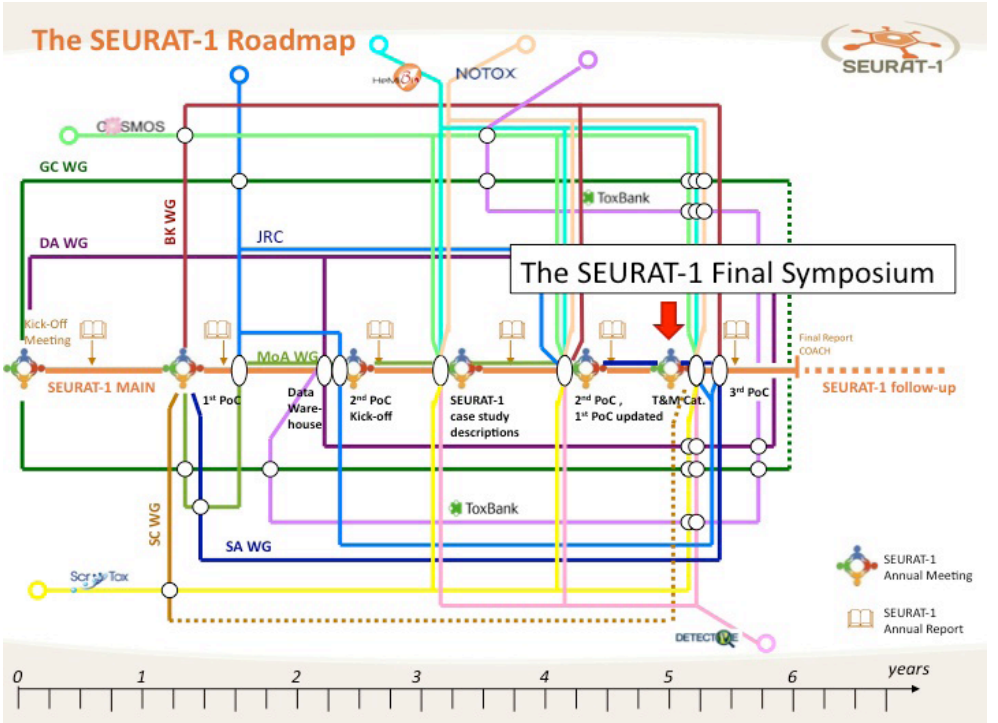

Figure 4.85 *The* ***SEURAT-1*** *roadmap illustrating the contributions from the projects, the Joint Research Centre and the Working Groups to the cluster-level objectives. GC WG = Gold Compound Working Group, DA WG = Data Analysis Working Group, BK WG = Biokinetics Working Group, MoA WG = Mode-of-Action Working Group, SC WG = Stem Cell Working Group, SA WG = Safety Assessment Working Group.*

The main roadmap, the separate roadmap lines and the progress-monitoring table, which is the basis for all the roadmaps, were all updated every six months and then presented to and discussed by the Scientific Expert Panel.

4.9.3 The SEURAT-1 Tools & Methods Catalogue

Elisabet Berggren

One of the most valuable outputs of **SEURAT-1** is the large portfolio of cutting-edge computational and *in vitro* tools and methods that underpin new animal-free approaches to safety assessment. Many of them were already applied in the **SEURAT-1** case studies to demonstrate their utility in predicting toxicity and supporting decision-making (see chapter 3 of this Annual Report). It is, therefore, important to clearly present the new tools and methods in their own right, as independent building blocks that can be assembled in a wide variety of ways for many different purposes. To this end, the ***SEURAT-1*** *Tools & Methods Catalogue* was developed *(http://publications.jrc.ec.europa.eu/repository/handle/JRC102532)*.

The ***SEURAT-1*** *Tools & Methods Catalogue* only includes non-confidential information in a summarised and easy-to-read format while the more detailed information can be retrieved from the ToxBank Data Warehouse *(https://services.toxbank.net/)*. The catalogue is disseminated via the public EURL ECVAM DataBase service on Alternative Methods (DB-ALM; *http://ecvam-dbalm.jrc.ec.europa.eu)*, developed and maintained by the European Commission's Joint Research Centre. The DB-ALM provides standardised curated descriptions of over 250 alternative methods intended for use both in biomedical research and regulatory safety assessment. The methods made available through the catalogue can be updated and completed at any time also after the finalisation of **SEURAT-1**.

4.9.4 The Model of Cross-Cluster Working Groups

The COACH Team

As briefly described in section 4.9.2, Working Groups were created to facilitate cross-cluster cooperation between projects and people. The overall motivation for establishing these cross-cluster working groups was to: (i) stimulate project interactions; (ii) assist the linkage of deliverables from different projects (in an effort to create the cluster-level roadmap); and (iii) capture the knowledge spread over more than 70 partners of the **SEURAT-1** Research Initiative. The challenge was to encourage collaborations not foreseen in the individual project deliverables lists and to find a way to broaden the reach of the **SEURAT-1** Research Initiative. It was therefore agreed by the **SEURAT-1** Scientific Expert Panel that a Working Group should have two aspects to its profile: one *Operational* aspect to deal with specific research questions and problems originating from project activities, and therefore finding common solutions on a cluster level; and a *Think Tank* aspect to encourage creativity and capture external expert views with the aim of achieving a broad multidisciplinary perspective.

A more detailed description about the establishment of the Working Groups, including Terms of References, is given in the second volume of the **SEURAT-1** Annual Report. *Table 4.5* provides an overview about the **SEURAT-1** Working Groups, including short descriptions (more detailed working group reports are given in the following sections).

Table 4.5 *Overview about the* ***SEURAT-1*** *Working Groups.*

Working Group	Co-leaders	WG Description
Gold Compound	Jeffrey Wiseman (ToxBank) Paul Jennings (DETECTIVE)	The goal for the Gold Compound Working Group was to achieve consensus across the **SEURAT-1** Research Initiative on the criteria for selecting, accepting and using test substances in the development of alternative testing methods for repeated dose systemic toxicity. Cross-project members and additional external experts collaborated on the discussion of compound selection, mechanisms and assays. A criterion for the compound selection was a preference for previously well-studied compounds for which there is a good understanding of modes-of-action.
Data Analysis	Glenn Myatt (ToxBank) Annette Kopp-Schneider (DETECTIVE)	The Data Analysis Working Group (DAWG) formulated best practices, standards and common approaches for programme data management and analysis, including topics such as vocabularies, protocols, ontologies, statistical analysis and integrated data analysis. The group also developed ideas and new approaches to data analysis that were required by research activities carried out under the programme. The DAWG also contributed to the discussions on the choice of biomarkers and approaches to the processing and analysis of associated '-omics' data.
Mode-of-Action	Mathieu Vinken (*HeMiBio* / DETECTIVE) Brigitte Landesmann (COACH)	The Mode-of-Action (MoA) Working Group assisted in achieving the **SEURAT-1** objective to formulate and implement a research strategy based on generating and applying knowledge of MoAs. The MoA Working Group identified known modes-of-action to support data analysis and outcomes from different projects. The Adverse Outcome Pathway framework approach was used as a practical tool to organise MoA information and capture interrelations in the cell by means of '-omics' and *in vitro* data, including dose dependencies. A special focus was made trying to link molecular initial events to possible adverse outcomes.
Biokinetics	Frédéric Bois (COSMOS) Emilio Benfenati (ToxBank)	The Biokinetics Working Group provided support to cluster activities in the paradigm shift from pure experimental approaches to a guided model-based approach. The Working Group assisted **SEURAT-1** projects and case studies to design *in vitro* and bioreactor models and experiments applied to those. To enable *in vitro* to *in vivo* extrapolation, partners provided the working group with concentration measurements and effects data from the *in vitro* experiments. The efforts of the Working Group gave strong support to achieve the **SEURAT-1** objective to develop highly innovative tools and methodology that can ultimately support regulatory safety assessment.
Stem Cells	Glyn Stacey (*SCR&Tox*) Anna Price (DETECTIVE/ *SCR&Tox*)	The aim of the Stem Cells Working Group was to standardise quality control issues of the cells used between different partners and projects. Three cross-consortia cell model subgroups have been identified: pluripotent stem cell lines (DETECTIVE, *SCR&Tox*), embroyd bodies (DETECTIVE, *SCR&Tox*) and differentiated cell lines (*HeMiBio*, DETECTIVE, *SCR&Tox*). The Stem Cell Working Group, with support from its subgroups, made it possible to evaluate the competence and robustness of the cell models used and also to ensure that results from different projects using the same cell models are comparable.

Safety Assessment	Andrew White (Unilever) Derek Knight (SEP)	The Safety Assessment Working Group aimed to bridge the gap between non-animal toxicity testing and safety assessment decision-making needs. Future safety assessment approaches should be based on comprehensive knowledge of the modes-of-action and pathways leading to adverse effects in humans, rather than on animal testing. This Working Group focused on applying the relevant information derived from the developing predictive systems across the projects to progress pragmatic solutions for addressing the safety decision needs. The group examined what approaches are useful for building confidence and understanding the uncertainty within a mechanistic framework (for example, biokinetic modelling in combination with dose response analysis of *in vitro* results). As such, the group acted as a facilitator to identify both key gaps in current knowledge and data needs for safety assessment, working across regulatory and science domains to ensure their generation, e.g. they worked with ToxBank to identify negatives that realistically help to define adaptive versus adverse effects.

4.9.5 Gold Compounds Working Group: Mechanism-Based Selection of Reference Compounds for the Development of *in vitro* Toxicity Testing Methods

Jeffrey Wiseman, Paul Jennings

Mechanistic understanding of chemical induced perturbation is at the heart of the much heralded paradigm shift in toxicology. Toxicogenomics and complementary '-omic' techniques have accelerated the discovery and delineation of a plethora of pathways which can aid in understanding mode of action of chemical toxins (*Jennings et al., 2013; Wilmes et al., 2013*). To expand this knowledge and to investigate the quality of the *in vitro* systems we developed in **SEURAT-1** and are currently developing in follow-up initiatives, we need to test more compounds. The question is which compounds should we test and why? The Gold Compound Working Group, co-chaired by the authors of this section, made a decision early in the project that **SEURAT-1** should focus on compounds with very well defined molecular mechanisms. The rationale is simple: if we know the mechanism of the chemical we are testing, we can assess if the novel *in vitro* systems and surrounding assays are fit-for-purpose. To this end the Gold Compounds Working Group got busy on building chemical lists and dossiers. After much discussions and debates, which are summarised in the previous volumes of this Annual Report, these chemicals were reduced to smaller manageable lists (*Table 4.6*). Information on these compounds is hosted by ToxBank on the compound wiki page *(http://www.toxbank.net/compound-wiki).*

Table 4.6 *Summary information for* ***SEURAT-1*** *standard reference compounds ('Gold Compounds').*

Hepatotoxins			
Toxicant	**Initiating Mechanism**	**Adverse Event of Interest**	**Wiki Table**
	Reactive Molecules		
Acetaminophen	Non-selective thiol reagent	Cytotoxicity	Yes
Iodoacetamide	Selective thiol reagent	Cytotoxicity	Yes
Allyl alcohol	Selective thiol reagent, energy source	Fibrosis	Yes
DMNQ	Redox cycling	Cytotoxicity	Yes
CCl_4	Free radical generator	Steatosis, fibrosis	Yes
Aflatoxin B1	Lysine reagent	Apoptosis	Yes
	Mitochondrial Disruption		
Antimycin A	Mitochondrial disruption, ROS	Cytotoxicity	No
Oligomycin A	Inhibition of complex V	Cytotoxicity	Yes
Rotenone	Inhibition of complex I	Cytotoxicity	Yes
FCCP	Proton gradient uncoupler	Cytotoxicity	Yes
	Promiscuous Binding		
Valproic acid	Membrane disruption, inhibition of fatty acid beta-oxidation	Steatosis	Yes
Chlorpromazine	Membrane disruption	Cholestasis	Yes
Amiodarone	Phospholipid binding, membrane disruption, inhibition of fatty acid beta-oxidation	Phospholipidosis, steatosis	Yes
	Selective Binding		
Methotrexate	Antifolate	Fibrosis	Yes
Bosentan	BSEP inhibitor	Cholestasis	Yes
Dirlotapide	Microsomal triglyceride transport inhibitor	Steatosis	Yes
Fluoxetine	Phospholipid binding	Phospholipidosis	Yes
Hygromycin B	Ribosome inhibitor	Cytotoxicity	Yes
	Nuclear Hormone Receptor Ligands		
T0901317	Dual LXR-PXR agonist	Steatosis	Yes
Rifampicin	PXR agonist	Negative control, steatosis	Yes
WY14643	PPARα agonist	Lipid metabolism disruption, proliferation	No
β-Naphthoflavone	AhR agonist	Lipid metabolism disruption	No
Tamoxifen	ER modulator	Epigenetics	Yes
Nephrotoxins			
$KBrO_3$	Strong oxidising agent	Cytotoxicity	Yes
Ochratoxin A	Cytoskeleton disruption	Epigenetics	Yes

Cardiotoxins			
Doxorubicin	Topoisomerase inhibitor, redox cycling	Repeated dose organ failure	Yes
Antimycin A	Mitochondrial disruption, ROS	Cytotoxicity	No
E4031	hERG antagonist	Torsade de Pointes	Yes
Carbachol	Cholinergic agonist	Cell phenotyping	Yes
Isoproterenol	Adrenergic agonist	Cell phenotyping	No
Nifedipine	L-type Ca channel antagonist	Cell phenotyping	No
Neurotoxins			
Naphthol AS-E phosphate	CREB inhibitor	Mechanistic standard	
Forskolin	CREB activator	Mechanistic standard	No
DAPT	Notch1 inhibitor	Mechanistic standard	No
Rapamycin	mTOR inhibitor	Mechanistic standard	No
GSK2334470	PDK1 inhibitor	Mechanistic standard	No
Akt1/2 inhibitor	AKT kinase inhibitor	Mechanistic standard	No
Nocodazole		Inhibition of neurite outgrowth	No
U0126		Inhibition of neurite outgrowth	No
Acrylamide		Inhibition of neurite outgrowth	No
Propofol		Inhibition of synaptogenesis	No
Lead(II) chloride		Inhibition of synaptogenesis	No
Chlorpyrifos	Organophosphate acetylcholinesterase inhibitor	Affecting cAMP signalling (CREB)	No
Diazinon	Organophosphate acetylcholinesterase inhibitor	Affecting cAMP signalling (CREB)	No
Dieldrin		Affecting cAMP signalling (CREB)	No
Ni^{2+}		Affecting cAMP signalling (CREB)	No
Tributyltin (TBT)		Affecting cAMP signalling (CREB)	No
Trimethyltin (TMT)		Affecting cAMP signalling (CREB)	No
PCB 153		Affecting Notch signalling	No
PCB 180		Affecting Notch signalling	No
Glutamate		Affecting PDK1/Akt /mTOR signalling	No
Generic Negative Controls			
D-Mannitol	NA	NA	No

In addition, we have supported these actions by conducting an extremely detailed mechanistic review on hepatotoxins and their mechanisms, focusing primarily to these gold compounds (*Jennings et al., 2014*). In the review, we divided compounds into different categories including chemically reactive compounds (alkylating and oxidising agents), compounds with specific cellular targets (mitochondrial toxins and specific enzyme inhibitors), compounds that interfere with lipid metabolism and compounds that disrupt the plasma membrane. We also discuss the molecular and cellular pathways that are perturbed and the cellular responses activated to redress these perturbations. We hope that this information will be valuable for scientists developing *in vitro* models, generating predictive assays and developing adverse outcome pathways.

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4.9.6 Data Analysis Working Group: Integrated Data Analysis

Glenn J. Myatt, Nina Jeliazkova, Barry Hardy, Annette Kopp-Schneider

The objective of the Data Analysis Working Group (DAWG) was to support the data analysis needs of the **SEURAT-1** Research Initiative, including data collection, integration, analysis, as well as experimental design. It provided a forum to discuss issues or problems within **SEURAT-1** as well as with other academic and industrial groups. This group discussed best practices, standards and common approaches including topics such as vocabularies, ontologies, statistical analysis, and integrated data analysis. The group also developed ideas and new approaches to data analysis required by emerging research activities carried out under the programme, such as the choice of biomarkers and approaches to the processing and analysis of associated '-omics' data. In the following, we demonstrate our approach in two

scenarios covering the integration of data from external resources to support the analyses of data obtained in **SEURAT-1** projects.

The integration of information from external resources with **SEURAT-1** generated information was an important task of the working group. One of these projects was to integrate the ToxCast (*US EPA, 2015*) and Tox21 (*US Department of Health and Human Services, 2015*) data with the **SEURAT-1** data to support a meta-analysis of the combined information and allow the application of these data in the context of the **SEURAT-1** case studies such as the read-across case study for safety assessment (see section 3.2). The first step in this process was to translate the data files and databases that have been made available by the US EPA into the ISA-TAB format to be uploaded into the ToxBank data warehouse. This includes the generation of information on the overall investigation, the study design as well as each assay that was performed. In addition, the processed endpoints were standardised based on the harmonisation proposed by the ToxBank consortium. The ToxCast/Tox21 release from 31 October 2014 contained raw dose response and processed data for 8599 chemical substances and 65 assay endpoints generated by 35 experimental protocols (Tox21 dataset) and raw and processed data for 1877 chemical substances; 821 assay endpoints (outputs), derived from 558 assay components (raw data readouts), generated from 342 experimental protocols (ToxCast dataset). The ToxCast/Tox21 data is organised in 7 levels and it was decided to only include Level5 data at this time (hitc, log AC10, log AC50 gain, log AC50 loss). The ISA-TAB study, assay and data files were generated by SQL queries from the ToxCast MySQL database[1]. In addition to generating the ISA-TAB files from the Tox21/ToxCast data, we also investigated a setup with ToxCast phase II data hosted in an AMBIT instance (an open-source cheminformatics data management system; *Ideaconsult, 2015*) with the data and structures available via REST API (JSON) to the ToxBank UI to query in the same fashion as it queries the COSMOS database.

Another project integrating external resources with **SEURAT-1** data was through a collaboration with the DETECTIVE consortium. As part of a publication (*Grinberg et al., 2014*), the TG-GATES transcriptomics data was analysed and the raw and processed data generated was converted to ISA-TAB. In addition, the toxicogenomics directory was made accessible through a wiki page (*ToxBank, 2015*). The ISA-TAB conversion included mapping the processed data onto the standardised format developed by the ToxBank project. To support access to this dataset as well as other '-omics' data in an integrated fashion, a new biomaterials search was developed to support the querying of genes (as well as other biomaterials). This search was developed in consultation with other projects to support identifying specific gene of interest from other experiments. The workflow of this study is shown in *Figure 4.86*.

1 http://www.epa.gov/ncct/toxcast/files/MySQL%20Database/MySQL_Database_v1.zip

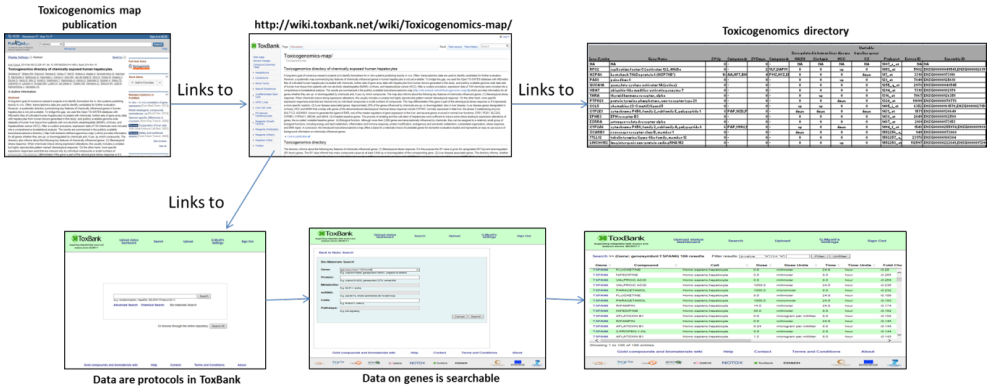

Figure 4.86 *Integration of data from TG-GATES transcriptomics database.*

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4.9.7 Mode-of-Action Working Group: Capturing Mode-of-Action Knowledge

Brigitte Landesmann, Mathieu Vinken

Following the second **SEURAT-1** Annual Meeting in February 2012, the Mode-of-Action Working Group (MAWG) was launched to facilitate cross-cluster cooperation between projects

and people and to assist in achieving the following **SEURAT-1** cluster-level objectives: (i) to formulate and to implement a research strategy based on generating and applying knowledge of modes-of-action (MoA); and (ii) to demonstrate proof-of-concept at multiple levels from theory to application.

In 2012 and 2013 the MAWG organised three workshops about the following topics: (i) on AOPs to liver toxicity in Ispra / Italy; (ii) on the exploration of existing data bases for MoA of repeated dose systemic toxicity in Tuebingen / Germany; and (iii) on the development of AOPs relevant to human neurotoxicity again in Ispra / Italy. These workshop activities were supported by the coordination project COACH.

During the **SEURAT-1** Annual Meeting in February 2014 in Barcelona, Spain, a joint session was organised together with the Gold Compound Working Group (GCWG). In this MAWG-GCWG event, different speakers from the **SEURAT-1** projects were asked to give a concise overview of past, on-going and future MAWG-related activities with respect to organ-specific (*i.e.* heart, liver, nervous system, kidney, skin) as well as cross-organ (*i.e.* mitochondrial) toxicity. Not only collaboration with the GCWG was discussed, but equally strategies to link the MAWG activities to the different **SEURAT-1** projects.

In 2014, focus has been put on the dissemination of activities and the practical elaboration and verification of established AOPs. The latter was mainly done in the context of the **SEURAT-1** case study programme. In the following years the main activities were:

- Active involvement in the practical performance and evaluation of the proof-of-concept case studies in the different **SEURAT-1** projects;
- Dissemination and communication of MAWG activities on international conferences, including presentation of developed AOPs;
- Generating new relevant AOPs and publication in peer-reviewed journals;
- Contribution to the development of an AOP knowledge base in collaboration with JRC, OECD and the US-EPA;
- Continuous refinement of the established AOPs;
- Project assistance in assay, biomarker and *in vitro* model development with respect to AOPs;
- Looking for opportunities to continue the AOP efforts after completion of the **SEURAT-1** Research Initiative.
- Continuing role of 'AOP-ambassadors'.

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4.9.8 Biokinetics Working Group: Biokinetic Modelling in Support of *ab initio* Predictions of Safety

Frédéric Y. Bois

Introduction

One of the **SEURAT-1** case studies was to explore the potentials and eventual pitfalls of a purely *ab initio* approach to toxicity predictions (see section 3.3). Assuming that only the chemical structure of a new molecule is known (along with the relationships between structure and toxicity for all other known molecules, if available), the questions are: can we first predict its absorption, distribution, metabolism and excretion (ADME) by humans as a prerequisite for the assessment of toxicological effects? Can we then, on the basis of predicted tissue concentrations relate toxic effects observed *in vitro* (hopefully on human cells) to expected *in vivo* effects?

This summary report addresses the first question, the prediction of ADME and tissue concentrations. The best tools for these aims is a combination of predictive chemistry (e.g., QSAR's) and physiologically based pharmacokinetic (PBPK) modelling (*Adler et al., 2011*), as currently used by the pharmaceutical industry for predicting the bioavailability of virtual molecules. However, PBPK modelling must be extended for cosmetic ingredients with dermal exposures and this requires validation datasets for known compounds. For that purpose we used methotrexate (MTX) and valproic acid (VPA), for which both pharmacokinetics and toxicity in humans are known. Arguably, these are not standard cosmetic ingredients, but we played the 'game' of considering them as entirely new molecules, for which we knew nothing, except their molecular structure (and *in vitro* toxicity on hepatocytes, but we focus here on pharmacokinetics). Therefore, in the following, the human data available were only used for validating the PBPK model predictions, but not for setting ADME parameter values.

PBPK Models: Parameter Values and Uncertainty Analyses

We used the generic PBPK model developed in the framework of COSMOS (see section 4.5.3.4). Briefly, the model includes distribution in the following tissues or organs: Venous blood, arterial blood, fat tissues, poorly perfused tissues (muscles), richly perfused tissues (viscera), gut, liver, and skin. Exposure can occur through the dermal route, ingestion or inhalation. The absorbed molecules can be excreted to urine or metabolised in liver. The model is coded as a set of ordinary differential equations, solved by numerical integration with the R 'deSolve' package. The scripts corresponding to the MTX and VPA implementations are available as KNIME workflows on the COSMOS web-portal.

While the structure of the PBPK model used and the list of its parameters are fixed, its parameter values were tailored to the molecule of interest (MTX or VPA), to the extent permitted by the sole knowledge of chemical structure and eventual *in vitro* tests of ADME properties. We were faced with two cases: For some parameters (*e.g.*, the partition coefficients, the total body clearance) we had *a priori* information from QSAR models (*ACD/LABS, 2015*) or *in vitro* experiments (even if very imprecise), while on others (in particular on the fraction metabolised) we had no information at all. Such uncertainties should be accounted for. That is why we decided to assign a statistical distribution to each model parameter, rather than a single value, and to perform Monte Carlo simulations (*Bois et al., 2010*) to estimate the impact of such parameter uncertainty on model predictions (*e.g.*, on the concentration of MTX or VPA in liver at any given time following exposure). For informed parameters we used distributions with a CV of 300%. For uninformed parameters we used uniform distributions between very wide bounds (*e.g.*, from 0 to 1 for the fraction absorbed by the gut or the fraction unbound in plasma). The correlation between partition coefficient estimates was taken into account. Monte Carlo simulations proceeded by sampling one random value (out of its assigned distribution) for each model parameter. The model was then run and the outputs (predictions)

recorded. Those two steps were iterated many times, and the collected output values formed a random sample, for which we computed the mean, the SD, and any percentile of interest. In short, Monte Carlo simulations give predictions' uncertainty provided that they are fed with parameter distributions reflecting uncertainty (or variability).

The following results were obtained that way and present not only the average (mean) predictions but also their 'confidence' intervals (in this case delineated by the 2.5th and 97.5th percentiles).

Results

Methotrexate: In a purely predictive framework we would just obtain predictions such as those given in *Figure 4.82* for liver (right panel) after a 10 mg oral dose. The uncertainty is large, and the upper 95% confidence bound lies one order of magnitude away from the median at the peak level. Simulations of repeated dosing (once a week) indicate no accumulation over time and the patterns shown on *Figure 4.87* repeat themselves every week. In the case of the venous blood (*Figure 4.87*, left panel) we have clinical data (*Stewart et al., 1990; Kozloski et al., 1992; Hroch et al., 2008*), which we can compare to the simulations. Those data were obtained at different doses (7.5, 10, and 15 mg). Since the model assumed linearity, the 7.5 and 15 mg data were simply rescaled by a factor of 0.75 and 0.67, respectively. The data fall within the 95% confidence limits and the decay slope is reasonable, but the peak blood concentration is underestimated by the median predictions. Some Monte Carlo predictions (grey curves) actually match the data quite well, indicating that more precise parameter estimates would probably improve our model predictions.

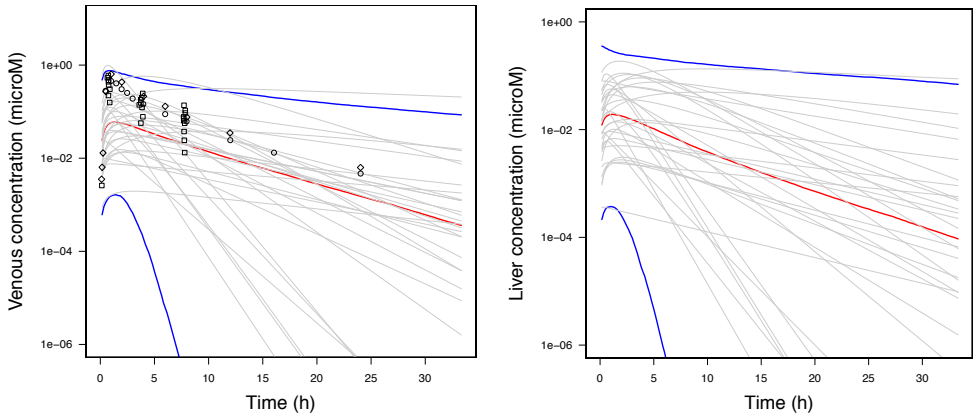

Figure 4.87 *PBPK model predictions of methotrexate venous (left panel) and liver (right panel) concentration in a human adult following a 10 mg ingestion. Red lines: median predictions; blue lines: 2.5 and 97.5 percentiles; grey lines: 20 random simulations. The comparison with measured concentrations is possible for venous blood (squares represent data from Hroch et al. (2008), circles represent data from Kozloski et al. (1992) and diamonds represent data from Stewart et al. (1990).*

If we want to improve the precision and accuracy of our model predictions we need to decide which parameters should be better estimated in priority (obviously we can decide to improve all parameter estimates if we have no time or budget constraints). Monte Carlo simulations can help us here too. Since sampled parameter values were recorded along with the predicted concentrations, it is easy to draw the plots shown on *Figure 4.88*. They show the relationship between the venous concentration of MTX, two hours after dosing (close to the peak time) and the values sampled for three parameters: the fraction absorbed by the gut, the absorption rate and hepatic clearance (actually renal clearance is as important as hepatic clearance). We have seen that the peak concentration is somewhat underestimated (*Figure 4.87*). Narrower ranges for those parameters would also narrow and improve the range of predicted concentrations. Note that the exercise is biased since we have human data which guide the way to improve the model predictions. In a purely predictive framework, we would have to decide which parameters to focus on, just on the basis of *Figure 4.88*. Because we care about the upper bound on peak concentrations we would focus on clearance, on the fraction absorbed and then on the absorption rate. The partition coefficients are less influential (data not shown).

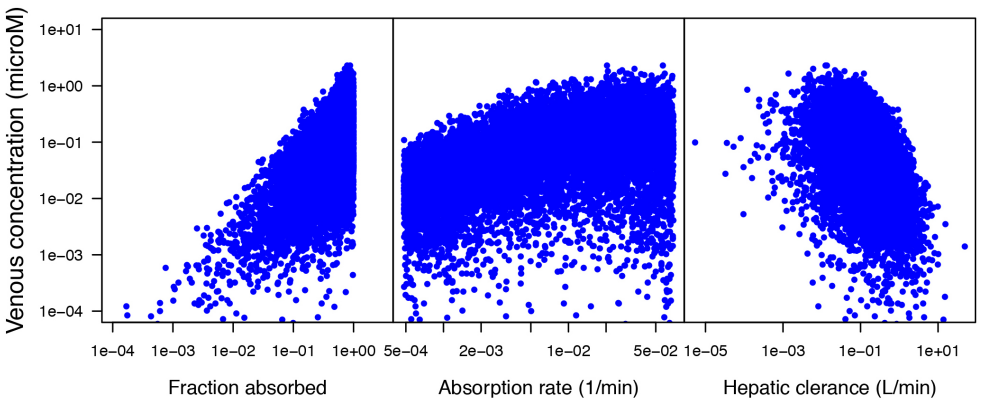

Figure 4.88 *Sensitivity analysis regarding three important parameters affecting the predicted methotrexate venous concentration in a human adult, 2 hours after a 10 mg ingestion. Each dot corresponds to the result of a Monte Carlo simulation (parameters were randomly sampled jointly).*

Valproic Acid: VPA would not accumulate when administered once a week. However, for therapeutic reasons, it is administered once or twice a day and a moderate accumulation is observed. Since we wanted to compare the predicted kinetics with clinical data, we simulated time courses over one week.

Figure 4.89 shows the model predictions for venous blood and liver VPA concentrations during repeated oral dosing (1000 mg every 12 hours). For venous blood, clinical data are available obtained at different doses (800, 900, and 1000 mg; *Perucca et al., 1978; Nitsche & Mascher, 1982*). Since the model is linear, the 800 and 900 mg data were rescaled. The lower bound uncertainty is large, but overall the uncertainty is lower than for MTX. The venous blood data fall within the 95% confidence limits and are reasonably well approximated by the model.

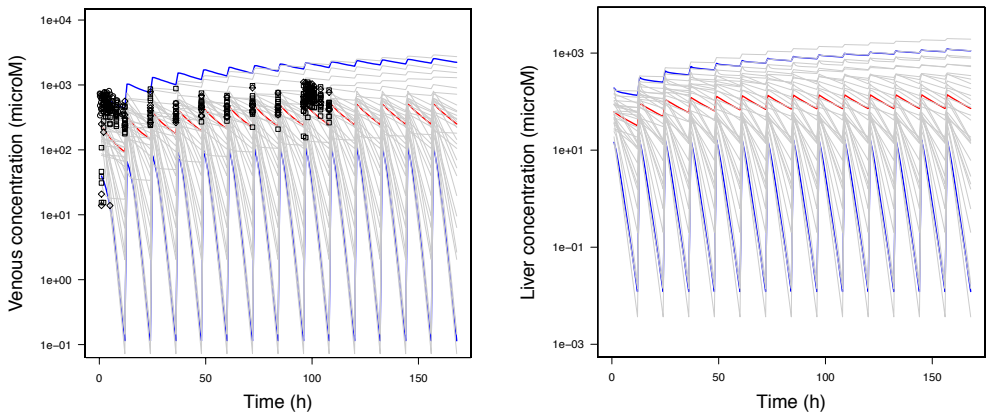

Figure 4.89 *PBPK model predictions of valproic acid venous (left panel) and liver (right panel) concentration in a human adult during repeated oral dosing (1000 mg every 12 hours). Red lines: median predictions; blue lines: 2.5 and 97.5 percentiles; grey lines: 20 random simulations. The comparison with measured concentrations is possible for venous blood (squares and diamonds represent data Nitsche & Mascher (1982) with doses of 900 and 1000 mg, respectively; circles represent data from Perucca et al. (1979)).*

The sensitivity of the predicted VPA venous blood concentration (at 100 hours, that is 2 hours after the 8th 1000 mg dose) is illustrated in *Figure 4.90* for three parameters: the fat - blood partition coefficient, the fraction absorbed by the gut, and the hepatic clearance rate. All partition coefficients are correlated, hence their estimates improve together. Getting a more precise estimate of either hepatic (shown here) or renal clearances would also narrow down our confidence intervals. Here we show the sensitivity to the fraction absorbed. For VPA, that fraction is rather well identified and within its plausible range the predictions are not very sensitive to these values. It would not make much sense to try to improve the estimate by further experiments or calculations.

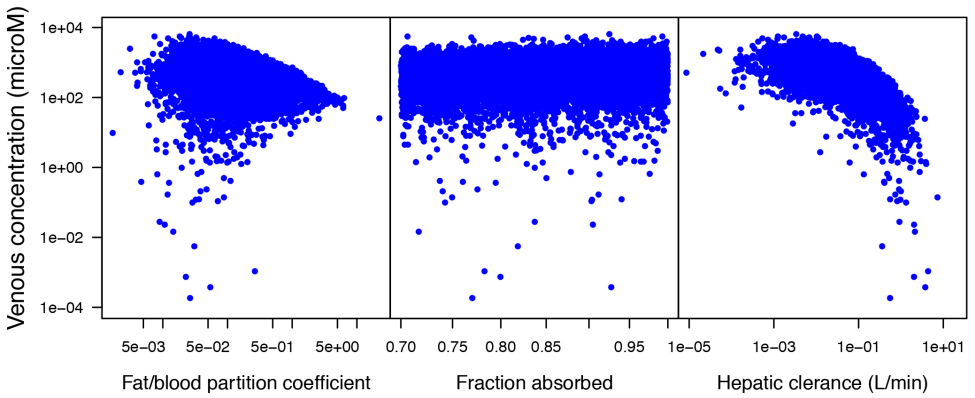

Figure 4.90 *Sensitivity analyses of three parameters relevant for the prediction of valproic acid venous concentration in a human adult, 2 hours after the 8th administration of 1000 mg per os. Each dot corresponds to the result of a Monte Carlo simulation (parameters were randomly sampled jointly).*

A third chemical, piperonyl butoxide, more relevant but less informed than MTX and VPA, was investigated in the **SEURAT-1** *ab initio* case-study (see section 3.3) and the PBPK model was used to predict the fate of this compound as well. The model can be used in the context of risk assessment: Given experimental *in vitro* dose-response data on liver effects, for example, we can now predict human *in vivo* liver dose for any consumer exposure scenario, and hence *in vivo* effects. We could also refine our parameter estimates prior to deciding a mode-of-action based on risk predictions, if such predictions are deemed too uncertain. Bayesian decision analysis can help to create such risk management strategies. The results of the Monte Carlo simulations we performed are directly usable in a Bayesian context.

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4.9.9 Stem Cell Working Group: Towards Good Stem Cell Culture Practice Principles

Glyn Stacey, Anna Price

Introduction and Objectives

SEURAT-1 has initiated a Stem Cell Working Group to support the development of good stem cell culture practice principles and to promote best practice in the development of standardised stem cell-based assays for predictive toxicology purposes across the **SEURAT-1** projects. It has had particular close interactions with *SCR&Tox* scientists and outputs on good practice have been posted on the ToxBank website.

The objectives of the working group were: (i) the identification of key areas of scientific development where reviews focusing on predictive toxicology would be helpful to the **SEURAT-1** objectives; and (ii) the definition of key criteria and steps required in the development of *in vitro* cell predictive toxicity assays adapted to high-content and high-throughput methods. In the development of stem cell-based toxicity assays, a range of cell lines are currently employed in a range of potential protocols. Nevertheless, we are still exploring the use of human pluripotent stem cells as biological resources for predictive toxicology. The study and definition of protocols for differentiation are developing rapidly but yet to reach regulatory approval in product safety testing. This complex matrix of different cells and methods makes it very difficult to draw comparisons across work in different laboratories and thus standardisation is very challenging.

Overview of Activities

The group compiled considerations regarding best practices on core toxicology assay procedures relating to the preparation, storage and use of test and control compounds. The latter activity on toxicology procedures is presented, along with other educational content on the ToxBank Data Warehouse. Key areas of consideration common to all partners in the **SEURAT-1** Research Initiative when sourcing tissues and cells were addressed and documents describing best practices were published via the ToxBank wiki on Biological Materials (*ToxBank, 2015*). This and other best practice documents on the ToxBank website provide templates against which an assessment can be made of the suitability of the biological material for partner projects. These activities were also included in a book chapter on quality control for hPSC lines and the development of hPSC-based assays (*Stacey et al., 2016*). Furthermore, the group has revisited the GCCP guidance (*Coecke et al., 2005*) with respect to stem cell culture and assay development standardisation and is coordinating with JRC activities to publish an updated guidance for stem cell lines.

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4.9.10 Safety Assessment Working Group: A Conceptual Framework to Combine Evidence

Derek J. Knight

Introduction

SEURAT-1 delivers computational and experimental tools and related knowledge that are critical components in predictive toxicology approaches. The proof-of-concept exercise to

demonstrate the potential of these tools when assembled in an integrated manner is at the theoretical, methodological and application levels by means of case studies. The Safety Assessment Working Group (SAWG) is to bridge the gap between knowledge and tools from **SEURAT-1** and practical safety assessment decision making needs of risk assessors. The SAWG has facilitated the three case studies at the proof-of-concept application level, described in detail elsewhere (see chapter 3):

- An *ab initio* risk assessment as a 'stretching target' that will highlight gaps for future research and development and illustrate overall progress made in **SEURAT-1**;
- Using evidence from new-approach methods to improve the robustness of 'read-across' within chemical categories to predict the toxicological properties on 'target' substances from the known toxicology of 'source' substances.
- A safety assessment using the Threshold of Toxicological Concern (TTC) approach.

Conceptual Framework to Combine Evidence

The **SEURAT-1** 'Conceptual Framework' sets out a structure to guide assessors in devising a fit-for-purpose Integrated Assessment and Testing Approach (IATA) that combines information from predictive tools with a stated protection goal. The overall outcome should be robust as it is not based on single pieces of evidence, rather a weight of evidence combined in a biologically-rational manner. The overall assessment results from combing all this information and evidence, including an assessment of the uncertainty associated with the prediction. It may be necessary to improve the assessment if the result is not fit for purpose. The SAWG has been involved in refining the Conceptual Framework (see *Figure 4.91*).

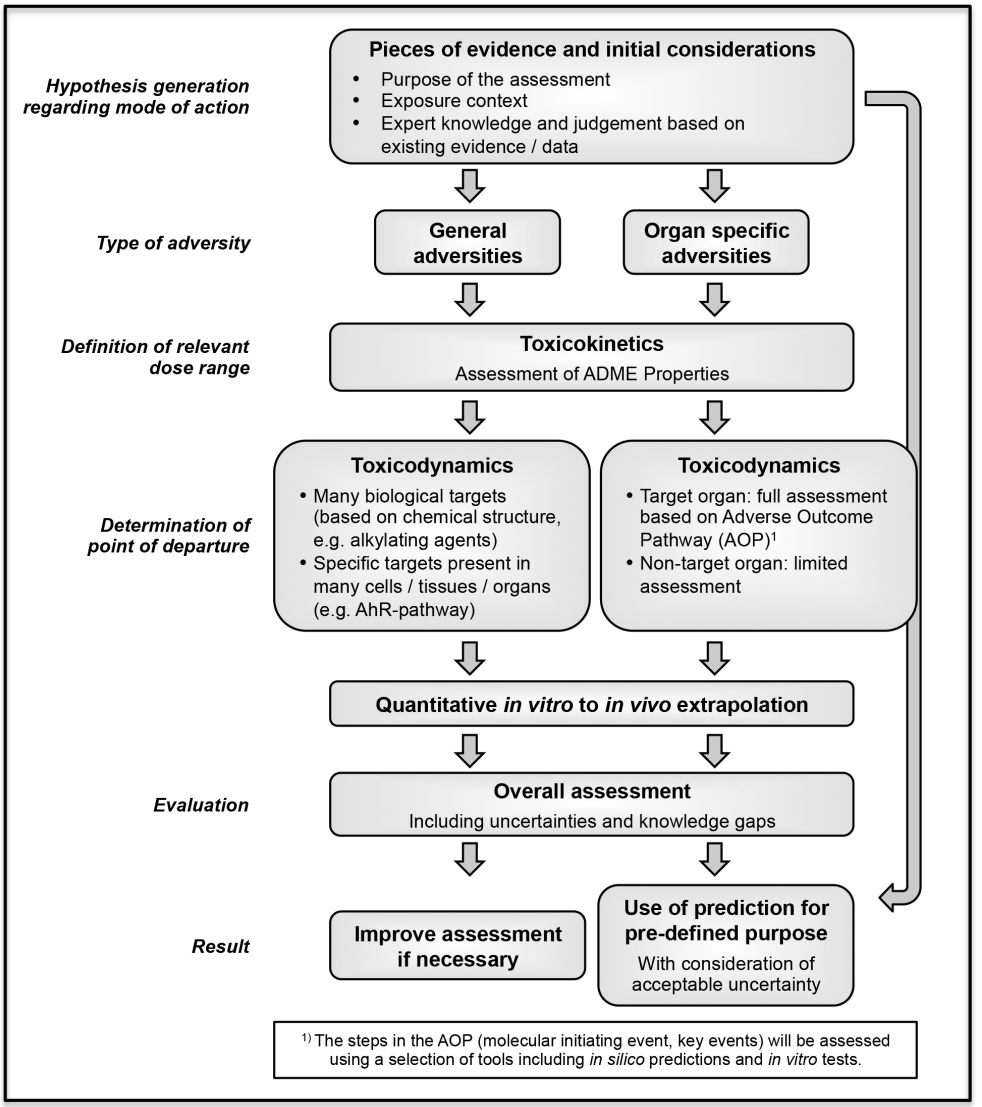


Figure 4.91 *'Conceptual framework' as a structure for assessors in devising a fit-for-purpose 'bespoke' Integrated Assessment Strategy for a particular case.*

Briefly, the Conceptual Framework consists of the following steps:

➠ Decide the degree of confidence needed for prediction (low degree of confidence may be acceptable in case of well-controlled and low human exposure);

➠ Examine existing knowledge (toxicological studies, 'read-across' from chemical or biological analogues, QSARs and structural alerts, expert judgement);

➠ Distinguish between 'general chemicals' (expected to be unselective in interacting with biological targets) and a drugs/pesticides (designed to be selectively biologically-active):

➠ Two parallel lines of consideration: (i) 'general' adverse effects not associated with a particular organ and (ii) organ based adverse effects;

➠ Consideration of toxicokinetics/toxicodynamics (for both lines of consideration);

➠ Effects on organs can be assessed by (several) AOPs incorporating existing knowledge and with new data as a combination of *in vitro* assays ('-omics' data etc.) and *in silico* predictions in a battery of tools.

Disclaimer

The views expressed in this paper are solely those of the author and the content of the paper does not represent an official position of the European Chemicals Agency.

4.10 The SEURAT-1 Final Symposium: Painting the Future Animal-Free Safety Assessment of Chemical Substances - Achievements of SEURAT-1

The COACH Team

4.10.1 Introduction and Programme

The **SEURAT-1** Symposium, held in Brussels on 4 December 2015, gathered 120 researchers, regulators, EU officials and industry representatives in the alternative methods field. The aim of the symposium was to communicate the main results achieved to a wide audience of stakeholders, as the cluster's six research projects concluded their activities.

The research strategy termed 'Safety Evaluation Ultimately Replacing Animal Testing' gave **SEURAT-1** its name, indicating that the initiative is the first in a long-term strategy where more steps are needed before the ultimate goal can be reached. The title of the Symposium, 'Painting the future animal-free safety assessment of chemical ingredients: Achievements of SEURAT-1', was a hint that the acronym of **SEURAT-1** also refers back to the French post-impressionist painter Georges Seurat (1859-1891), known for his innovative painting technique called 'pointillism'. Just as Georges Seurat's pictures develop from numerous small, coloured dots, which can only be meaningfully interpreted from some distance, the **SEURAT-1** research has delivered new techniques and elements of scientific knowledge forming the individual stones which, together, pave the way towards the ultimate goal of a better science that does not need to use experimental animals. The Symposium reported on the achievements of **SEURAT-1** in this direction.

The Symposium was a great opportunity to learn about recent achievements and their impact in the field of alternative testing strategies. It allowed participants to network with renowned experts and get acquainted with the activities of other ongoing and future initiatives. High-level presentations showcased **SEURAT-1**'s success stories in a practical, accessible manner, while an exhibition allowed for deeper discussions. Furthermore, the participants learned how the extensive research efforts over the last 5 years can translate into solutions for safety assessment ultimately replacing animal testing. Other related ongoing and future initiatives from the EU and the US also contributed, showcasing progress in the field and stimulating exchange and networking.

The detailed programme is given in *Table 4.7*.

***Table 4.7**: Programme of the **SEURAT-1** Final Symposium.*

Plenary sessions	
Chair: Ian Cotgreave, Swetox	
10:00 – 10:30	Opening addresses from the European Commission and Cosmetics Europe *Speakers: Ruxandra Draghia-Akli, European Commission, Director of the Health Directorate at DG RTD* *John Chave, Cosmetics Europe, Director-General* *Lowri Evans, European Commission, Director-General of DG GROW*
10:30–11:40	**High-level presentation on major achievements in SEURAT-1** What is SEURAT-1? *Speaker: Michael Schwarz, University of Tübingen* What has SEURAT-1 achieved? *Speaker: Mark Cronin, Liverpool John Moores University* What is the impact of SEURAT-1 in the international context? *Speaker: Rusty Thomas, U.S. Environmental Protection Agency*
11:40-12:10	**Consequences and next steps** EU-ToxRisk: a new safety sciences flagship programme on the horizon *Speaker: Bob van de Water, Leiden University* SEURAT-1 - Continuing the Successful Journey *Horst Wenck, Beiersdorf, Cosmetics Europe*
12:10–12:30	**Concluding remarks** *Speakers: Ian Cotgreave, Swetox* *Derek Knight, European Chemical Agency* *Arnd Hoeveler, European Commission, Head of Unit Innovative tools, technologies and concepts in health research at DG RTD*

Exhibition	
12:30–14:00	**Finger lunch & Exhibition**
14:00–16:00	**Exhibition composed of two parts**
	Guided educational tour: The visitors will be introduced into the field of non-animal-based toxicity testing by a series of stations aligned in the order of the "SEURAT-1 conceptual framework".
	Scientific exhibition: The scientific results will be displayed on posters and in hands-on demonstrations presented by scientists from the SEURAT-1 cluster projects and other EU and US initiatives.
	SEURAT-1 film: During the exhibition, the SEURAT-1 film will be shown in the auditorium
16:00–18:00	**Coffee & networking**

Overall, the symposium managed to communicate both achievements of the largest ever EU initiative to support the development of non-animal methods to be applied in chemical safety assessment, and also the philosophy developed within the project to tackle difficulties in bridging science and regulatory implementation.

4.10.2 Presentations

The opening addresses were made by Ruxandra Draghia-Akli (European Commission, Director of the Health Directorate, DG RTD), John Chave (Director-General of Cosmetics Europe) and Lowri Evans (European Commission, Director-General of DG GROW). They all stressed the importance of developing more efficient safety assessment of chemicals based on non-animal methods, and how **SEURAT-1** had successfully made the first step towards the challenge of tackling systemic toxicity after repeated long-term exposure.

The first plenary session focussed on the impact of the **SEURAT-1** Research Initiative on science and society. Michael Schwarz (University of Tübingen, COACH) explained how the mission and objectives of the **SEURAT-1** Research Initiative had been achieved and how a new culture of multi-expert collaboration in the scientific world underpinning method development for safety assessment had been created. Mark Cronin (Liverpool John Moores University, COSMOS) provided the audience with an overview of main achievements in the various **SEURAT-1** projects and the overall cluster level outcome. Finally, Rusty Thomas (U.S. EPA) placed the **SEURAT-1** Research Initiative and its outcome into an international

perspective, stressing the importance of international collaboration and supplementary efforts to faster make progress towards modern, animal-free assessment of chemicals.

The plenary continued with two presentations looking at how the outcome and lessons learned from **SEURAT-1** will form the basis for further research. Bob van de Water (Leiden University) presented the new Horizon 2020 flagship on the horizon of innovative safety science, EU-ToxRisk, starting its activities in 2016 (see also section 5.4). Horst Wenck (Cosmetics Europe) commended the success of **SEURAT-1** and said that the outcome fed directly into the Cosmetics Europe Long Range Scientific Strategy currently being finalised.

The two co-chairs of the **SEURAT-1** Scientific Expert Panel (see *Table 1.1* in Chapter 1), Ian Cotgreave (Swetox) and Derek Knight (ECHA), shared their thoughts on the **SEURAT-1** experience with the audience. They saw the initiative as an enormous multi-expertise training event leading to comprehensive added value, especially as regards safety assessment case studies. The read-across case studies strengthened with data from alternative methods were already fit for regulatory use. The morning plenary session was concluded by Arnd Hoevelver (European Commission, Health Directorate, DG RTD) telling the audience that he was impressed by the innovative spirit of **SEURAT-1**, which had resulted in solid ground for EU-ToxRisk to build on further. The audience was then invited to lunch in the exhibition area, where all **SEURAT-1** projects presented posters on their main achievements.

4.10.3 Poster Session

The scientific exhibition consisted of posters and hands-on demonstrations presented by scientists from the projects of the **SEURAT-1** Research Initiative and other EU and US initiatives. It included eight stands as follows:

- The six **SEURAT-1** Research and Technology development projects (6 stands);
- The **SEURAT-1** Case Studies;
- ToxCast (U.S. EPA's Toxicity Forecaster): a US research initiative which generates data and predictive models on thousands of chemicals of interest to the EPA. ToxCast showed high-throughput screening and *in silico* computer simulation methods.

The poster abstracts were included in the Symposium booklet which was handed out to delegates at the event. Since the **SEURAT-1** project achievements and case studies are presented as part of this final volume of the **SEURAT-1** Annual Reports (see chapter 3 and sections 4.2-4.7), we will repeat only the two abstracts from the ToxCast stand in the following. The electronic version of the booklet, including all abstracts, can be found on the **SEURAT-1** website *(http://www.seurat-1.eu/media/SEURAT-1_Symposium-booklet.pdf)*.

EDSP21: High-throughput Screening and Prioritization Supporting the EPA Endocrine Disruptor Screening Program

Presenter: Richard Judson (U.S. Environmental Protection Agency, Office of Research and Development, National Center for Computational Toxicology, Research Triangle Park, North Carolina, USA

The U.S. Environmental Protection Agency (EPA) is developing multiple high-throughput *in vitro* assays and computational models to enhance the ability of the EPA to evaluate chemicals for their ability to be endocrine disruptors. Under the U.S. Endocrine Disruptor Screening Program (EDSP), there are as many as 10,000 chemicals that must be evaluated for their ability to perturb the estrogen, androgen and thyroid signalling systems. Using the current EDSP Tier 1 battery of 11 *in vitro* and *in vivo* assays, evaluating this chemical universe would take many decades. As an alternative, EPA is focusing on using combinations of *in vitro* assays, plus computer models that integrate data across the assays, to identify chemicals that are potential endocrine disruptors. The initial focus is on prioritization of chemicals to be tested in the Tier 1 battery, but in selected cases, the original low-throughput tests will be replaced with higher throughput versions. The first example of this EDSP21 approach is a model that combines data from 18 estrogen receptor (ER) assays to derive a single score for each chemical. Those with high scores are deemed to be highly likely to interact with the estrogen receptor. This model was validated against both *in vitro* literature data, and data from the guideline *in vivo* uterotrophic assay. The performance was high enough that the EPA is allowing the results of the *in vitro* model to be used in lieu of the Tier 1 *in vitro* ER assays and the uterotrophic test. A total of 1800 chemicals have been evaluated in the ER model, and 1000 more are in progress. A similar model for androgen receptor signalling is underway. There is also active research into developing similar approaches for the thyroid hormone signalling system. All of the data from the EDSP21 program is being made publicly available through the EDSP21 dashboard: *http://actor.epa.gov/edsp21*.

The views expressed in this presentation are those of the author and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency.

In Silico Dynamics: Computer Simulation in a Virtual Embryo

Presenter: Thomas B. Knudsen (U.S. Environmental Protection Agency, Office of Research and Development, National Center for Computational Toxicology, Research Triangle Park, North Carolina, USA)

Evaluating and assessing impacts to development is an Agency priority (EPA's Children's Environmental Health Research Roadmap); however, the quantity of chemicals needing assessment and challenges of species extrapolation require alternative approaches to traditional animal studies. One approach is to profile the human exposure universe of chemicals

with HTS assays (*in vitro*) and then build computational (*in silico*) models that integrate these data with biological knowledge representing human development. Imputing HTS data into spatially-dynamic computer models of developmental signalling networks can then be used to simulate how an embryonic system might respond to a disturbance in the maternal environment. An *in silico* strategy with virtual tissue models can yield theoretical answers to relevant questions that are not attainable experimentally. This exhibition will demonstrate a workflow to build a cell agent-based computer model, seed it with HTS data, analyse cellular response networks and emergent properties, and compare simulations to adverse outcomes. This exploratory platform may be useful to evaluate chemical effects on development, such as disruption of cardiovascular development (angiodysplasia), palatal fusion (cleft palate), limb outgrowth (ectrodactyly) and urethral fusion (hypospadias) among other systems. Simulations of AOPs for embryonic disruption in a 'Virtual Tissue Laboratory System' can be built with biological information and parameterised with *in vitro* data for chemical prioritisation and early lifestage exposure considerations.

The views expressed in this presentation are those of the author and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency.

4.10.4 Guided Educational Tour and SEURAT-1 Film

There was also the opportunity to take part in a guided educational tour, which was developed to guide interested participants, who are not necessarily experts in the field, through a virtual 'case' in which non-animal testing tools and methods are combined to answer the question of whether a new compound (a new ingredient in a shampoo, for example, called chemical X) is safe for consumers when used in a defined exposure context. The tour was organised as a series of stations addressing the various steps required to answer a safety assessment question. The **SEURAT-1** conceptual framework (see section 4.9.10) was used as the backbone of this exercise, and 13 stations covered the definition of the case and the exposure and effect scenarios to be considered as part of the safety assessment procedure up to the description of the uncertainties as intrinsic properties of the methods used (see *Figure 4.92*). Posters were combined with hands-on exercises to showcase how available non-animal testing tools and methods can be applied in the safety assessment context.

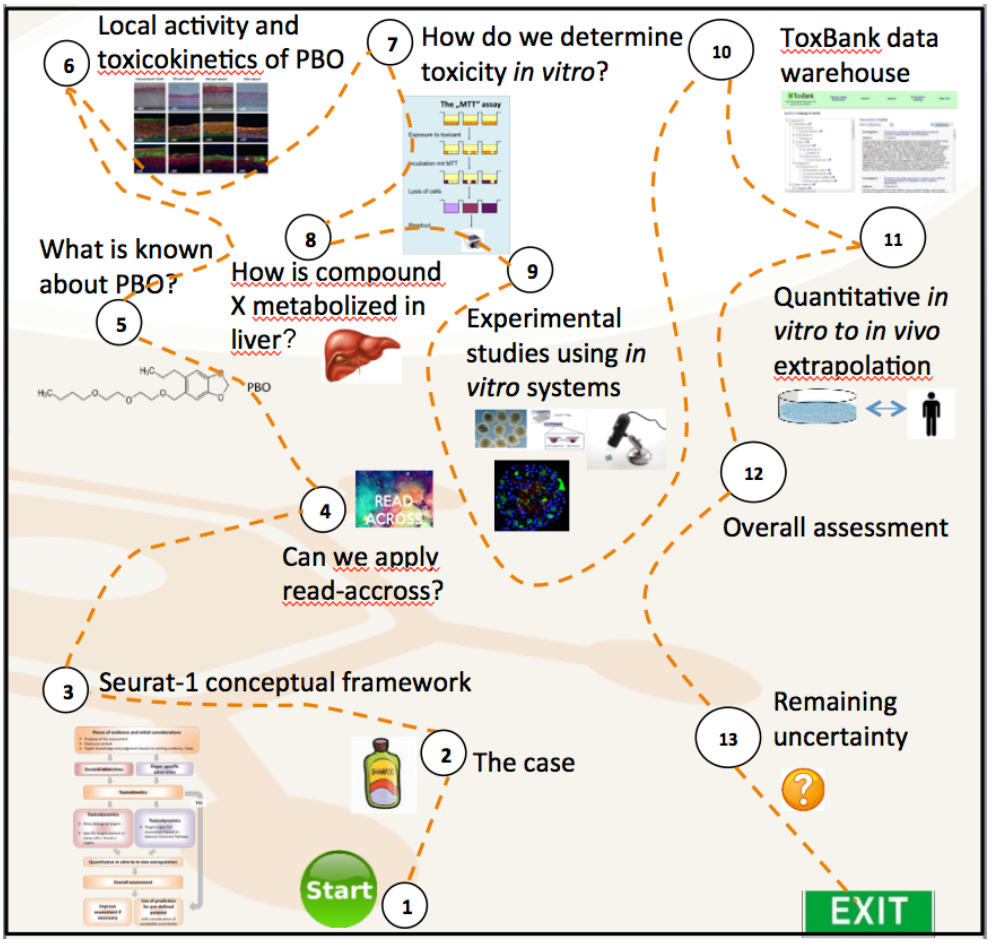


Figure 4.92 *Map of the Guided Educational Tour stations.*

This guided educational tour was summarised in a booklet which is publicly available on the **SEURAT-1** homepage *(http://www.seurat-1.eu/media/Guided%20Tour%20Booklet.pdf).*

Furthermore, a new film summarising the **SEURAT-1** strategy and achievements from the scientific projects in plain language was produced and projected in the auditorium. The 25-minute film, entitled 'SEURAT-1 – Testing Chemicals without Animals', can be watched on the **SEURAT-1** homepage, as well as on YouTube *(https://www.youtube.com/watch?v=Ymzsh9p5pwM).*

4.11 Other Outreach Activities

*The **COACH** Team*

4.11.1 Training Activities

Training activities have been essential for **SEURAT-1** from the very outset. At the instigation of COACH, a common cluster-level training strategy was developed at the beginning of **SEURAT-1**, going beyond the project-level training activities planned in the individual project work-plans. COACH analysed the training activities of the individual projects, drew up a proposal for a cluster training concept, and initiated the **SEURAT-1** Training Task Force (STTF). The STTF became an important cluster task force, meeting annually to discuss the training programme's implementation in the most efficient way.

The first **SEURAT-1** Summer School, held in Portugal in 2012, was the first accomplishment of the STTF. As planned in the training strategy, the next year (2013) was dedicated to individual project training programmes focusing on project level training needs. During 2013, COACH started preparing the organisation of the next cluster-level training activities for 2014 by carefully analysing feedback from the first summer school. Based on this input, the STTF agreed to take the opportunity suggested by the European Society of Toxicology *In Vitro* (ESTIV) and organise a joint summer school in June 2014. This second **SEURAT-1** Summer School, organised in conjunction with ESTIV, turned out to be a very fruitful collaboration, bringing a new dimension to the cluster training activities. In addition to the scientific discussions and networking between participants on their ongoing research and development work, the event brought visibility to **SEURAT-1** and enabled advanced dissemination activities. The feedback received from participants on-site was extremely positive, especially as they could learn something of all different research domains within **SEURAT-1**.

The concept was carried forward to the follow-up project EU-ToxRisk, and the first Summer School under the umbrella of this new project was held in June 2016.

4.11.2 Workshops

Within **SEURAT-1**, a number of workshops were organised by the various Working Groups (see section 4.9.5-4.9.10). Participants were experts (both internal and external) in the respective fields, as the intention was to discuss open questions in the field on a high level and provide suggestions for future activities. In principal, the workshops were intended to be used as a starting point for collaborations between cluster projects of the **SEURAT-1** Research Initiative and other related international activities. Particular attention was given to the mechanistic aspect of the **SEURAT-1** Research Initiative and the formulation of case studies to be addressed by the projects. Under this cluster-level perspective the most important workshops were:

- Mechanisms Underlying Repeated Dose Systemic Toxicity, 14-15 November 2011 at the European Commissions 'Joint Research Centre' (JRC) in Ispra, Italy;
- Exploring Existing Databases for Modes of Action of Repeated Dose Systemic Toxicity, 13-14 November 2011 at the University of Tuebingen, Germany;
- The Development of Case Studies to Define Fit for Purpose Safety Risk Assessment of Repeated Dose Systemic Toxicity, 13-14 November 2011 at the University of Tuebingen, Germany;
- Describing Mode-of-Action in Liver Toxicity Using Adverse Outcome Pathways, 24-25 November 2012 at the European Commissions 'Joint Research Centre' (JRC) in Ispra, Italy;
- Using Mechanistic Information in Developing the Concept of the Adverse Outcome Pathways Relevant to Human Neurotoxicity Evaluation, 21-22 March 2013 at the University of Tuebingen, Germany;
- **SEURAT-1** Meets Tox21, 25-27 June 2013 at the European Commissions 'Joint Research Centre' (JRC) in Ispra, Italy;
- The Read-Across Case Study for Safety Assessment Contributing to the **SEURAT-1** Proof-of-Concept, 29-30 April 2014 at the European Commissions 'Joint Research Centre' (JRC) in Ispra, Italy;
- The *Ab Initio* Case Study for Safety Assessment Contributing to the **SEURAT-1** Proof-of-Concept, 9-10 October 2014 at the European Commissions 'Joint Research Centre' (JRC) in Ispra, Italy.

Following this route, a workshop addressing the application of the methods developed within **SEURAT-1** in the regulatory context was organised by COACH partner JRC in the final year of the **SEURAT-1** Research Initiative. This expert workshop was held in Ispra, Italy, on 6-7 October 2015 with the aim of reviewing progress in the read-across case study (see also section 3.2). The question discussed at this workshop was whether the read-across arguments could be improved sufficiently for regulatory acceptance and where further enhancement could be sought.

While the focus of the workshops in the first years was to stimulate collaborations between the various **SEURAT-1** projects by means defining case studies, the motivation in the later years was more centred on the implementation of the developed tools and methods in the regulatory context. COACH thus significantly supported the organisation of the workshop organised by the European Chemicals Agency (ECHA) on 19 and 20 April 2016 in Helsinki. The workshop was entitled 'New Approach Methodologies in Regulatory Science', and a summary report can be found in section 2.3.

Furthermore, a collaboration workshop of EURAL ECVAM, **SEURAT-1** and EU-ToxRisk, entitled 'Exploring the Regulatory Use of Alternative Approaches in Toxicology for the Safety

Assessment of Chemicals', was held on 9 and 10 November 2016 in Ispra (Italy). An outline of this event is summarised in section 5.3.

4.11.3 Conferences

51st Congress of the European Societies of Toxicology (EUROTOX 2015)

The fifth volume of the **SEURAT-1** Annual Report was launched at the 51st Congress of the European Societies of Toxicology (EUROTOX) in Porto, Portugal, in September 2015. Several dissemination activities accompanied the book launch, ensuring high visibility of **SEURAT-1** at this major event in the field of toxicology, including:

- A specific **SEURAT-1** workshop entitled 'New approaches to repeated dose toxicity assessment - are we ready to replace animal testing?' The session started with an overview of the **SEURAT-1** approach given by Maurice Whelan (JRC). This was followed by a presentation from Steven Wink (LU) about the high-throughput reporter platform developed in DETECTIVE. Jan Hengstler (IFADO) then gave an overview of toxicity assessment in 3D liver models compared to intact livers. Leo van Grunsven further presented the use of adverse outcome pathways in liver fibrosis using a 3D co-culture system representing the functions of the liver. Finally, George Daston (Procter & Gamble) presented how data on non-standard methods for chemical risk assessment can be exploited.
- The NOTOX project held a sponsored symposium session presenting results of the project with presentations from several NOTOX partners focusing on models and methods for hepatotoxicity assessment (see section 4.9.1).
- A **SEURAT-1** display within the JRC ECVAM booth was available to all EUROTOX visitors throughout the congress. Its main objective was to attract stakeholders and other participants interested in the research initiative, but also those previously unaware of its existence, and to inform them of **SEURAT-1** activities via direct discussions and promotional hand-outs, including leaflets, USB keys, Annual Reports and roll-up banners. The fifth volume of the Annual Report also included a 'save-the-date' postcard for the upcoming **SEURAT-1** Symposium (see previous section 4.10). Discussions and bilateral interviews between visitors and key **SEURAT-1** members attending EUROTOX were encouraged to enhance interactions and networking. The stand was also a strategic meeting point for all members of **SEURAT-1** who attended the event.

The Society of Toxicology's (SOT) 55th Annual Meeting

A presentation entitled 'SEURAT/EU Tox-Risk Update', delivered by Michael Schwarz (COACH), took place during the 55th SOT Annual Meeting held in New Orleans, United States, in March 2016.

Other conferences

During the period covered by this annual report, the **SEURAT-1** Research Initiative was further represented via its projects at a number of international conferences.

Further activities and conferences during the last year are summarised in *Table 4.8.*

***Table 4.8** Presence of the **SEURAT-1** Research Initiative at international conferences and workshops.*

Conference	Date	Place	Contribution	Project
54th SOT Annual Meeting	22-26 March 2015	San Diego, USA	Poster and oral presentations	COACH COSMOS
50th Annual Meeting of the European Association for the Study of the Liver (EASL)	22-26 April 2015	Vienna, Austria	Poster and oral presentations	*HeMiBio* NOTOX
25th Annual Meeting of the Society of Environmental Toxicology and Chemistry (SETAC)	3-7 May 2015	Barcelona, Spain	Poster and oral presentations	COACH COSMOS
2nd International Conference Of Alternatives to Animal Experimentation	8-9 May 2015	Lisbon, Portugal	Oral presentation	COSMOS *HeMiBio* NOTOX
8th International Symposium on Computational Methods in Toxicology and Pharmacology Integrating Internet Resources	21-25 June 2015	Chios, Greece	Poster and oral Presentation	COSMOS
International Society for Stem Cell Research (ISSCR)	24-27 June 2015	Stockholm, Sweden	Poster and oral Presentations	*HeMiBio* *SCR&Tox*
Center for Alternatives to Animal Testing (CAAT) Read-Across Workshop	8 October 2015	Baltimore, USA	Oral Presentation	COSMOS
51st Congress of the European Societies of Toxicology	13-16 September 2015	Porto, Portugal	Workshop Session & JRC booth	All
19th European Congress on Alternatives to Animal Testing	20-23 September 2015	Linz, Austria	Poster and oral Presentations	COACH DETECTIVE ToxBank
OPENTOX EURO 2015	30 September - 2 October 2015	Dublin, Ireland	Poster and oral presentation	ToxBank
18th International Symposium on Cells of the Hepatic Sinusoid	11-13 November 2015	Asilomar, USA	Poster and oral presentation	*HeMiBio*
The Liver Meeting of the American Association for the Study of Liver Diseases (AASLD)	13 November 2015	San Francisco, USA	Poster and oral presentation	*HeMiBio*

SOT 55th Annual Meeting	13-17 March 2016	New Orleans, USA	Poster and oral presentation	COACH COSMOS
The International Liver Congress™ of the European Association for the Study of the Liver (EASL)	13-17 April 2016	Barcelona, Spain	Poster and oral presentations	*HeMiBio*
ECHA Topical Scientific Workshop - New Approach Methodologies in Regulatory Science	19-20 April 2016	Helsinki, Finland	Poster and oral presentations	COACH COSMOS
17th International Conference on QSAR in Environmental and Health Sciences	13-17 June 2016	Miami Beach, USA	Poster and oral presentations	COSMOS
2nd Annual Organ-on-a-Chip World Congress & 3D-Culture Conference	7-8 July 2016	Boston, USA	Poster and oral Presentations	NOTOX
20th European Congress on Alternatives to Animal Testing	24-27 August 2016	Linz, Austria	Oral Presentation	COACH
Regulations & Safety in Cosmetics Conference	21-22 September 2016	Munich, Germany	Oral Presentation	COACH

4.11.4 SEURAT-1 Public Website

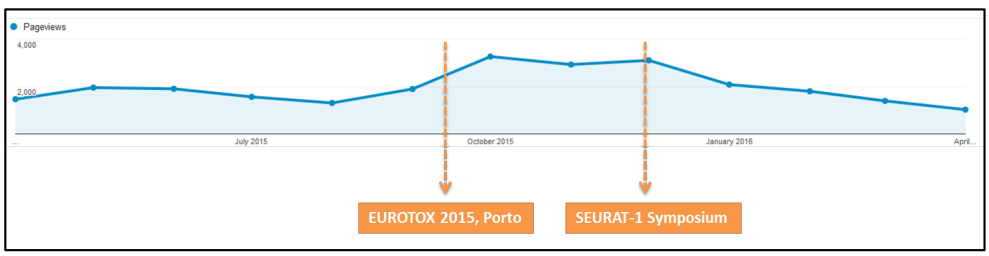
The **SEURAT-1** public website *(http://www.seurat-1.eu)*, one of the key channels of **SEURAT-1** outreach activities, was launched in 2011. The website disseminates information from the cluster to a large audience of stakeholders, scientists and the general public, and serves as a source of statistically important information regarding the impact of the dissemination activity.

The content of the website (general overview of the **SEURAT-1** Research Initiative, detailed information about the objectives and results, its future vision and strategy, the work structure, overviews of the seven cluster projects involved and their contributions) is updated regularly. New cluster-level and individual projects' dissemination material, such as the COACH film, is immediately published in the 'Publications' section; the 'Who is Who' section is now a

well-known depository of important information about the experts involved in **SEURAT-1**; the 'Bibliography' contains articles about **SEURAT-1** and the domain of alternative testing in general; and the section 'Online library', launched in early 2015, lists the scientific publications resulting from **SEURAT-1** research work. The website also provides information on upcoming events and training activities and other important news within the cluster.

In 2016, as part of mass media outreach activities, a 'Press corner' was created targeting the general public in particular. It contains press releases and articles about **SEURAT-1,** as well as links to various information sources (films, project brochures, FAQs, etc.).

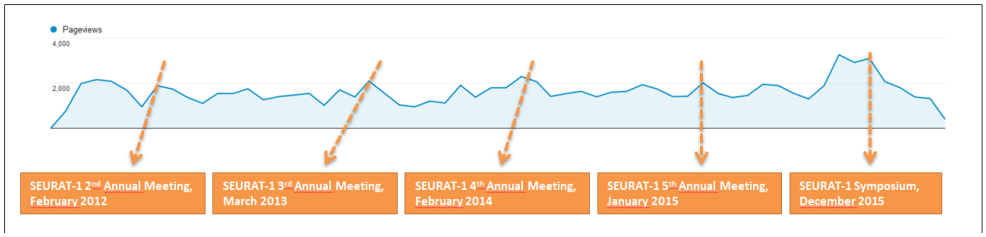
The following figure (*Figure 4.93*) gives an overview of visits to the public website for the period from April 2015 until April 2016. The busiest period in terms of site visitors was between the 51st Congress of the European Societies of Toxicology (EUROTOX 2015) and the **SEURAT-1** Final Symposium.



	Country	Sessions	% Sessions
1.	France	1,339	11.91%
2.	Belgium	1,327	11.80%
3.	United States	1,325	11.79%
4.	Germany	1,276	11.35%
5.	United Kingdom	1,029	9.15%
6.	Italy	699	6.22%
7.	Japan	466	4.14%
8.	Netherlands	381	3.39%
9.	China	267	2.37%
10.	Bulgaria	250	2.22%

Figure 4.93 SEURAT-1 *public website statistics for the past year (April 2015 to April 2016): number of site visitors and visitor countries (source: Google Analytics).*

Figure 4.94 summarises visits to the public website for the period from June 2011, which marked the launch of the website, until April 2016. This covers almost the total duration of the **SEURAT-1** Research Initiative. The total number of page views since the launch is 96,650. The busiest periods in terms of site visitors were linked with the **SEURAT-1** annual meetings.



	Country	Sessions	% Sessions
1.	France	5,144	13.41%
2.	Germany	4,915	12.81%
3.	United Kingdom	4,150	10.82%
4.	United States	4,067	10.60%
5.	Belgium	3,328	8.68%
6.	Italy	2,961	7.72%
7.	Japan	1,267	3.30%
8.	Brazil	1,251	3.26%
9.	Netherlands	926	2.41%
10.	Spain	860	2.24%

Figure 4.94 SEURAT-1 *public website statistics for the period from June 2011 until April 2016: number of site visitors and visitor countries (source: Google Analytics).*

4.11.5 SEURAT-1 Dissemination Materials

Various items of dissemination material were created or updated during 2015 and early 2016. In particular, a *new video* was produced explaining in a simple language the alternative methods developed in **SEURAT-1** and how they will affect ongoing efforts in the field of the replacement of animal testing (*Figure 4.95*). The video was launched at the **SEURAT-1** Final Symposium on 4 December 2016 (see section 4.10.4). The feedback on the **SEURAT-1** video was extremely positive.

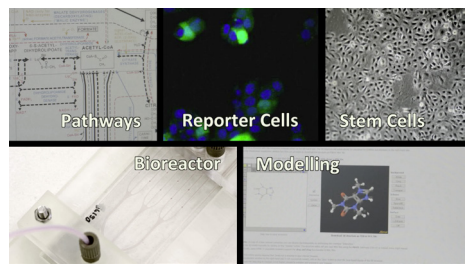


Figure 4.95 *Screenshots from the new* ***SEURAT-1*** *video. The video summarises the achievements of the* ***SEURAT-1*** *Research Initiative and can be watched on YouTube (https://www.youtube.com/watch?v=Ymzsh9p5pwM#t=45).*

In connection with the **SEURAT-1** Final Symposium, COACH produced a *new leaflet* with highlights from the **SEURAT-1** Research Initiative, both for each project and on cluster level. A *booklet* was created for and distributed in advance of the Final Symposium, including the programme, a map of the guided tour and abstracts from the scientific exhibition (see also section 4.10). In addition, *new USB keys* were produced and distributed at the Final Symposium (incorporated into the badge holder), which included the five volumes of the Annual Report, the **SEURAT-1** highlights leaflet, and the **SEURAT-1** strategy paper (published earlier).

Moreover, following the success of the guided tour held at the **SEURAT-1** Final Symposium (see section 4.10.4), a stand-alone *guided tour brochure* was produced by COACH including the posters used in the tour and associated explanations. This brochure was and will be disseminated in numerous workshops and conferences during the last year of the COACH project. The posters presented at the Guided Tour were also reused in several events, including the Cosmetics Europe Week in June 2016 and the EURL ECVAM / **SEURAT-1** / EU-ToxRisk stakeholders' workshop in Ispra, Italy, in November 2016.

4.11.6 Outreach in Mass Media

In 2015, COACH invested important efforts in the communication on and representation of the **SEURAT-1** Research Initiative in mass media, with the support from i) a communication consultant; and ii) a freelance journalist with publication experience in the alternative testing domain. COACH has achieved the objectives of informing the general public through the following activities:

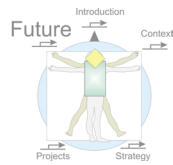
- Producing a detailed plan for dissemination in mass media, including the definition of the main objectives, procedures for validation of dissemination materials, concrete actions and associated timetables and budgets;
- Contact with over 70 journals and magazines, including newspapers from across Europe (supported by the communication expert);
- Four press releases written in accessible language were published in 2015:
  - The first, published in April 2015, intended to attract the attention of science journalists to the **SEURAT-1** Research Initiative;
  - The second was published in June 2015, further to the European Citizen's Initiative 'Stop Vivisection', and gave **SEURAT-1**'s perspective on the issue;
  - The third press release was published in September 2015, in connection with the EUROTOX 2015 congress, to communicate about the achievements presented at EUROTOX and provide information on the upcoming **SEURAT-1** Final Symposium on 4 December 2015;

- The fourth press release, published on the occasion of the **SEURAT-1** Final Symposium, highlighted the most significant results of the initiative.

All press releases were sent to the 70 contacts made earlier and were also posted on the Alpha Galileo web service linking research with the media *(http://www.alphagalileo.org)*. Press offices at partner organisations also received the press releases for further distribution in their networks.

➠ A press corner was created on the **SEURAT-1** website, where all press releases and other information considered useful for journalists were posted.

Overall, the **SEURAT-1** dissemination strategy was successfully implemented and increased the visibility of the research initaitve substantially.



5 PREPARING FOR THE FUTURE

"Nothing is less predictable than the development of an active scientific field."

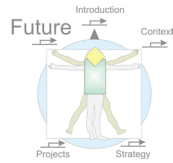
Charles Francis Richter

5.1 Introduction

Tilman Gocht, Michael Schwarz

Taking into account the complexity of the problems to be solved and the broadness of the expertise needed to address the underlying scientific questions, it was clear from the beginning that the **SEURAT-1** Research Initiative would not be able to finalise the necessary work to fully replace animal testing in the area of repeated dose systemic toxicity within the lifetime of the research programme. Indeed, moving from animal testing to mode-of-action based *in vitro* assays for improved human safety assessment requires the combined efforts of European and other international activities. The **SEURAT-1** Research Initiative operated in a very dynamic field of research, and a number of related research projects in different parts of the world were also active at the same time. This chapter provides an overview of these parallel research programmes by presenting short descriptions and collaborations that were set up with the **SEURAT-1** Research Initiative.

The aim was, in fact, to establish close international cooperation over the course of **SEURAT-1**, and to advance scientific progress in this field of research by using the synergy of a collaborative approach. This served as a basis for the identification of knowledge gaps that need to be addressed in the future. In the 4th Annual Report, we highlighted the collaboration with the related initiative in the USA, Tox21, which was set up in 2013 and culminated in the involvement of Tox21 researchers in the **SEURAT-1** read-across case study (see section 3.2). In 2014, the focus was on exploring options for collaborations with the IMI MIP-DILI project and, in the last year, the follow-up project to the **SEURAT-1** Research Initiative was selected and started in January 2016, under the umbrella of the European Commission's funding scheme, Horizon 2020. The follow-up project is called 'EU-ToxRisk – An Integrated European 'Flagship' Programme Driving Mechanism-based Toxicity Testing and Risk Assessment for the 21st Century' and one of the major tasks in 2016 was to support this new consortium during the initial phase and hand over the results and achievements of the **SEURAT-1** Research Initiative. These efforts culminated in a collaboration workshop between the **SEURAT-1** Research Initiative, the EU-ToxRisk Project and EURL ECVAM entitled 'Exploring the Regulatory Use of Alternative Approaches in Toxicology for the Safety Assessment of Chemicals', which was held on 9 and 10 November 2016 in Ispra (Italy). A brief description of this workshop is given in section 5.3. The final section of this chapter then provides an overview of the goals and approach of this new initiative that takes over the challenging long-term strategic target, SEURAT ('Safety Evaluation Ultimately Replacing Animal Testing'). The project need not start from the beginning, as the **SEURAT-1** Research Initiative left a solid foundation on which the EU-ToxRisk Project can build its activities. This section 5.4 completes this sixth and final **SEURAT-1** Annual Report and the entire book series, and it is the hope of the editors that the



book series will serve as a valuable resource not only for the EU-ToxRisk Project but also for other related research initiatives working towards the strategic target SEURAT.

5.2 Related International Activities

Tilman Gocht, Michael Schwarz

The following sections provide an overview of parallel research activities and highlight collaborations between **SEURAT-1** and other consortia. The descriptions have been kept very brief and were, in parts, taken directly from published descriptions of corresponding projects. The sources used are given at the end of each project summary (in general, this refers to a public webpage). Only currently running activities (research projects as well as institutions) or those that ended in 2015 are considered in this compilation.

5.2.1 European Activities

EU Horizon 2020: The EU Framework Programme for Research and Innovation

The European Commission's current funding scheme, Horizon 2020, combines the aspects of three separate initiatives into one single programme: It is the follow-up programme of the 7th Research Framework Programme (FP7), incorporating innovation aspects from the Competitiveness and Innovation Framework Programme (CIP) and the EU contribution to the European Institute of Innovation and Technology (EIT). In total, €80 billion in funding will be made available between 2014 and 2020.

Besides highlighting excellent science, Horizon 2020 prioritises industrial leadership and plans to provide investments in key industrial areas, including biotechnology. Societal challenges are the third priority for future investments under Horizon 2020, reflecting the policy priorities of the Europe 2020 strategy. Major concerns shared by citizens in Europe and elsewhere are addressed, and the area of 'Health, Demographic Changes and Wellbeing' was identified as one of seven societal challenges on which funding is focused (although EU support of health-related research and innovation is not limited to this particular societal challenge). Topics to be addressed include the integration of molecular biological, epidemiological and toxicological approaches, as well as the integration of toxicological testing to seek alternatives to animal testing and to improve human safety assessment. Uptake of research activities by the market is key to the success of applications for funding under Horizon 2020, as this will establish a new focus on innovation-related activities that bridge the gap between fundamental research

and the development of new knowledge-driven products and their implementation into the market.

The work programmes for the years 2014–2015 and for the years 2016–2017 were published and grouped according to the seven societal challenges. The call for proposals that was most relevant to the **SEURAT-1** Research Initiative was published within the work programme of the societal challenge 'Health, Demographic Change and Wellbeing' in the work programme 2014–2015:

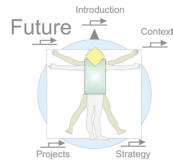
PHC 33 – 2015: New approaches to improve predictive human safety testing (within the area 'Improving health information, data exploitation and providing an evidence base for health policies and regulation'): Proposals should focus on approaches that improve the efficiency of predictive toxicological testing to address key areas of concern for human health and meet regulatory requirements. Proposals should capitalise on advances in all relevant fields of science to understand complex biological pathways of toxicological relevance and to identify early markers predictive of toxicological effects in humans. The objectives are:

- To develop and validate routine, non-animal approaches for toxicity testing of chemical substances (excluding radio-chemicals);
- To develop methodologies for confirmatory testing of mechanistic hypotheses to improve understanding of toxicity mechanisms.

International cooperation has been identified as key to success in research and innovation and, consequently, cooperation was encouraged with similar initiatives in the USA and elsewhere. The expected impact comprises the following issues:

- More effective, faster and cheaper toxicological testing to better predict human risk and meet regulatory needs;
- Improved toxicological knowledge to encourage 'read-across' between chemical substances for use in different research and regulatory domains;
- Commercial exploitation of the developed toxicological testing methods and assessment approaches, products and services;
- Advancement of international co-operation in the field of predictive toxicology and human safety testing;
- Reduced use of laboratory animals in safety testing.

The date for the submission of proposals was 24 February 2015. The successful consortium was selected subsequently and the project started on 1 January 2016. It is the EU-ToxRisk Project ('EU-ToxRisk – An Integrated European 'Flagship' Programme Driving Mechanism-based Toxicity Testing and Risk Assessment for the 21st century') and since the relationships between the objectives, the expected impacts listed above and the outcomes of the **SEURAT-1**



Research Initiative are obvious, COACH contacted the successful consortium at an early stage. Exchange activities culminated in the organisation of a joint workshop (see section 5.3). Further details about the most important **SEURAT-1** follow-up activity can be found in section 5.4.

The work programme of the societal challenge 'Health, Demographic Change and Wellbeing' for the years 2016–2017 has a very strong focus on personalised medicine and is less relevant for the field of the development of alternatives to animal testing. However, relationships exist between **SEURAT-1** activities and calls for proposals published under the societal challenge 'Nanotechnologies, Advanced Materials, Biotechnology and Advanced Manufacturing and Processing', particularly in the area 'Science-Based Risk Assessment and Management of Nanotechnologies, Advanced Materials and Biotechnologies'. Besides others, the goal in this area is to develop adequate knowledge of interactions between engineered nano-materials and Biota to engineer-out or reduce non-acceptable risks through the usage of such materials. Perception of risks resulting from chronic or acute exposure should be based on science-based risk analysis. The most relevant call for the **SEURAT-1** Research Initiative (or follow-up projects) in this area is

NMBP-29-2017: Advanced and realistic models and assays for nanomaterial hazard assessment': The call recognises that the classical toxicological testing paradigm so far focusing on *in vivo* testing is gradually but steadily shifting towards *in vitro* and *in silico* testing approaches. Proposals should address knowledge gaps with respect to long-term effects (low doses, chronic exposure), both for human health and the environment, and should also consider the toxicokinetics. High priority should be on the development of *in vitro* tests with special emphasis on the creation of appropriate biological models (co-culture models, 3D cultures or primary cell models). The improved predictive power of *in vitro* and *in silico* approaches as compared with *in vivo* systems should be demonstrated as a basis for the regulatory acceptance of the new approaches. These activities may mirror the **SEURAT-1** work on chemicals in the field of particle toxicity.

Furthermore, two other calls in this working area may be of relevance for **SEURAT-1** follow-up projects:

- *NMBP-26-2016: 'Analytical techniques and tools in support of nanomaterial risk assessment'* (the aim is to develop new, or further improve, relevant analytical methods and corresponding equipment, relevant to hazard and exposure testing strategies, that enable characterisation of ensembles of nanomaterial properties including longer term fate of particles following their interactions with living systems);

- *NMBP-28-2017: 'Framework and strategies for nanomaterial characterisation, classification, grouping and read-across for risk analysis* (the aim is to support grouping of engineered nano-materials for further risk analysis, to help in

developing intelligent testing strategies and identifying 'properties of concern of engineered nano-materials' that need to be tested more thoroughly).

More information: http://ec.europa.eu/programmes/horizon2020/

EU FP7: 7th Framework Programme of the European Union represented by the European Commission

Funding in the field of predictive toxicology within the previous European Union's funding scheme for research and innovation, FP7, which was active until 2013, was organised within the Health Theme. Besides the **SEURAT-1** Research Initiative, a number of projects were active during the funding period of **SEURAT-1**, the last one is still active and is briefly described in the following:

HeCaToS *(Hepatic and Cardiac Toxicity Systems Modelling)*: HeCaToS is a collaborative large-scale integrated project funded within the European Commission's 7th Framework Programme (FP7) under the Health Theme. HeCaToS started in 2013 and will run until 2018. A total of 14 European participants from different scientific sectors (academia and industry) are working on this project. The overall goal is the development of integrative *in silico* tools for predicting human liver and heart toxicity.

The overall objective of HeCaToS is to develop an integrated framework for modelling toxic perturbations in liver and heart across multiple scales. Advances in computational chemistry and systems toxicology will be combined for this purpose and case studies based on biopsies from patients suffering from liver injuries or cardiomyopathies due to adverse drug effects will be developed. Particular attention will focus on adverse outcome pathways related to mitochondrial deregulations and immunological dysfunctions.

Scientific Coordinator: Jos Kleinjans (University of Maastricht, The Netherlands)

More information: http://www.hecatos.eu/

*Cooperation with **SEURAT-1***: Given the focus on liver and heart toxicity and adverse outcome pathways related to specific diseases, the relevance to the **SEURAT-1** Research Initiative is obvious. The scientific coordinator of HeCaToS, Jos Kleinjans, was invited to the fourth **SEURAT-1** Annual Meeting held in February 2014, where he gave an overview presentation about the objectives of and methods used in the HeCaToS Project as a starting point for identifying areas of cooperation.

IMI: Innovative Medicines Initiative

The Innovative Medicines Initiative Joint Undertaking (IMI JU) is a pan-European public–

private partnership between the European Commission and the European Federation of Pharmaceutical Industries and Associations (EFPIA), driving collaboration between all relevant stakeholders, including large and small biopharmaceutical and healthcare companies, regulators, academia and patients to improve the drug development process. Typical IMI consortia consist of partners from academia and industry including SMEs. The IMI research projects that are selected for funding through open calls for proposals must adhere to the four major axes of research defined in the strategic research agenda for the second funding period (IMI 2) from 2014 – 2024: (i) target validation and biomarker research (efficacy and safety); (ii) adoption of innovative clinical trial paradigms; (iii) innovative medicines; and (iv) patient-tailored adherence programmes. In total, four calls for proposals were launched in 2014 (IMI 2 Call 1 – Call 4), another four calls in 2015 (IMI 2 Call 5 – Call 8) and one call in 2016 until April (IMI 2 Call 9). The goals of IMI 2 are to develop next generation vaccines, medicines and treatments. The most relevant IMI projects for the **SEURAT-1** activities are briefly described below.

More information: http://www.imi.europa.eu/

The topic 'Validation of translational imaging methods in drug safety assessment (TRISTAN)' was addressed in the IMI 2 Call 7 and stage 2 proposals were under review at the end of 2016.

More information: https://www.imi.europa.eu/content/imi-2-call-7-0

The topic 'Development of Quantitative System Toxicology (QST) approaches to improve the understanding of the safety of new medicines' was addressed in the IMI 2 Call 6. The submission deadline for stage 2 proposals was 14 June 2016 and the selection of the successful consortium was ongoing at the end of 2016.

More information: https://www.imi.europa.eu/content/stage-1-17

EBiSC (*European Bank for induced pluripotent Stem Cells*): EBiSC is funded by the Innovative Medicines Initiative Joint Undertaking (IMI JU). The project started in 2014 and did run until end of 2016. 25 partner institutions were involved in EBiSC. The overall goal of the project was to establish a European iPS cell bank that should become the 'go-to' resource for the characterisation, storage and distribution of high quality iPS cells. EBiSC is a centralised, not-for-profit iPSC bank providing researchers across academia and industry with access to scalable, cost-efficient and consistent, high quality tools for new medicines development. The project established a robust, reliable supply chain from the generation of cell lines, over the specification, to internationally accepted quality criteria and their world-wide distribution to any qualified user.

The mission is that academic institutions, biotech companies, and large pharmaceutical companies can store and access high-quality, well-characterised iPS cells covering a range of disease areas as well as cells from healthy donors. The bank provides standardised protocols for the storage, retrieval, culture, and differentiation into different cells types, plus a searchable catalogue where cells can be requested based on specific characteristics or disease areas. Samples of the cell lines in the catalogue can be shipped to scientists around the world. The project partners themselves generated and deposited the first lines to the bank, but the project also received deposits from other projects and organisations. Once the initial collection of cell lines was in place, the project quickly started distributing cells to the scientific community. The project did then scale up its activities to become fully operational by 2016. The objective was for the initiative to be self-sustaining financially by 2019 and become an independent legal entity, distributing iPS cells worldwide on a not-for-profit basis.

Scientific Coordinator: Timothy Allsopp (Pfizer Ltd, Basel, UK)

More information: http://www.ebisc.org/

*Cooperation with **SEURAT-1***: The **SEURAT-1** Stem Cell Working Group, comprised of researchers from the *SCR&Tox* and ToxBank projects, shared the goal of offering well-characterised cell-lines and standard operating procedures. There is an overlap between the EBiSC consortium and **SEURAT-1** projects, which ensured exchange activities.

StemBANCC *(Stem cells for biological assays of novel drugs and predictive toxicology):* The IMI project StemBANCC started in 2012 and did run for five years. In total, 35 European participants are involved in StemBANCC with the aim of generating 1,500 high-quality human iPS cell lines from 500 people that can be used by researchers to study a range of diseases and test for drug efficacy and safety. Mainly skin and blood samples were taken from patients with certain diseases, people who displayed adverse reactions to drugs, and healthy individuals. The cells were re-programmed until they reach their pluripotent status and characterised in terms of their genetic, protein and metabolic profiles. All cell lines also underwent a rigorous quality check. The project also investigated the use of these cell lines for toxicity testing and generated liver, heart, neuron and kidney cells for this purpose.

A key objective of StemBANCC was to deliver a bio-repository of well-characterised human iPSCs from different disease groups. Key components in the work programme included: (i) the provision of biomaterials and bio-data; (ii) cellular phenotypic discovery; and (iii) assay development and validation.

Project Coordinator: Martin Graf (F. Hoffmann-La Roche Ltd, Basel, Switzerland)

More information: http://stembancc.org/

MIP-DILI *(Mechanism-based integrated systems for predicting drug-induced liver injury):* Another IMI project is MIP-DILI, which started in 2012 and did run for five years. MIP-DILI brought together 26 partners from academia and industry with the aim of developing improved tools for liver toxicity testing in the early stages of the drug development process. This requires a deepened understanding of the science behind drug-induced liver injury and the use of that knowledge to overcome the many drawbacks of the tests currently used.

Cultures of liver cells in one-dimensional and three-dimensional configurations were evaluated; the latter integrated different types of liver cells to form three-dimensional units that accurately mimic human liver physiology. Natural differences between patients were taken into account through the generation of iPS cell lines from patients who were particularly sensitive to drug-induced liver injury. The objectives of MIP-DILI were to

- Identify and validate an improved panel of *in vitro* 'best practice assays' for predicting DILI in the human population;
- Explore and understand the relationship between *in vitro* assay signals and DILI *in vivo*, in preclinical test species and in humans;
- Develop and validate novel systems modelling approaches that integrate multiple preclinical data types to improve prediction of DILI in humans;
- Enhance shared understanding of the value and limitations of new and existing approaches for DILI hazard identification and risk assessment between academia, pharmaceutical and regulatory agencies.

Project coordinator: Kevin Park, University of Liverpool, UK

More information: http://www.mip-dili.eu/

*Cooperation with **SEURAT-1***: The importance of drug-induced liver injury within the **SEURAT-1** Research Initiative is clearly shown in the theoretical mode-of-action descriptions as part of the **SEURAT-1** proof-of-concept case studies. Liver fibrosis, cholestasis and steatosis are addressed in these case studies, and the elucidation of mechanisms as the basis for the development of toxicity testing is the focus of interest. Exchange activities were initiated through the invitation of the MIP-DILI coordinator, Kevin Park, to the **SEURAT-1** workshop 'Mechanisms underlying repeated dose systemic toxicity' held in November 2011 in Ispra and culminated in a telephone conference between **SEURAT-1** and MIP-DILI partners in December 2014, in which possible areas of collaboration were further explored.

eTOX *(Integrating bioinformatics and chemoinformatics approaches for the development of expert systems allowing the in silico prediction of toxicities):* eTOX (also funded by IMI) was started in 2010 and did run for five years. The consortium comprised 25 partners. The aims of eTOX were to develop (i) a drug safety database from the pharmaceutical industry

legacy toxicology reports and public toxicology data, and (ii) innovative *in silico* strategies and novel software tools to better predict the toxicological profiles of small molecules in the early stages of the drug development pipeline. This was achieved by jointly storing and exploiting private data from the participating European Federation of Pharmaceutical Industries and Associations (EFPIA) companies, as well as publicly available data, and by coordinating the efforts of specialists from EFPIA pharmaceutical companies, relevant SMEs and academic institutions. The strategy included a synergetic integration of innovative approaches in the following areas:

- Database construction and management, including procedures and tools for protecting sensitive data;
- Ontologies and text mining techniques, with the purpose of facilitating knowledge extraction from legacy preclinical reports and biomedical literature;
- Chemistry- and structure-based approaches for the molecular description of the studied compounds, as well as their interactions with the anti-targets responsible for the secondary pharmacologies;
- Prediction of DMPK features, since they are often related to the toxicological events;
- Systems biology approaches in order to cope with the complex biological mechanisms that govern *in vivo* toxicological problems;
- Computational genomics to afford the inter-species and inter-individual variability that complicates the interpretation of experimental and clinical outcomes;
- Sophisticated statistical analysis tools required to derive the inevitably highly-multivariate QSAR models;
- Development and validation (according to the OECD principles) of QSARs, integrative models, expert systems and meta-tools.

Project coordinator: Francois Pognan, Novartis, Basel, Switzerland

More information: http://www.e-tox.net/

*Cooperation with **SEURAT-1***: eTOX operated in many fields that were related to the **SEURAT-1** Research Initiative. A representative of eTOX was invited to the **SEURAT-1** workshop 'Exploring Existing Databases for Modes-of-Action of Repeated Dose Systemic Toxicity' held in 2012. The databases and tools compiled and developed within eTOX is an important resource for identifying key events within an adverse outcome pathway. Thus, database mining was identified as an important field for collaboration with eTOX and it was agreed that eTOX could provide some support in the refinement of mode-of-action descriptions through the elucidation

of additional key events. This led to a collaboration between the **SEURAT-1** project COSMOS and eTOX regarding the development of computational profilers for hepatotoxicity and mining of repeated dose toxicity data.

SAFE-T *(Safer and Faster Evidence-based Translation):* Another IMI project was SAFE-T, which started in 2009 and was terminated in June 2015. Overall, 20 partner organisations worked together to improve the drug development process through the development of tools for the prediction, detection and monitoring of drug-induced injuries to the kidney, liver and vascular system, using markers in patients' blood and/or urine. The ultimate goal was to identify a set of biomarkers for each of the three organ toxicities that are more specific, more sensitive and more predictive than those currently available, and to gain regulatory acceptance for routine use of these biomarkers in drug development.

The specific objectives were to:

- Evaluate the utility of safety biomarkers for monitoring organ safety in humans;
- Develop assays and devices for clinical application of safety biomarkers;
- Gather sufficient evidence to qualify safety biomarkers in clinical drug development and in translational contexts in cooperation with the health authorities, such as the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA);
- Gain evidence for how safety markers may also be used in the diagnosis of diseases and in clinical practice.

Project coordinator: Michael Merz, Novartis, Basel, Switzerland

More information: http://www.imi-safe-t.eu/

Important Institutions that are Active in SEURAT-1-Related Fields

NC3R^s: The British 'National Centre for the Replacement, Refinement and Reduction of Animals in Research' (NC3R^s), together with Innovate UK, is developing a work programme to accelerate the development and application of non-animal technologies. The programme delivered the following activities:

- The publication of an UK roadmap for non-animal technologies in 2015. This supports industry, research councils, academia and government in establishing a more connected and focussed non-animal technology sector in the UK;
- Two competitions have been run which will provide funding for (i) feasibility

studies and (ii) collaborative research and development;

➠ A Non-Animal Technologies Special Interest Group (NAT SIG) has been set up to connect the research community, shape the UK non-animal technologies sector and provide access to the latest funding opportunities, events and news.

Regarding the funding opportunities, the business sector was invited to apply for funding of up to £1 million to develop new ways to minimise the use of animals in research based on a call for proposals published in August 2016 within the framework of the feasibility studies. The application deadline was 9 November 2016 and the addressed challenges included, besides others, the topic of 'developing reliable predictions for chemical toxicity'. In the framework of collaborative research and development, the Biotechnology and Biological Sciences Research Council, the Engineering and Physical Sciences Research Council and the Medical Research Council made available £6 million. A key aim of the funding is to harness the commercial potential of technologies in this area, including fields related to biological-, tissue engineering and imaging (e.g. cellular engineering and '-omics' technologies), manufacturing-related fields (e.g. high-throughput technologies and microfluidics) and information and communication technology-related fields (e.g. *in silico* approaches and data mining). Ultimately, the aim is to produce better tests and systems that more accurately predict efficacy, safety and environmental effects of new chemicals.

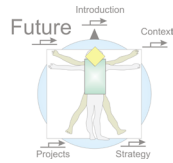
More information: http://www.nc3rs.org.uk/

CEFIC *(The European Chemical Industry Council)*: CEFIC represents 29,000 large, medium and small chemical companies in Europe and it is the forum and voice of the chemical industry in Europe. Most importantly for **SEURAT-1** is the 'Long-range Research Initiative' (CEFIC LRI), which was established as an integral part of CEFIC's innovation strategy to improve the regulatory framework of the chemical industry in Europe. The focus is on gaps in the industry's knowledge and understanding that are critical for risk assessment. Areas where scientific knowledge relevant for both the industry and regulators should be enhanced were identified. Funding is being made available for research in these areas through requests for proposals.

The most relevant request for proposals to the **SEURAT-1** Research Initiative in 2016 was:

➠ LRI-ECO36: Building improved *in vitro* exposure assessment capability (Deadline: 31 August 2016; objectives: (i) development of analytical tools capable of accurately quantifying *in vitro* bioassay dose(exposure)-response relationships for chemicals with a broad range of physicochemical properties; (ii) based on a small number of case studies, provide guidance on how to best assess the behaviour and exposure of non-stable chemicals within *in vitro* systems and application towards extrapolating between *in vitro* to *in vivo* systems.

More information: http://www.cefic-lri.org/ and http://cefic-lri.org/request-for-proposals/



LRI-AIMT5: Towards Building an AOP Prenatal Developmental Toxicity Ontology: This call was published in 2015 as part of the CEFIC LRI and the primary objective of the selected project is to establish a novel Developmental Toxicity Ontology, organising information about Modes of Action (MoA) and their relationships with Adverse developmental toxicity Outcomes (AO) through an understanding of normal embryology. The construction of a developmental toxicity ontology will be undertaken by integrating MoA and AOP information from three different perspectives, the biological, the chemical and the toxicological perspective. Information from these three points of view will be integrated into a formal Developmental Toxicity Ontology. The project started in April 2016 and will run until April 2018.

Project coordinator: Aldert Piersma, RIVM, National Institute for Public Health and the Environment, Bilthoven, The Netherlands

More information: http://cefic-lri.org/projects/aimt5-bulding-a-prenatal-developmental-toxicity-ontology-integrating-existing-biological-chemical-in-silico-models-and-in-vitro-methods-and-data-aiming-at-an-alternative-integrated-aopmoa-framew/

ECO30-ARC: Expanding the applicability domain of the chemical activity approach for hazard and risk assessment: This call was published in 2014 as part of the CEFIC LRI. The project started in February 2015 and will run until January 2017. The objectives are (i) to link the toxicity of chemicals to their chemical activity; (ii) to expand *and critically evaluate* the applicability domain of chemical activity for interpreting toxicity data; and (iii) to facilitate the application of chemical activity within chemical hazard and risk assessments.

Project coordinator: Jon Arnot, ARC Arnot Research & Consulting Inc., Toronto, Canada

More information: http://cefic-lri.org/projects/eco30-arc-expanding-the-applicability-domain-of-the-chemical-activity-approach-for-hazard-and-risk-assessment/

EURL ECVAM (*European Union Reference Laboratory for Alternatives to Animal Testing*): The European Commission's involvement in activities targeted toward the validation of alternative approaches to animal testing started in 1991, with the launch of ECVAM (the European Centre for the Validation of Alternative Methods), hosted by the Joint Research Centre, Institute for Health and Consumer Protection (IHCP). As of 2011, ECVAM's tasks were assigned to EURL ECVAM, and it is now part of the 'Systems Toxicology Unit' (STU) of the IHCP. Today, ECVAM provides the institutional basis to fulfil the requirements of the 'Directive 2010/63/EU on the protection of animals used for scientific purposes'. Following this, the aim of EURL ECVAM is twofold:

- To promote the scientific and regulatory acceptance of non-animal tests that are of importance to biomedical sciences, through research, test development

and validation as well as the establishment of a specialised database service;

➠ To coordinate at the European level the independent evaluation of the relevance and reliability of non-animal tests for specific purposes, so that chemicals and products of various kinds (including medicines, vaccines, medical devices, cosmetics, household products and agricultural products) can be manufactured, transported and used more economically and safely, while the current reliance on animal-based test procedures is progressively reduced.

EURL ECVAM collaborates with its closest partners in the field of validation through the 'International Collaboration on Alternative Test Methods' (ICATM). This agreement is intended to intensify communication and collaboration during the planning and execution of validation studies on alternative methods, during peer review of these studies and with respect to the development of test method recommendations.

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

*Cooperation with **SEURAT-1***: The JRC (host institution of EURL ECVAM) was a key partner in several research projects (*SCR&Tox*, DETECTIVE, COSMOS) as well as in the coordination project COACH of the **SEURAT-1** Research Initiative. The experimental work in **SEURAT-1** aimed at developing new test methods entering the pre-validation stage and, therefore, the involvement of ECVAM at an early stage was essential for the success of these activities. Furthermore, ECVAM supported the definition of cluster-level case studies, demonstrating that the new methods developed within **SEURAT-1** are fit for purpose.

ECHA *(European Chemicals Agency)*: ECHA is the driving force among regulatory authorities in implementing the EU's chemicals legislation for the benefit of human health and the environment as well as for innovation and competitiveness. ECHA helps companies to comply with the legislation, advances the safe use of chemicals, provides information on chemicals and addresses chemicals of concern. ECHA was founded in 2007 and is based in Helsinki, Finland. ECHA's work helps to ensure that chemicals are used safely and that the most hazardous chemicals are replaced by safer alternatives.

ECHA's most relevant field of activity for the **SEURAT-1** Research Initiative is the implementation of the REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation. REACH entered into force in 2007 and was adopted not only to improve the protection of human health and the environment from the risks posed by chemicals (while enhancing the competitiveness of the EU chemicals industry), but also to promote alternative methods for the hazard assessment of substances in order to reduce the number of tests on animals.

More information: http://echa.europa.eu/

*Cooperation with **SEURAT-1***: The regulatory perspective on human safety assessment of chemicals within **SEURAT-1** was ensured through the engagement of an ECHA representative in the **SEURAT-1** Scientific Expert Panel (SEP). He was actively involved in the case study planning as a co-leader of the **SEURAT-1** Safety Assessment Working Group. The **SEURAT-1** read across case study for safety assessment (see section 3.2) contributed significantly to the Topical Scientific Workshop entitled 'New Approach Methodologies in Regulatory Science' organised by ECHA, which was held on 19-20 April 2016 in Helsinki/Finland (see section 2.3).

OECD *(Organisation for Economic Co-Operation and Development)*: The OECD Guidelines for the Testing of Chemicals are a collection of the most relevant internationally agreed testing methods used for the safety assessment of chemicals. Different OECD working groups have been established, addressing the various approaches in the field of toxicity testing, which will be briefly discussed below.

The (Quantitative) Structure-Activity Relationship [(Q)SAR] Project was launched in the early 1990s. This project has focused on the acceptance of (Q)SAR approaches for the evaluation of chemicals, focusing since 2004 on the development of the OECD (Q)SAR Toolbox. This software was created for use by governmental agencies and stakeholders in the chemical industry in order to bridge the data gaps in (eco)toxicology. Version 2 of the Toolbox was released in 2010. It can be used for the identification of potential toxic mechanisms of chemicals, including their metabolites. The Toolbox comprises all regulatory endpoints and contains 'mechanistic profilers' for the identification of relevant mechanisms or modes-of-action.

The 'Molecular Screening for Characterisation Individual Chemicals and Chemical Categories Project' (Molecular Screening Project) was established in 2007 by the OECD in cooperation with the International Program on Chemical Safety (IPCS). The aim is to develop a strategy for prioritising further testing of chemicals, based on the molecular properties that are linked to potential toxicity. High-throughput screening (HTS) using *in vitro* assays and selected chemicals are applied for the evaluation of specific pathways.

The emerging area of toxicogenomics is also being addressed by the OECD in collaboration with IPCS. The objectives are to: (i) identify new biomarkers that are representative for specific pathways; and (ii) conduct surveys on existing toxicogenomic tools. The overall goal of these activities is the development of a strategy regarding the future application of toxicogenomics in the context of regulatory chemical safety assessment.

Finally, the OECD is very active in the field of adverse outcome pathway (AOP) developments, and has released some key documents outlining basic rules for establishing new AOPs as well as proposals for a common terminology (ontology) in this dynamic field.

More information: http://www.oecd.org/env/testguidelines

*Cooperation with **SEURAT-1***: Members from the **SEURAT-1** projects COSMOS and the JRC collaborated actively with the OECD in developing the AOP framework. The prototype AOPs developed and investigated within **SEURAT-1** as a result of work within the **SEURAT-1** projects DETECTIVE and *HeMiBio* feed directly into the respective current OECD activities. Furthermore, COSMOS actively contributed to the QSAR Toolbox Project through the development of approaches to group molecules for the prediction of chronic toxicity.

CAAT-Europe *(The Center for Alternatives to Animal Testing – Europe):* CAAT-Europe was founded in 2009 as a transatlantic joint venture between the Johns Hopkins Bloomberg School of Public Health, Baltimore, USA, and the University of Konstanz. The University of Konstanz has 20 years of experience in the field of alternatives to animal testing. CAAT-Europe critically evaluates *in vivo*, *in vitro* and *in silico* approaches. The aim is to bring together organisations within the industrial and academic sectors that are involved in the development of toxicity tests in order to serve the needs for establishing alternative methods.

The objectives of CAAT-Europe are to: (i) bring together industry and academic sectors to address the need for human-relevant methods; (ii) make use of funds strategically to fill gaps in the development and implementation of alternative methods; (iii) coordinate workshops and information days in Europe on relevant developments in the area of alternatives and toxicology; (iv) develop strategic projects with sponsors to promote human science and 'new toxicology'; (v) develop a joint education programme between the Johns Hopkins University and the University of Konstanz; (vi) set up transatlantic consortia for international research projects on alternative methods; and (vii) support *ALTEX* as the official journal of CAAT, the European Society for Alternatives to Animal Testing (EUSAAT), and the Transatlantic Think Tank for Toxicology (t4).

More information: http://cms.uni-konstanz.de/leist/caat-europe/

*Cooperation with **SEURAT-1***: Researchers from the **SEURAT-1** Research Initiative contributed as invited speakers to several workshops and symposia organised by the CAAT. The CAAT Europe Office and the **SEURAT-1** Office (COACH) exchanged information on a regular basis about planned activities.

EBTC Europe *(Evidence-Based Toxicology Collaboration)*: Following the effort in the US of creating an Evidence-based Toxicology Collaboration (EBTC) in 2011 (see below), a European counterpart to adapt Evidence-based Medicine (EBM) principles to toxicology has recently started. The kick-off meeting of EBTC Europe took place in conjunction with the EUROTOX Congress 2012.

More information: http://ebtox.com/eu-kickoff.html

SCCS *(Scientific Committee on Consumer Safety)*: The SCCS is a part of the European Commission's Directorate General for Health and Consumers. It provides opinions on health and safety risks of non-food consumer products (such as cosmetic products and their ingredients) and services (such as artificial sun tanning). The SCCP releases the 'Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation', which is regularly updated according to scientific progress made.

More information: http://ec.europa.eu/health/scientific_committees/consumer_safety/

Cooperation with ***SEURAT-1***: Vera Rogiers (Vrije Universiteit Brussel, Belgium; active in the **SEURAT-1** projects *HeMiBio* and DETECTIVE) is an external expert in the SCCS working group on cosmetic ingredients.

EFSA *(European Food Safety Authority)*: As a consequence of a series of food crises, the European Food Safety Authority (EFSA) was set up in 2002 by the European Union as an independent agency for risk assessment and risk communication, covering all aspects associated with the food chain. EFSA aims to provide appropriate, consistent, accurate and timely communications on food safety issues to all stakeholders and the public at large, based on the Authority's risk assessments and scientific expertise. Nearly 460 people are currently engaged at EFSA, working in different food-related scientific fields, such as food and feed safety, nutrition, animal health and welfare, and plant protection. EFSA plays a major role in Europe's food safety system by providing independent scientific advice and assessing all risks concerning the food chain.

More information: http://www.efsa.europa.eu/

5.2.2 International Activities

USA

Tox21: The 'Toxicology in the 21st Century' (Tox21) programme is a joint initiative of the US EPA, the National Toxicology Programme of the National Institute of Environmental Health Sciences (NIEHS), the National Institutes of Health (NIH), the National Center for Advancing Translational Sciences (NCATS), US FDA, and is organised under the umbrella of the EPA's Computational Toxicology Research Programme. Tox21 aims to develop high-throughput decision support tools for prioritising the thousands of chemicals that need toxicity testing. In this context, Tox21 develops, validates and translates innovative chemical testing methods that characterise toxicity pathways. The knowledge about toxicity pathways will then be used for prioritisation of chemicals that need to be further tested as well as the development of innovative *in silico* methods.

The general approach is to screen a large number of chemicals (approximately 10,000) using high-throughput screening assays at the NIH NCATS using innovative robotic technology. These data are then used to research, develop, validate and translate innovative chemical testing methods that characterise toxicity pathways. Ways to use new tools to identify chemically induced biological activity mechanisms are being explored. This knowledge will then be used to prioritise the chemicals that need more extensive toxicological evaluation (i.e., the need for additional information). The experimental work is being accompanied by the development of models that can be used to more effectively predict how chemicals will affect biological responses. The different methods should be effectively combined as a toolbox of innovative chemical testing methods. Fifty or more ToxCast™ (see below) high-throughput screening assays in this enlarged chemical library should be conducted every year for the next several years. Finally, the challenge of being able to provide the data generated from the innovative chemical testing methods to risk assessors for making decisions about protecting human health and environment is being addressed.

Four different working groups were established within Tox21: (i) Assays/Pathways Group, which is responsible for identifying key toxicity pathways/assays, incorporating hepatic metabolism into *in vitro* assays, and establishing methods that account for interactions between compounds and pathways, as well as between cells (cell-to-cell interactions); (ii) Compounds Group, which is responsible for quality control issues and the establishment of two libraries, one containing the chemical structures of the 10,000 chemicals to be tested within Tox21, and another comprising water-soluble compounds and mixtures to be tested in the future; (iii) Bioinformatics Group, which is responsible for interpreting data (response within and across assays and endpoints respectively, and response patterns and relationships with adverse outcomes in *in vivo* tests) and ensuring the accessibility of data by the public; and (iv) Targeted Testing Group, which is responsible for evaluating the *in silico* methods and prioritisation schemes.

Scientific Coordinator: Russell Thomas (Director of EPA's National Center for Computational Toxicology, Research Triangle Park, USA)

More information: http://www.epa.gov/ncct/Tox21/

*Cooperation with **SEURAT-1***: A joint meeting between **SEURAT-1** and Tox21 occurred in June 2013 in Ispra, Italy. Common interests as a basis for future collaboration were discussed on this occasion. In the meantime, exchange activities were implemented on the level of the **SEURAT-1** proof-of-concept case studies and culminated in the **SEURAT-1** read-across case study for safety assessment (see section 3.2).

ToxCast™ *(Screening Chemicals to Predict Toxicity Faster and Better)*: The EPA launched ToxCast in 2007 as an important component of their Computational Toxicology Research Programme for chemical screening. The aim is to develop a cost-effective approach for

prioritising the vast number of chemicals that still need toxicity testing, and to predict the potential toxicity of chemicals. ToxCast uses advanced scientific tools to help understand how the processes of the human body are impacted by exposure to chemicals and to determine which exposures are most likely to lead to adverse health effects. ToxCast is being developed in phases:

- Phase I (Proof of Concept) was completed in 2009 and it profiled roughly 300 well-studied chemicals (primarily pesticides) through the use of over 500 high-throughput screening assays. The chemicals screened in phase I already had extensive toxicity testing results from traditional chemical tests, mostly animal tests. Data from animal studies can be searched and queried using the EPA's Toxicity Reference Database (ToxRefDB, see below). Having both the ToxCast and animal testing results allows the EPA to compare results and determine if both screening processes make similar predictions.

- Phase II involved the profiling of approximately 2000 additional chemicals, most of them with limited toxicity data as compared with phase I chemicals. Selected chemicals from a broad range of sources, including drugs, food additives, 'green' chemicals, industrial chemicals and consumer products, and nanomaterials were investigated in over 700 high-throughput assays that cover a range of high-level cell responses and approximately 300 signaling pathways. Phase II was completed in 2013.

ToxCast research is ongoing to determine which assays under what conditions may lead to toxicological responses. The data are fed into the ToxCast database (ToxCastDB) and used for the elucidation of toxicity signatures. The results of this research can then be used to inform the context in which decision makers can use the data (e.g. in the context of the EPA's Endocrine Disruption Screening Programme). As ToxCast screens more chemicals, the EPA will be able to determine which combinations of high-throughput assays are best used as indicators for different types of potential toxicity that can lead to health effects such as chronic diseases. All ToxCast chemical data is publicly available for anyone to access and use through user-friendly web applications called interactive Chemical Safety for Sustainability Dashboards. Users can select the chemicals and data of interests and then explore and export this information.

Director: Russell Thomas (Director of EPA's National Center for Computational Toxicology, Research Triangle Park, USA)

More information: http://www.epa.gov/ncct/toxcast/

*Cooperation with **SEURAT-1***: The **SEURAT-1** project COSMOS established a collaboration on the mutual use of repeated dose toxicity data in the respective data bases. Further collaborations are described above (see entry under Tox21).

Virtual Tissue Models: The Virtual Tissues Research Project was also established as a component of the EPA's Computational Toxicology Research Programme. The aim is to estimate the potential of chemicals to cause chronic diseases, such as cancer, by means of a large-scale computer model that simulates dynamic biological processes.

The mechanistic understanding of chemical effect networks will serve as the basis for modelling the key molecular, cellular and circulatory systems. The Virtual Tissues team includes an interdisciplinary team of toxicologists, computer engineers, programmers, bioinformaticians, biologists, mathematicians, and other experts. The team aims to use a selection of everyday chemicals with known health effects in animals to develop methods to use vast collections of data, biological knowledge-bases and high-tech computer modelling to build computer-based virtual models.

More information: https://www.epa.gov/chemical-research/virtual-tissue-models-predicting-how-chemicals-impact-development

Other components of the Computational Toxicology Research Programme: In addition to the above-mentioned projects that operate in the related fields of the **SEURAT-1** Research Initiative, the Computational Toxicology Research Programme also comprises further components that will be just briefly mentioned here:

The EPA's online warehouse is called **ACToR** (Aggregated Computational Toxicology Resource). Comprising all publicly available chemical toxicity data, it can be used to find data on potential chemical risks to human health and the environment.

The **ExpoCast™** project focuses on the environmental fate of chemicals to assess exposure routes. The project is closely related to ToxCast with the common goal of establishing a list of priority chemicals to be further tested and/or regulated.

The Interactive Chemical Safety for Sustainability (**iCSS**) dashboard provides an interactive tool to explore rapid, automated (or *in vitro* high-throughput) chemical screening data generated by the ToxCast™ project (see above) or other components of the Tox21 collaboration.

CPCat (Chemical and Product Categories) is a database containing information mapping more than 43,000 chemicals to a set of terms categorising their usage or function.

Finally, the aim of **EDSP21** (Endocrine Disruption Screening Programme for the 21st Century) is to evaluate chemicals for endocrine-related activity.

More information: http://www.epa.gov/ncct/

DrugMatrix *(A toxicogenomics and tissue library hosted by the National Toxicology Programme)*: DrugMatrix is the scientific communities' largest molecular toxicology reference

database and informatics system. DrugMatrix contains a graphic user interface for rapid scoring of genomic signatures of toxicity. The database is populated with the comprehensive results of thousands of highly controlled and standardised toxicological experiments, in which rats or primary rat hepatocytes were systematically treated with therapeutic, industrial and environmental chemicals at both non-toxic and toxic doses and multiple exposure durations. The heart of the DrugMatrix database is large-scale gene expression data generated by extracting RNA from the toxicologically relevant organs and tissues and applying the RNA to the GE Codelink™ 10,000 gene rat array and, more recently, the Affymetrix whole genome 230 2.0 rat GeneChip® array. DrugMatrix contains toxicogenomic profiles for 638 different compounds.

DrugMatrix is publicly available. The primary value that DrugMatrix provides to the toxicology community is in its capacity to use toxicogenomic data to perform rapid toxicological evaluations. Further value is provided by DrugMatrix ontologies that help characterise mechanisms of pharmacological/toxicological action and identify potential human toxicities.

More information: https://ntp.niehs.nih.gov/drugmatrix/index.html

*Cooperation with **SEURAT-1***: A representative of the DrugMatrix project was invited to the **SEURAT-1** workshop 'Exploring Existing Databases for Modes-of-Action of Repeated Dose Systemic Toxicity', held in Tuebingen in 2012. The DrugMatrix database tools are an important resource for identifying key events within AOPs.

Tissue Chip for Drug Screening: To help streamline the therapeutic development pipeline, the National Center for Advancing Translational Sciences as part of the National Institutes of Health (NIH), in collaboration with the Defense Advanced Research Projects Agency and the US Food and Drug Administration, is leading an initiative to improve the process for predicting whether drugs will be safe in humans. The Tissue Chip for Drug Screening initiative aims to develop 3D human tissue chips that accurately model the structure and function of human organs, such as the lung, liver and heart. Once developed, researchers can use these models to predict whether a candidate drug, vaccine or biologic agent is safe or toxic in humans, and in a faster and more cost-effective way than current methods. The ultimate goal is to combine all major organ systems to form a so-called human-body-on-a-chip.

In the first funding phase (2012-2014), the NIH issued 19 awards, 11 of which supported studies to develop 3D cellular microsystems that represent a number of human organ systems. The additional seven awards explored the potential of stem and progenitor cells to differentiate into multiple cell types that represent the cellular architecture within organ systems. These could act as a source of cells to populate tissue chips.

The goal for the second funding phase (2014-2017) was to further refine the technology and begin organ-chip integration by means of renewable cell sources and bioengineered

microsystems that successfully demonstrated physiological function in the first phase.

More information: http://www.ncats.nih.gov/tissuechip/about

NICEATM – ICCVAM *(National Toxicology Programme Interagency Center for the Evaluation of Alternative Toxicological Methods - Interagency Coordinating Committee on the Validation of Alternative Methods)*: ICCVAM is an interagency committee of representatives from 15 US federal regulatory and research agencies that require, use, generate or disseminate toxicological and safety testing information. ICCVAM conducts technical evaluations of new, revised and alternative safety testing methods with regulatory applicability. ICCVAM also promotes the scientific validation and regulatory acceptance of safety testing methods that more accurately assess the safety and health hazards of chemicals and products and that reduce, refine (enhance animal well-being and lessen or avoid pain and distress) or replace animal use. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM also conducts independent validation studies to assess the usefulness and limitations of new, revised and alternative test methods and strategies.

ICCVAM has contributed to the approval or endorsement of 43 alternative safety-testing methods by federal regulatory agencies and international organisations since its establishment in 1997. ICCVAM has also identified critical research, development and validation efforts needed to further advance numerous other alternative methods.

In May 2012, ICCVAM published a five-year plan for the years 2013 to 2017 with the overall aim to better align ICCVAM and NICEATM with the vision laid out by the National Academy of Sciences in the 2007 NRC Report *Toxicity Testing in the 21st Century: A Vision and A Strategy*, while simultaneously fulfilling the mission of ICCVAM to implement the 3Rs of toxicity testing (i.e., replace, reduce, and refine) in accordance with the ICCVAM Authorization Act of 2000. The initial steps towards this new strategic direction are to: (i) set priorities and identify areas for scientific focus for immediate resource investment (i.e. investment into projects where there is a high likelihood of success within a reasonable timeframe of 1-5 years for implementation into regulatory use, such as acute oral and dermal toxicity testing or skin sensitization); (ii) develop plans to improve communications between stakeholders and the public (e.g. through focused workshops); and (iii) explore new paradigms for the validation and utilisation of alternative toxicological methods.

More information: http://iccvam.niehs.nih.gov/

PSTC *(Predictive Safety Testing Consortium)*: The PSTC is a public–private partnership supervised by the Critical Path Institute (C-Path) as an independent, non-profit institute, which was created by the University of Arizona and the US FDA in 2005. The PSTC provides

a platform for pharmaceutical companies to share and validate each other's safety testing methods with consultation from the FDA, its European counterpart, the European Medicines Agency (EMA), and the Japanese Pharmaceutical and Medical Devices Agency (PMDA). Since 2013, PSTC collaborates with the IMI project SAFE-T (see above) on the development of important new drug safety tests.

The mission of PSTC is to identify new and improved safety testing methods and submit them for formal regulatory qualification by the FDA, EMA and PMDA. Currently, the PSTC has 18 corporate members with the same goal: to find improved safety testing methods. The members share their internally developed methods and test these methods developed by one another across the consortium. Ten EMA and twenty-eight FDA scientists serve as advisors along with more than 250 participating scientists. C-Path leads the collaborative process and collects and summarises the data.

President and CEO: Martha A. Brumfield (Critical Path Institute, Tucson, USA)

More information: https://c-path.org

ILSI / HESI *(International Life Sciences Institute / Health and Environmental Sciences Institute)*: ILSI is a non-profit, worldwide organisation whose mission is to provide science that improves human health and well-being and safeguards the environment. ILSI is located in Washington D.C., USA. HESI was established in 1989 as a global branch of ILSI. The intention of ILSI / HESI is to bring together different research groups from industry, government and academia to advance the understanding of scientific issues in the field of human health, toxicology, risk assessment and the environment. It develops scientific programmes through committees that organise, support and execute projects, including collaborative laboratory studies, development and analysis of databases as well as workshops and conferences. The goal is always to address and reach consensus on scientific questions that have the potential to be resolved through creative application of intellectual and financial resources.

HESI created the RISK21 project, which developed a framework for integrating animal-free testing methods into human health risk assessment. An overview of this framework and the RISK21 project is given in section 2.4 of this Annual Report.

Executive Director: Syril Pettit (Health and Environmental Science Institute, Washington D.C., USA)

More information: http://www.ilsi.org/; http://www.hesiglobal.org/

*Cooperation with **SEURAT-1***: The **SEURAT-1** project COSMOS has established two expert groups with ILSI Europe: Expert Group 1 for the development of criteria to be applied in the extension of the current TTC approach to cosmetics-related chemicals, and Expert Group 2 for the evaluation of oral-to-dermal extrapolation.

CAAT *(Centre for Alternatives to Animal Testing)*: The Centre for Alternatives to Animal Testing (CAAT) is located within the Johns Hopkins Bloomberg School of Public Health in Baltimore. It was established in 1981 through a grant from the Cosmetic, Toiletry, and Fragrance Association (CTFA) (now the Personal Care Products Council). Similarly to the European counterpart described above, CAAT's vision is to be a leading force in the development and use of methods following the 3R's principle (reduction, refinement and replacement) in all involved sectors (research, testing and education). Consequently, CAAT supports research for the development and validation of new *in vitro* test methods and other alternatives, organises discussion to enhance acceptance of such new methods, distributes information to academia, government, industry and the general public (for instance through the *ALTEX* journal), and organises training courses in the application of innovative methods in toxicity testing.

The Doerenkamp-Zbinden Foundation (DZF) and CAAT are collaborating to establish the Transatlantic Think Tank for Toxicology (t^4). t^4 prepares and/or commissions high-quality analyses of toxicological problems and orchestrate workshops, reports, and review papers designed to bring to fruition the innovative approaches outlined in the report of the National Academy of Science (*Toxicity Testing and Assessment in the 21st Century*).

More information: http://caat.jhsph.edu/

EBTC *(Evidence-Based Toxicology Collaboration)*: The Evidence-Based Toxicology Collaboration has taken up the challenge of translating evidence-based approaches from medicine to toxicology. The Collaboration has closely coordinated steering committees in the US and Europe with members drawn from government agencies, academia and industry. The EBTC will further the conceptual development of evidence-based toxicology, set priorities, raise awareness and create working groups. Four Work Groups are currently active: (i) the Zebrafish Embryotoxicity Test Work Group (formed in late 2012 to carry out a systematic review of the Zebrafish Embryo Test as a predictor of developmental toxicity); (ii) the Methods Work Group (with the goal to identify and adapt methods from evidence-based medicine and health care that are applicable to evidence-based toxicology, as well as to develop new methods as necessary); (iii) the Empirical Risk of Bias Tool Work Group (with the goal to develop a new set of risk of bias tools suitable for *in vitro*, high-throughput studies); the Tox21 Data Analysis Work Group (with the goal to study how the mechanistic data from ToxCast and Tox21 can be used in safety assessment). The Governance and Work Processes Work Group, which was established in 2013 to identify, recommend and implement appropriate administrative structures and procedures to facilitate the activities of the EBTC, has completed its work in 2015 with the formation of the Board of Trustees and setting up governance processes. The Work Groups produce guidance documents – tailored to toxicology – on conducting systematic reviews and their components, including data appraisal and data synthesis, as well as on the application of evidence-based tools to various toxicological practices, such as assessing

the hazards and risks of exposure to individual chemicals and evaluating the performance of toxicological test methods. The EBTC will also undertake case studies to illustrate how evidence-based approaches can address these topics. The EBTC will evolve into an umbrella organisation facilitating the application of evidence-based approaches to toxicology.

More information: http://www.ebtox.com/

JAPAN

JaCVAM *(Japanese Center for the Validation of Alternative Methods)*: JaCVAM is part of the Office for New Testing Method Assessment in the Division of Pharmacology of the Japanese National Biological Safety Research Centre (NBSRC) and the National Institute of Health Sciences (NIHS). JaCVAM is responsible for the evaluation of innovative testing methods following the 3Rs principle in the field of chemical toxicity screening and thereby for chemical safety assessment in Japan. JaCVAM's agenda also comprises the establishment of guidelines for alternative testing methods, with special emphasis on international collaborations for the development of harmonised experimental protocols (e.g., correlation with OECD guidelines). For that, JaCVAM organises international workshops and disseminates the respective information regarding alternative testing methods. Furthermore, representatives of the US National Toxicology Programme, Health Canada, Japan (JaCVAM) and the EU (ECVAM) signed a memorandum of cooperation in 2009 with the aim of establishing an International Cooperation on Alternative Test Methods (ICATM). This was done in order

"to expand and strengthen cooperation, collaboration and communication among national validation organisations on the scientific validation and evaluation of new alternative testing methods proposed for regulatory health and safety assessments" (Memorandum of Cooperation, *http://jacvam.jp/en_effort/en_icatm.html*).

The original agreement was expanded in March 2011 to include the South Korea in the ICATM.

More information: http://jacvam.jp

TG-GATEs *(Genomics Assisted Toxicity Evaluation System)*: TG-GATEs was a project of the Laboratory of Toxicogenomics Informatics hosted by the Japanese National Institute of Biomedical Innovation. The first five-year collaborative studies in the Toxicogenomics Project by the government and pharmaceutical companies started in 2002, in which rats were exposed to chemicals (mainly medicines) and gene expression in the liver (kidney in some cases) was measured by Affimetrix's GeneChip and collected together with classical toxicological data. Experiments were also done with rat and human hepatocytes and more than eight hundred million gene expressions for more than 150 chemicals were obtained by 2007. The data were combined with analysis and prediction systems established under the name of TG-GATEs (Genomics Assisted Toxicity Evaluation system). In order to utilise this system effectively,

the second stage of the Toxicogenomics Informatics Project was started in 2007 and was completed in 2011. This project discovered over 30 different safety biomarkers using TG-GATEs. The additional data obtained in this second phase for verifying the biomarkers and analysing their mechanisms has been incorporated into TG-GATEs.

More information: http://toxico.nibiohn.go.jp/english/

*Cooperation with **SEURAT-1***: Data collected by TG-GATEs is publicly available and the **SEURAT-1** case studies made use of this important resource.

5.2.3 Meetings and Symposia

FOCUS ON ALTERNATIVE TESTING

EPAA *(European Partnership for Alternative Approaches to Animal Testing)*: The EPAA is a collaboration between the European Commission, European trade associations and companies from several industrial sectors. The vision of EPAA is the replacement, reduction and refinement (3Rs) of animal use for meeting regulatory requirements through better and more predictive science. Consequently, EPAA is active in research as well as in regulation. In the field of regulation the goal of EPAA is to improve the implementation of 3Rs in European regulatory testing and decision-making. In the field of research, EPAA is exploring opportunities to prioritise, promote and implement future research in the field of the 3Rs.

Furthermore, the EPAA organises an annual conference and workshops supporting the development of alternative approaches to animal testing. The 2015 annual conference was entitled 'A Decade of Support to the 3Rs' and was held in Brussels on 1 December 2015 and the 2016 annual conference entitled 'Science-based Regulation' took again place in Brussels on 5 December 2016. Most important for the **SEURAT-1** Research Initiative was the organisation of the first **SEURAT-1** stakeholder event in September 2013, which presented latest success stories in non-animal methods for human safety assessment of chemicals and was used to launch the third **SEURAT-1** Annual Report.

More information: http://ec.europa.eu/growth/sectors/chemicals/epaa/

ecopa *(european concsensus-platform for alternatives)*: Similarly, ecopa has been established to stimulate research into alternatives to animal experimentation and enforce the acceptance of alternatives in experimental practice. The ambition is to act as a pan-European platform, integrating people from different sectors, such as animal welfare, industry, academia and governmental institutions. As one of its main activities, ecopa supports the organisation of workshops in the field.

More information: http://www.ecopa.eu/

EUSAAT 2016: 16th Annual Congress of the European Society for Alternatives to Animal Testing

Date: 24–27 August 2016

Location: Linz, Austria

The goals of the European Society for Alternatives to Animal Testing (EUSAAT) are to support: (i) the dissemination and validation of alternative methods to animal testing; (ii) the promotion of research in the field of the 3Rs (refine, reduce, replace); (iii) the reduction of the use of animals for tests in the field of education and continuing education; (iv) the reduction of sufferance and stress of laboratory animals by better breeding, keeping, test planning and other accompanying measures; (v) the provision of specialist guidance and expert opinions for public and private organisations, companies and universities; (vi) suitable information for the public and the media.

Main themes of the congress for discussion were:

- EU 3R Projects in Toxicology and other 3R Funding Institutions;
- Global Cooperation on Implementing the 3Rs;
- Implementing Dir 2010/63/EU;
- Novel Approaches in Efficacy and Safety Testing;
- Stem Cells and Reproductive Toxicity;
- Efficacy and Safety Testing of Drugs, Biological and Vaccines;
- Disease Models *in vivo* and *in vitro*;
- Refinement and Welfare;
- Replacement of Animals in Research an Use of Non-Animal Methods and Technologies;
- 3Rs in Academia and Education;
- Ethical and Legal Issues.

More information: http://www.eusaat.org/

5th Annual Meeting of the American Society for Cellular and Computational Toxicology (ASCCT)

Date: 29–30 September 2016

Location: Research Trinagle Park, North Carolina, USA

The ASCCT is a scientific society dedicated to the promotion of toxicology testing and research that reduces and replaces the use of animals. The 'Physicians Committee for Responsible Medicine' and the 'Institute for *In Vitro* Sciences' formed the ASCCT in 2010 to foster cooperation and dialogue among North American scientists, regulators and non-governmental organisations from the pharmaceutical, chemical, pesticide and consumer product sectors.

The main session topics for the ASCCT 2015 meeting were:

- Read Across;
- Stem Cells.

More information: http://www.ascctox.org/meetings.cfm

ESTIV2016: International Conference of the European Society of Toxicology *In Vitro*

Date: 17–20 October 2016

Location: Juan-les-Pins, France

The European Society of Toxicology *In Vitro* (ESTIV) is the Europe's leading organisation working to strengthen the scientific network of *in vitro* toxicologists and promotes *in vitro* toxicology, both scientifically and educationally, in all European countries. The conference aims to create a forum for scientists to discuss and exchange knowledge in a uniquely friendly atmosphere. The objective of the congress was also to promote interaction between junior and senior scientists, students and toxicologists from European companies, government and universities involved in the development and use of *in vitro* methods in toxicology and toxicity testing.

The scientific programme of the ESTIV2016 congress was organised around the motto '*In Vitro* Toxicology for Safety Assessment'. Emphasis was specifically on how new technologies can strengthen the interpretation and application of *in vitro* methods in toxicological research and risk assessment. Session themes were:

- New Developments in Cell Bioengineering and Self Assembly;
- Extrapolation Dose, Modeling and Biodistribution;
- Systemic Toxicity;
- Endocrine Disruptors;
- Biopharmaceuticals;
- Emerging Technologies for *in vitro* Tissue/Organ Formation and Toxicity Testing;

- Regulatory Updates;
- Mixtures.

More information: http://www.estiv2016.com/index.php

IVTS: *In Vitro* Toxicology Society

Date: 14–15 November 2016

Location: Glasgow, UK

The IVTS was founded under a constitution in 1988 for scientists active in the study, practice or development of *in vitro* toxicology. The aims of the IVTS are (i) to encourage alternative approaches to animal testing and promoting the 3Rs; (ii) to provide a forum for discussion for scientists actively involved in the study, practice or development of *in vitro* toxicology; (iii) to arrange scientific meetings on the subject of *in vitro* toxicology and its practical applications; (iv) to promote an exacting scientific approach to the practice of *in vitro* toxicology; and (v) to encourage participation of new student scientists in the field of *in vitro* toxicology.

The IVTS hosted their annual meeting entitled 'Championing *in vitro* and Computational Toxicology' in November, with topics ranging from regulatory issues through to innovative *in vitro* models and *in silico* tools, providing an excellent forum for sharing information and cross-sector and cross-disciplinary collaboration. The main session topics for the IVTS 2016 meeting were:

- Systems Pharmacology;
- Tissue Engineering;
- Nanotoxicology.

More information: http://www.ivts.org.uk/site/annual-meeting-2016/

CCT: Contemporary Concepts in Toxicology Meetings

The Society of Toxicology (SOT) conducts Contemporary Concepts in Toxicology (CCT) Meetings to achieve the SOT Strategic Objective of providing tools and resources to members that will enhance their professional and scientific development, as well as to provide an opportunity for building improved understanding of and dialogue around emerging science critical to advancing the practice of toxicology.

Latest CCT Meetings were:

- The Use of Cardiomyocytes for the Assessment of Proarrhythmic Risk (25–26 October 2016 In Arlington, Virginia, USA);

- Toxicoepigenetics: The Interface of Epigenetics and Risk Assessment (2–4 November 2016 in Tysons, Virginia, USA);
- Metabolic Syndrome and Associated Diseases: From the Bench to the Clinic (11 March 2017, Baltimore, Maryland, USA).

More information: http://www.toxicology.org/events/shm/cct/meetings.asp

European Commission: Non-Animal Approaches - The Way Forward

Date: 6–7 December 2016

Location: Brussels, Belgium

The European Commission organised a scientific conference in Brussels to engage the scientific community and relevant stakeholders in a debate on how to exploit cutting edge advances in biomedical and other research in the development of scientifically valid non-animal approaches (alternatives to animal testing). The conference brought together people from different countries, scientific disciplines and sectors with expertise in research involving animals and / or non-animal alternative approaches. Through scientific presentations and panel discussions attendees explored the current benefits and limitations of different models, and considered how to improve the quality and predictivity of models used in research, including approaches to accelerate non-animal alternatives.

Participants were invited to discuss and propose recommendations to the European Commission, research community and other stakeholders about:

- The Validity of Animal Models Today in Different Sectors;
- Current State of Play and Way(s) Forward for Non-Animal Approaches in Different Areas of Research and Testing;
- Most Promising Technologies and Approaches which offer Prospects for Significantly Reducing and Replacing Animals in Research and Development;
- Best Practice to Advance Research Integrity, Funding and Reporting.

The conference was announced in the European Commission Communication responding to the European Citizens Initiative 'Stop Vivisection' and is one of four actions that should contribute towards the goal of, ultimately, phasing out animal testing. The other three actions are: (1) accelerating progress in the Three R's through knowledge sharing, (2) development, validation and implementation of new alternative approaches, (3) enforcement of compliance with the 3R's principle and alignment of relevant sector legislation. The conference included a session to report progress on these three actions.

This final **SEURAT-1** Annual Report was launched during the conference.

More information:
http://www.euconf.eu/non-animal-approaches-the-way-forward/en/registration/index.html

Mondial Research Group meeting on Reduced Animal Testing

Date: 26–27 January 2017

Location: London, UK

Although most experiments performed on animals are regarded as important for furthering of human and veterinary science, there is a strong movement from within the scientific community to develop methods that do not rely on animals. However, it still may take a long time before all animal experiments can be replaced. The meeting focused, therefore, on options to reduce both the number and suffering of experimental animals.

Main themes for discussion were:

- An in-depth Study of the 3Rs;
- Relative and Absolute Replacement Models;
- Difficulties of Extrapolating Results to the Human Situation;
- *In vitro* Methods: Replacement or Addition to Animal Testing;
- Computer Modelling, Biochemical Techniques and *in vitro* Methods;
- The Refinement and Reduction of Suffering of Experimental Animals before, during and after an Experiment.

More information: www.mondialresearchgroup.com/

12th Annual International Conference on Predictive Human Toxicity and ADME/Tox Studies

Date: 26–27 January 2017

Location: Brussels, Belgium

This meeting brought together scientific experts from the Biotech and Pharmaceutical Industry to present and discuss recent advances towards accurately predicting human toxicities and screening earlier in the drug discovery process. The latest and novel technologies used in the field of Predictive Toxicology and ADME/Tox were explored.

An extensive short course on ‘Evaluation of ADMET Drug Properties in Drug Discovery and Development’ was held the two days prior to the conference.

More information: www.mondialresearchgroup.com/

10th World Congress on Alternatives and Animal Use in the Life Sciences

Date: 20–24 August 2017

Location: Seattle, USA

The Congress provides a forum supporting the ethical use of animals in chemical testing, as well as scientific exchange regarding the development of innovative experimental methods. The motto of the 10th World Congress is 'The Three Rs in Action'. The congress promises to be a cutting edge scientific meeting with emphasis on the latest technologies for reduction and replacement of animals and innovations in approaches to ethics, animal welfare and public policy. Its placement in Seattle offers a broader reach to countries in Asia that are strengthening their focus on the Three Rs. The occurrence of the 10th in a series of World Congresses in the area of Alternatives and Animal Use in the Life Sciences is a milestone and correlates with the 10th anniversary of the National Research Council report on Toxicity Testing in the 21st Century – A Vision and a Strategy – a perfect occasion to take stock on the progress.

More information: http://www.wc10seattle.org/

OTHERS IN THE FIELD

43rd Annual Meeting of the Japanese Society of Toxicology (JSOT)

Date: 29 June – 1 July 2016

Location: Nagoya, Japan

The continual integration of new knowledge and technology within the field of toxicology, has created a growing need for greater scientific precision during investigations that could contribute to the protection of people from various chemical hazards. JSOT annual meetings have been a forum for participants to convey their latest scientific achievements in the field of toxicology. Continuing along this line, the main theme for the 43rd Annual Meeting was the 'Toxicology for Health Promotion'.

Main themes for discussion included:

- Oxidative Stress and Signal Transduction;
- Lung Toxicology;
- Novel approaches for the mechanistic analysis and biomarker identification of toxicity;
- Safety Evaluation of Pharmaceuticals Using the Microminipig;
- Organelle Toxicology;

- Quality and safety of regenerative medicine and cell therapy in Japan – Novel approach toward the risk minimisation of patients;
- Recent advances in cadmium toxicology - from molecular mechanisms to epidemiology.

More information: http://jsot2016.jp/index_en.html

52nd Congress of the European Societies of Toxicology

Date: 04–07 September 2016

Location: Seville, Spain

The Federation of European Toxicologists & European Societies of Toxicology (EUROTOX), with about 7,000 members of different countries, was founded in 1985. EUROTOX organises an annual congress presenting topics covering the latest scientific and regulatory developments with the aim of encouraging future work in toxicology (scientifically as well as educationally). The aim of the 2016 conference was 'Protecting Public and Environmental Health by Understanding and Communicating Toxicology'.

Main themes for discussion include:

- Regulatory Toxicology;
- Human and Environmental Risk Assessment;
- *in vitro* Models;
- Computational Toxicology;
- '-Omics' Technologies;
- Mechanisms of Toxicity;
- Organ Toxicities.

More information: http://www.eurotox2016.com/

OpenTox Euro 2016

Date: 24–28 September 2016

Location: Rheinfelden, Germany

The OpenTox Euro series of meetings emerged as a continuation of the EU-funded Health-FP7 project 'OpenTox' which was completed successfully in August 2011. The ambitious plan is to further develop OpenTox as an infrastructure and community with annual events held in

Europe and the USA in the area of predictive toxicology and related fields.

The OpenTox Euro 2016 meeting placed particular emphasis on the following topics:

- Computational Modelling;
- Translational Bioinformatics;
- Advances in Cheminformatics;
- Development & Application of AOPs;
- Open Science Applications;
- AOP & Protocol Ontologies;
- Emerging Methods and Practice.

More information: http://www.opentox.net/events/opentox-euro-2016

56th Annual Meeting of the Society of Toxicology (SOT)

Date: 12–16 March 2017

Location: Baltimore, USA

The SOT Annual Meeting is the most comprehensive forum for highlighting premier scientific presentations that span the discipline of toxicology. From the essential knowledge to the latest advances, the scientific sessions, including platform sessions, poster presentations, and plenary talks, provide access to the important information of the field.

More information: http://www.toxicology.org/events/am/AM2017/

OpenTox Asia 2017

Date: 17–18 May 2017

Location: Seoul, Korea

The OpenTox Asia 2017 meeting will focus on the following themes:

- International Collaboration;
- Data Management and Integration;
- Computational Science and Modelling;
- Application of Stem Cell Biology to Alternative Testing Methods;
- Application of Integrated Testing Methods & Weight of Evidence to Nanotechnology;
- Emerging approaches to Industrial & Regulatory Risk Assessment;

More information: http://www.opentox.net/events/opentox-asia-2017

53rd Congress of the European Societies of Toxicology

Date: 10–13 September 2017

Location: Bratislava, Slovakia

The motto of the EUROTOX 2017 is 'Connecting for a Safer Future'. In addition to presenting cutting-edge research, the EUROTOX annual meetings offer an unparalleled venue for renewing professional relationships, networking and remaining up to date in our challenging and expanding discipline.

More information: http://www.eurotox2017.com/

5.3 SEURAT-1 Meets EU-ToxRisk

Elisabet Berggren

A collaboration workshop of EURL ECVAM, **SEURAT-1** and EU-ToxRisk, entitled 'Exploring the Regulatory Use of Alternative Approaches in Toxicology for the Safety Assessment of Chemicals', was held on 9 and 10 November 2016 in Ispra (Italy). This workshop explored the possible regulatory use of alternative (non-animal) approaches to systemic toxicity that are intended for the hazard and safety assessment of chemicals used in a variety of sectors. Questions addressed included:

- What types of alternative approaches are emerging from scientific research?
- What are their strengths and limitations for different regulatory purposes?
- How should alternative approaches be evaluated to understand their value?

The aim was also to identify and discuss issues that need addressing collectively to build regulator and end-user confidence in alternative approaches, including:

- The importance of characterising and comparing sources of uncertainty associated with both conventional and alternative approaches and how they influence decision-making;
- The possibility of ensuring equivalent or better levels of human health protection through the use of alternative approaches that generate non-conventional hazard data;
- The need for a longer-term strategy to incrementally refine and deploy new approaches in parallel with appropriate developments in regulatory practice.

Emphasis was on the **SEURAT-1** case studies that operated at the application level (i.e. the read-across case study and the *ab initio* case study, see section 3.2 and 3.3, respectively) and the case studies planned in the context of the EU-ToxRisk-Project. A summary report about the aims and the strategy of this research initiative following up **SEURAT-1** is given in the following section.

5.4 EU-ToxRisk - An Integrated European 'Flagship' Programme Driving Mechanism-based Toxicity Testing and Risk Assessment for the 21st Century

Mardas Daneshian

Modern approaches for risk assessment, such as suggested by the **SEURAT-1** Research Initiative (*Gocht et al., 2015*) and the Risk21 group (*Pastoor et al., 2014*), integrate an iterative refinement of exposure estimation and hazard characterisation. Moreover, the human relevance of non-animal approaches involving human cells, tissues and organoids as well as advanced *in silico* methodologies such as read-across are the main pillars of the modern risk assessment approaches. EU-ToxRisk, a large Horizon2020-funded project (2016-2022), aims at furthering the field of human-relevant and human safety science.

The vision of the EU-ToxRisk Project is to bring about the required paradigm shift in toxicology towards an animal-free safety assessment. The EU-ToxRisk mission is to evolve an adverse outcome pathway (AOP) mechanism-based toxicity testing approach integrating all relevant *in vitro* and *in silico* technologies required for the assessment of chemical safety for humans. The proof-of-concept for the new mechanism-based concept, provided in this project for the areas of repeated dose toxicity focusing on liver, kidney, lung and nervous system and developmental/reproductive toxicity (DART), is to enhance the field of animal-free chemical safety assessment. The project will give guidance for the universal application of the new mechanism-based concept, and allow pushing the entire field forward in an integrated manner, in contrast to the numerous, often dispersed single approaches conducted in the past. The resulting validated test and assessment strategy will fit the current regulatory framework and impact on the future development of safety sciences and regulatory risk assessment.

The drive of this project, as applied also for **SEURAT-1**, is the paradigm shift in toxicology towards a human relevant, animal-free safety assessment across industry sectors and for regulatory purposes. In defiance of this paradigm shift, safety evaluation is today still largely based on

animal testing. However, there is consensus among academia, industry and regulators that the sensitivity and specificity of animal-based safety testing too often lead to wrong predictions of human chemical adversities (*Olson et al., 2000*; *Basketter et al., 2012*; *Leist et al., 2008*). Whilst false-negative results compromise human safety, false-positive animal tests and use of unnecessarily large safety factors may lead to the loss of beneficial and safe chemicals and drugs. In addition, ethical issues (animals use) and economic considerations (high costs, time delay by testing) demand a paradigm shift, away from 'black box' animal testing towards a toxicological assessment based on human cell responses and a comprehensive mechanistic understanding of cause-consequence relationships of chemical adverse effects.

EU-ToxRisk takes on the challenge for this paradigm shift to provide proof-of-concept for an animal-free chemical safety testing approach, thereby directly addressing two complementary critical regulatory needs: (i) a rapid improvement of read across approaches; and (ii) a new 'Adverse Outcome Pathway' (AOP)-based *ab initio* quantitative risk assessment approach. EU-ToxRisk combines cutting-edge complementary methods (*in silico* models and *in vitro* tests) in an integrated approach for testing and assessment (IATA) based on mechanistic understanding of toxicity to fulfil these two key regulatory needs.

Recent developments in cell biology, '-omics' technologies and systems biology now facilitate the mapping of metabolic and signalling pathways affected by xenobiotics, and to understand the complex chains of events linking cause with effect. This potential has been recognised in the US NRC 2007 report: Toxicity testing in the 21st Century: A vision and a strategy (*Bhattacharya et al., 2011*; *Leist et al., 2008*). Its key concept is that most xenobiotic toxicities are related to effects on a limited number of physiological pathways required for normal cellular maintenance, regulation or adaptation. These physiological pathways form an interactive network that controls cell fate and function. Within these networks AOPs represent routes of sequential biological changes leading to adverse effects. Defining these AOPs allow toxicologists moving away from a 'black box approach', investigating so-called 'apical endpoints', towards an approach where effects are mechanistically understood, allowing prevention and monitoring. There is still a major knowledge gap in the understanding of the complex cause-effect network features of AOPs. Lack of quantitative AOPs, incorporating response dynamics and counter-regulation, may explain why the existing qualitative descriptions of AOPs at the level of signal transduction and metabolic pathways alone have not delivered a quantitative understanding of AOPs, allowing clear-cut application in a regulatory setting. Also, dose-effect relationships have not yet been incorporated in the equation. A systematic, quantitative systems toxicology-based understanding of hazard and its extrapolation from *in vitro* to *in vivo* is essential to drive a paradigm shift in chemical safety testing to meet regulatory requirements. EU-ToxRisk will establish human-relevant testing strategies aligned along validated knowledge of AOPs, and implemented in IATAs to meet risk assessment purposes.

The unique feature of the EU-ToxRisk Project is that it takes an advanced unified and holistic approach. It intends to evolve a new era for European safety sciences, by (i) building on

achievements and lessons learnt from **SEURAT-1**, past FP7 and IMI projects, allowing for a 'hot start' in the project; (ii) bundling under one roof different cutting edge technologies, such as biology models from **SEURAT-1** and *in silico* models from eTOX, together with the additional components of absorption, distribution, metabolism and excretion (ADME) and risk assessment; and (iii) engaging with all key stakeholders, at European and international level, to ensure optimum use of resources and enhance impact. A basis for this integrated strategy is EU-ToxRisk's strong and decisive management structure that will integrate the diverse technological approaches and stakeholders from different industry sectors (cosmetics, (agro-) chemicals and pharmaceuticals), as well as regulatory bodies. EU-ToxRisk's novel quantitative hazard and AOP-based risk assessment strategies, based on the best human relevant 'technology/knowledge', will provide confidence in the prediction and will therefore find wide application in various regulatory contexts, across industry sectors, and for different population groups i.e. patients, workers, consumers, and the public at large.

The EU-ToxRisk programme aims to provide innovative solutions for an animal-free mechanism-based toxicity testing and risk assessment paradigm. These solutions should fulfil both industry risk assessment need and the regulatory needs. The EU-ToxRisk strategy was carefully designed to integrate all essential scientific approaches necessary to predict human hazard (*Figure 5.1*). These strategic project approaches include: (i) integrate *in vitro* and *in silico* hazard data with ADME predictions; (ii) provide clear rules for implementation of mechanistic data and ADME in read-across procedures; (iii) fully integrate computational toxicology and experimental *in vitro* test systems in IATAs; (iv) provide quantitative concentration-response measures of AOP key events (and their relationships) and their use in risk assessment; (v) provide tools for the extrapolation of hazard information and associated uncertainties to the human situation; (vi) involve industry and regulators to ensure a need-driven project design.

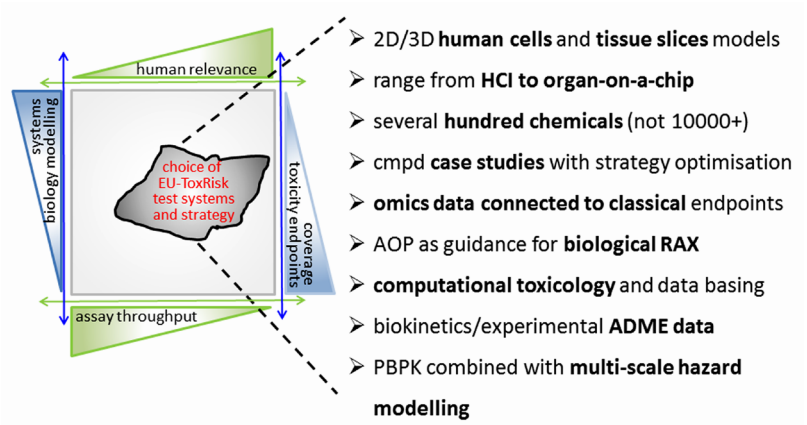


Figure 5.1 *Strategic choices of project strategy, of toxicological focus and of technologies applied.*

To provide these solutions and proof-of concept for their functioning, the available resources need to be used optimally. This has forced strategic choices to balance and position what the project itself will deliver directly and which approaches to leave for later follow-up. In short, the experimental data generation will be based on non-animal, human-relevant test systems that provide AOP information and quantitative information on key event activation. For repeated dose toxicity, the focus is on liver, kidney, lung and nervous system. Advanced technologies, including high throughput transcriptomics, RNA interference, and high-throughput microscopy, will provide the mechanistic underpinning of AOPs and key events. Complex test systems like (multi)organ-on-the chip and precision cut human organ slices will support the translation of *in vitro* to *in vivo*. The project will then combine *in silico* tools and *in vitro* assays based on human cells by computational modelling approaches to provide quantitative data on the activation of AOP key events. This information will be used, in combination with toxicokinetics data and *in vitro-in vivo* extrapolation ((q)IVIVE) algorithms, as a basis for hazard assessment (*Figure 5.2*).

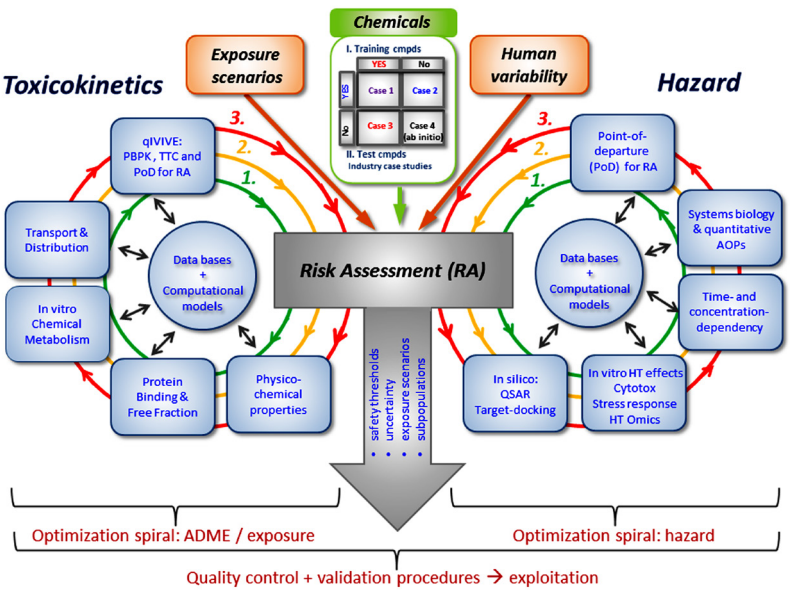


Figure 5.2 *The strategy of EU-ToxRisk to fill data gaps for risk assessment will be tiered, and based on two pillars - hazard and toxicokinetics data. Risk assessors will use available information on case study chemicals and define data gaps; then in silico tools will be used to predict ADME properties and hazard; this new information package will be re-evaluated by risk assessors, and exposure scenarios will be considered for data needs. If the data are not sufficient, a new layer of additional information will be collected (next circle, here numbered 1-3 corresponding to 1-n), e.g. experimental human ADME data, and full concentration-response testing in a KE battery.*

The project had an excellent start, which was helped by extensive preparations by the scientific steering team and the coordinator of the project, Professor Bob van de Water, already in 2015, in order to optimise and refine the information flow and work processes of the project. This contributed to the positive and enthusiastic atmosphere within the EU-ToxRisk consortium. The spirit of all participants of all fourteen work packages is optimistic and stimulating. If the project keeps up this level of dynamics, a lot is to be expected for the near future and the coming 6 years.

References

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Glossary

This glossary was compiled from various existing reference documents coming from different sources. When possible, we used the *Glossary of Reference Terms for Alternative Test Methods and their Validation* published by *Ferrario et al.* (*2014*) in ALTEX (Vol. 31, Is. 3, p. 319-335) as a point of reference (an internet-based version of this published compilation can be found at: *http://altweb.jhsph.edu/resources/validation_glossary.html*).

3Rs Reduction, replacement, refinement - defined by Russel & Birch 1959. Reduction is any means of lowering the number of animals used to obtain information of a given amount and precision. Refinement is any development that refines procedures to lessen or eliminate pain or distress to animals, or enhances animal well-being. Replacement is any scientific method employing non-sentient material, which may replace methods which use conscious living vertebrates.

AAT Alternatives to Animal Testing

AC Activity concentration (AC10 / AC50 = activity concentration at 10% / 50% of response)

ADME Absorption, Distribution, Metabolism, and Elimination. ADME describes the disposition of a pharmaceutical compound within an organism (see also TK, toxicokinetics).

ADMET Absorption, Distribution, Metabolism, Excretion, and Toxicokinetics of a compound.

ALF Acute liver failure

ALT Alanine aminotransferase. The serum ALT activity is regarded as a reliable and sensitive marker of liver disease.

Analogue and / or category approach The terms category approach and analogue approach describe techniques for grouping chemicals. The term analogue approach is used when the grouping is based on a very limited number of chemicals, where trends in properties are not apparent.

A chemical category is a group of chemicals whose physicochemical and human health and/or environmental toxicological properties and/or environmental fate properties are likely to be similar or follow a regular pattern as a result of structural similarity (or other similarity characteristic). In principle, there should be sufficient members in the chemical category, to enable the detection of trends across endpoints. As the number of chemicals being grouped into a category increases, the potential for developing hypotheses and making generalisations about the trends will also increase, and hence increase the robustness of the evaluation.

AOP An AOP is a sequence of events from the exposure of an individual or population to a chemical substance through a final adverse (toxic) effect at the individual level (for human

health) or population level (for ecotoxicological endpoints). The key events in an AOP should be definable and make sense from a physiological and biochemical perspective. AOPs incorporate the toxicity pathway and mode of action for an adverse effect. AOPs may be related to other mechanisms and pathways as well as to detoxification routes.

APAP Acetaminophen (paracetamol), standard reference compound from the **SEURAT-1** Gold Compound list.

API Application Programming Interface: a particular set of commands, functions and protocols that programmers can use to develop software programs that interact with services and resources provided by another particular software program that also implements that API.

AUC Area under the curve

Authentication Confirmation of the identity of a user.

Authorisation Provision of controlled access to resources to a user based on the access permissions they have for the resources.

BAC recombineering: A bacterial artificial chromosome (BAC) is a DNA construct used for transforming and cloning in bacteria, usually *Escheria coli*. Recombineering (recombination-mediated genetic engineering) is a genetic and molecular biology technique that has been developed in *E. coli* and now is expanding to other bacteria species and is used to modify DNA in a precise and simple manner.

BAL Bioartificial liver

BLAST Basic Local Alignment Search Tool

BMD Benchmark Dose: dose levels corresponding to specific response levels, or benchmark responses, near the low end of the observable range of the data. BMDs are obtained from dose-response modelling and can serve as possible points of departure (PODs) for linear or nonlinear extrapolation of health effects data and/or as bases for comparison of dose-response results across studies/chemicals/endpoints. In terms of statistics, the BMD is the calculated lower 95% confidence limit on the dose that produces a defined response (called the benchmark response or BMR, usually 5% or 10%) of an adverse effect compared to background, often defined as 0% or 5%.

BMDL A lower one-sided confidence limit on the benchmark dose (BMD).

CAS Chemical Abstract Service

Category formation The process of forming a group of chemicals – often termed a category – on a rational basis, such as having a similar chemical structure or mechanism of action.

Cell Index A dimensionless parameter derived as a relative change in measured electrical impedance to represent cell status.

CET Cryo-electron tomography

Cell viability (Equivalent to cell mortality) Number of cells that survives upon a given concentration of a compound.

Chemical category see Analogue and / or category approach.

ChIP Chromatin Immuno-Precipitation, antibody based enrichement analysis of genomic regions to analyse the presence or relative distribution of histone-modifications and histone variants at and across genomic regions

CI Cell Index

Clearance Elimination of a compound by an organ.

CLP Classification, Labelling and Packaging Regulation, i.e. (EC) No 1272/2008.

CNS Central nervous system.

Computational Chemistry Computational chemistry is a discipline using mathematical methods for the calculation of molecular properties or for the simulation of molecular behaviour.

CSR Chemical Safety Report in the context of EU regulations of chemicals (see REACH, CLP)

CSRML Chemical Subgraph Representation Markup Language

CTF Contrast Transfer Function

CTFA Cosmetic Toiletries and Fragrance Association

CYP Cytochrome-P450

DBD DNA Binding Domain

DEB Dynamic Energy Budget. The theory aims to identify simple quantitative rules for the organization of metabolism of individual organisms that can be understood from basic first principles. The word ‘dynamic’ refers to the life cycle perspective of the theory, where the budget changes dynamically over time.

DILI Drug-induced liver injury

DNEL Derived no effect level

DPRA Direct Peptide Reactivity Assay

EB Embryoid body

EC Endothelial cell

EC SCCS European Commission Scientific Committee on Consumer Safety (see entry under 'SCCS')

EC_{50} (median effective concentration): Statistically derived median concentration of a substance in an environmental medium expected to produce a certain effect in 50% of test organisms in a given population under a defined set of conditions.

ECG Electrocardiogram

ECHA European Chemicals Agency

ECM extracellular matrix

ecopa European Consensus Platform for 3R Alternatives

ENCODE ENCyclopedia Of DNA Elements, NHGRI programme to identify all functional elements in the human genome sequence in the human genome *http://genome.ucsc.edu/ENCODE/*

ECVAM European Centre for the Validation of Alternative Methods

EM Electron microscopy

ER stress Endoplasmatic Reticulum stress

ESC, ES cells See pluripotent stem cells. ES cells are obtained by derivation from the inner cell mass of the embryo at the blastocyst stage (5.5 to 7.5 days after fertilization in the Human).

EST Embryonic stem cell test

ESTIV European Society of Toxicology *In vitro*

Expert system for predicting toxicity This is a broadly used term for any formal system, generally computer-based, which enables a user to obtain rational predictions about the properties or biological activity of chemicals. Expert systems may be classified as knowledge-based (when the rules are based on expert knowledge), induction rule-based (when statistical methods are used to automatically derive the rules) or hybrid (when both approaches are present). One or more databases may additionally be integrated in the system.

FDA U.S. Food and Drug Administration (TG)

FP 7 Seventh Framework Programme for Research and Technological Development of the European Union

fup Fraction unbound to protein

GCCP Good Cell Culture Practice

GDH Glutamate dehydrogenase

Gesicles Methodology for producing proteins and transferring them to target cells, based upon the introduction in producing cells of the gene encoding the viral fusiogenic protein VSVG. Vesicles ("Gesicles" where the G stands for the G viral protein) formed and released by those producing cells are, then, both much more numerous and very prone to fusion with cell membranes. Engineering producing cells with constructs encoding proteins of interest leads to packing of well translated and processed proteins in gesicles, providing a way to produce and transfer proteins into target cells where normal function has been well demonstrated.

GFP Green fluorescent protein

GLP Good laboratory practice: A set of principles that provide a framework within which laboratory studies are planned, performed, monitored, recorded, reported, and archived. GLP helps to assure regulatory authorities that the data submitted are a true reflection of the results obtained during the study, and can therefore be relied upon when making risk/safety assessments.

GMP Good manufacturing practice

GO Gene Ontology

Gold Compound: A well characterised compound for toxicity testing.

GSH Gluthatione

HBV Hepatitis B virus

HCC Hepatocellular carcinoma

hCMC human embryonic stem cell related cardiomyocyte clusters

HCV Hepatitis C virus

HepG2 BAC-GFP A Hep G2 reporter cell line containing the fluorescent moiety (GFP) and a selected gene marker in a Bacterial artificial chromosome (see BAC)

Hep G2cells A HCC derived human hepato-carcinoma cell line (ATCC No. HB-8065) from liver tissue of a 15 year old Caucasian American male with a well differentiated hepatocellular carcinoma.

HepaRG cell line HepaRG is an immortalized cell line of the liver that can be differentiated into hepatocytes which retain many characteristics of primary human hepatocytes.

hES cell Human embryonic stem cell

hiPS cell Human induced pluripotent stem cell

hitc Hit-call. Parameter used to determine the activity for the concentration series ind a dose-response curve.

HLC Hepatocyte like cell

HOMO Highest Occupied Molecular Orbital

HPC Hepatic progenitor cells

HSC Hepatic stellate cells

HSEC Hepatic sinusoidal endothelial cells

hSKP human skin-derived precursors

HTS High-Throughput-Screening: The use of robotics-based technology to screen large sets of substances for specific activities.

IATA Integrated Assessment and Testing Approaches. Combination of approaches in a weight of evidence (see WoE) as a rational integration of tests data and predictions coming from various data domains (e.g., *in silico* models, computational chemistry, high content and high throughput bioassays, genomics, human exposure, pharmacokinetics, etc.) in order to better understand the likely biological targets of chemicals.

IC10 10% inhibitory concentration

INCI International Nomenclature of Cosmetic Ingredients

***In silico* methods for toxicity prediction** The use of computer-based methods e.g. databases, (Q)SARs, read-across etc to retrieve or estimate toxicological effects of chemicals. These do not require the testing of a chemical (and hence can be termed non-testing information).

Intermediate precursors Cells that are committed to a specific lineage but are not terminally fully differentiated and exhibit the capacity to self-renew without changes in phenotype for a number of passages when grown in culture with specific cocktails of cytokines (e.g. EGF/FGF2 for neural precursors). Intermediate precursors can be terminally differentiated into discrete populations of their lineage. For *SCR&Tox* purposes, intermediate precursor populations are currently available in the neural, mesodermal and keratinocyte lineages

Interoperability The ability of two or more systems or components to exchange information and to correctly use the information that has been exchanged. More generally, it is a property of a system, whose interfaces are completely understood, to work with other systems without any restricted access or implementation.

IPA Ingenuity Pathway Analysis. IPA is a software tool that enables biologists and bioinformaticians to identify the biological mechanisms, pathways, and functions most relevant to their experimental datasets or genes of interest

iPSC, iPS cells See pluripotent stem cells. iPS cells are most commonly obtained nowadays by transferring into replicative donors' cells (e.g. dermic fibroblasts) genes encoding 4

transcription factors (in the original technique, designed by S. Yamanaka, c-Myc, Oct4, Klf4, Sox2). Because current techniques rely on transgene expression, they "alter" cell homeostasis, potentially in a definitive manner. Alternative methods – referred to in the *SCR&Tox* project as "clean reprogrammation" – are therefore actively sought.

IRIS Integrated Risk Information System

ISA-TAB Investigation-Study-Assay TAB delimited format. The ISAcreator software is used to create archives containing experiment descriptions as well as the raw data of an investigation. An archive typically includes all the work that is part of a publication. The archive contains three tables describing the experimental set-up in a hierarchical fashion. The upper level table in the ISA-TAB archive is known as an Investigation. An Investigation contains one or more Studies. Studies share the use of similar biological materials, e.g. same types of treatments and cells that are investigated for instance using different technologies. A Study contains one or more Assays. Each assay is technology-specific and common features associated with a particular technology (e.g. affymetrix microarrays) are captured in **SEURAT-1**/ToxBank assay templates. An Assay contains links to one or more data files. The table contains links to these data files and details about the protocols that were used to derive them.

iTRAQ Isobaric Tag for Relative and Absolute Quantitation

ITS Integrated Testing Strategy. In the context of safety assessment, an integrated testing strategy is a methodology which integrates information for toxicological evaluation from more than one source, thus facilitating decision-making. This should be achieved whilst taking into consideration the principles of the 3 R's (reduction, refinement, and replacement).

IVIVE *In Vitro* Concentration to *In Vivo* Dose Extrapolation

JNK c-Jun NH(2)-terminal protein kinase pathway

KE Key Eevents: Steps along the pathway that represent intermediate events, typically at the different levels of biological organisation which are experimentally or toxicologically associated with an adverse outcome pathway.

KEGG Kyoto Encyclopedia of Genes and Genomes or KEGG is a collection of online databases dealing with genomes and enzymatic pathways. The database was created to improve understanding of functions and utilities of the biological systems, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies. Further information and access to the databse: *http://www.genome.jp/kegg/*.

KNIME Konstanz Information Miner

Lattice-based model Single-cell based model comprising different classes: (i) each lattice site can be occupied by at most one cell (for cells with homogenous size and shape and fixed positions); (ii) a cell may span many lattice sites (for migrating cells with complex shapes); (iii)

lattice sites can be occupied by many cells (for growing cell populations). Lattice models are rule based and do not directly represent the physical reality.

Lattice-free model Represent deformable spheres or ellipses. In some approaches each cell is mimicked by an aggregate of many spheres. Compared with lattice-based models, off-lattice models permit to better directly represent the physical reality.

LBD Ligand Binding Domain

LBP Ligand Binding Pocket

lin-log kinetics Reaction rates are linearly dependent on enzyme concentration and on the logarithm of concentrations. Rates are defined with respect to a reference state.

Linked Data A method of publishing structured data, so that it can be interlinked and become more useful. It builds upon standard Web technologies, but rather than using them to serve web pages for human readers, it extends them to share information in a way that can be read automatically by computers. This enables data from different sources to be connected and queried.

Linked Resources Linked Data approach expanded to all resources including for compounds, biomaterials, assays, algorithms, models, analysis, validation and reports.

LLNA Local Lymph Node Assay. This assay is a murine model developed to evaluate the skin sensitization potential of chemicals.

LOAEL Lowest Observed Adverse Effect Level: Lowest concentration or amount of a substance (dose), found by experiment or observation, which causes an adverse effect on morphology, functional capacity, growth, development, or lifespan of a target organism distinguishable from normal (control) organisms of the same species and strain under defined conditions of exposure.

LOEL Lowest Observed Effect Level: Lowest concentration or amount of a substance (dose), found by experiment or observation, that causes any alteration in morphology, functional capacity, growth, development, or lifespan of target organisms distinguishable from normal (control) organisms of the same species and strain under the same defined conditions of exposure.

LSEC Liver sinusoidal endothelial cells

LUMO Lowest Unoccupied Molecular Orbital

LXR Liver X Receptor

MEA Microelectrode array

Mechanism of toxic action The mechanism of toxic action is the molecular sequence of

events leading from the absorption of an effective dose of a chemical to the production of a specific toxicological response in the target organ or organism. It is the specific biochemical interactions through which a substance produces its effect. Mechanism of action refers to a detailed description, often at molecular level, of the means by which an agent causes a disease state or other adverse effect.

MeDIP profile Methylated DNA immuno-precipitation - a method to analyse the DNA methylation across the genome using antibodies directed against modified cytosines (e.g. 5-methylcytosine or 5-hydroxymethylcytosine). Profiling across the genome involved either subsequent next-generation sequencing MeDIP-Seq or array (MeDIP-Chip) technologies.

Meganucleases Endonucleases, either natural or specifically engineered, that are capable of identifying a very discrete region of the DNA and to cut it, resulting in the disruption of a specific sequence with the potential insertion of a construct of interest. One construct used in *SCR&Tox* is a so-called “landing pad”, i.e. a sequence that has been engineered in order to facilitate homologous recombination of various gene constructs that will be secondarily introduced into cells that carry the “landing pad”. Flanking regions of the “landing pad” have been engineered in order to allow meganucleases to retrieve the entire region, leaving no scar in the host genome.

MID Moulded interconnect device

MIE Molecular Initiating Event, which is the initial point of chemical-biological interaction within the organism that starts the pathway leading to an adverse outcome.

miRNA MicroRNA

MoA The Mode of Action relates to the events including, and downstream of, the toxicity pathway. These could lead to an adverse effect in an individual.

MoE The Margin of Exposure is a term used in risk assessment approaches. It is the ratio of the no-observed-adverse-effect level (NOAEL) or the benchmark dose (BMD) to the estimated exposure dose or concentration.

MRM Multiple Reaction Monitoring (MRM), simultaneous quantification of a large number of peptides (several hundreds) in transcriptomics (Toxicoproteomics).

mRNA Messenger RNA

MS Mass spectrometry

M.SssI DNA methyltransferase from Spiroplasma sp. with the DNA sequence specificity CpG.

MTT assay Assays for measuring the activity of enzymes that reduce 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or close dyes (XTT, MTS, WSTs) to formazan dyes, giving a purple color. Used to assess the viability (cell counting) and the proliferation of cells (cell culture assays), as well as cytotoxicity.

NAM New Approach Methodologies supporting the -> 3Rs principles.

NIH reference map Epigenome reference map: A program launched by the NIH to uncover the epigenomic landscape across human cells (*http://www.roadmapepigenomics.org/)*

NMR Nuclear magnetic resonance

NOAEC No observed adverse effect concentration (see NOAEL).

NOAEL No observed adverse effect level: Greatest concentration or amount of a substance, found by experiment or observation, which causes no detectable adverse alteration of morphology, functional capacity, growth, development, or lifespan of the target organism under defined conditions of exposure.

NOEL No observed effect level: Greatest concentration or amount of a substance, found by experiment or observation, that causes no alterations of morphology, functional capacity, growth, development, or lifespan of target organisms distinguishable from those observed in normal (control) organisms of the same species and strain under the same defined conditions of exposure.

Non-testing information Non-testing data can be generated by three main approaches: a) grouping approaches, which include read-across and chemical category formation; (quantitative) structure-activity relationships ((Q)SARs); and c) expert systems.

NR Nuclear Receptor(s). A class of proteins found within cells that are responsible for sensing distinct molecules. In response, these receptors work with other proteins to regulate the expression of specific genes, thereby controlling the development, homeostasis, and metabolism of the organism.

NTP National Toxicological Program

OED Oral Equivalent Dose. The dose which results in *in vivo* concentrations corresponding to the *in vitro* effective concentration of interest.

OECD Organisation for Economic Co-operation and Development

OECD Principles for the Validation of (Q)SARs A series of rules to assist in the evaluation of a (Q)SAR for use for regulatory purposes. These state that to facilitate the consideration of a (Q)SAR model for regulatory purposes, it should be associated with the following information:

i) a defined endpoint

ii) an unambiguous algorithm

iii) a defined domain of applicability

iv) appropriate measures of goodness-of-fit, robustness and predictivity

v) a mechanistic interpretation, if possible (COSMOS)

OECD QSAR Application Toolbox Software tool (under development) that allows the user to: a) make (Q)SAR estimations for single chemicals; b) receive summary information on the validation results of the model according to the OECD validation principles; c) receive a list of analogues, together with their (Q)SAR estimates; d) receive estimates for metabolite activation/detoxification information. The Toolbox is freely downloadable from www.qsartoolbox.org

OFAS Office of Food Additive Safety (US FDA)

Ontology An ontology is a formal representation of knowledge as a set of concepts within a domain, and the relationships between those concepts. Domain experts are required to specify an ontology. Computer scientists use ontologies to reason about entities within that domain in the creation of user applications.

PAFA Priority-based Assessment of Food Additives

PAMPA assay Parallel Artificial Membrane Permeation assay. A method which determines the permeability of substances from a donor compartment, through a lipid-infused artificial membrane into an acceptor compartment.

PBPK models Physiologically-based Pharmacokinetic models. These models apply a realistic mathematical description of physiology and biochemistry to simulate ADME (Absorption, Distribution, Metabolism, Excretion) processes and assess the distribution of chemicals and their metabolites in the body throughout time. They are particularly adapted to interspecies extrapolation and can be calibrated based on *in vivo*, *in vitro* or *in silico* data.

PBTK Physiologically-Based Toxicokinetics

PCA Principal component analysis

PCPC Personal Care Product Council

PDB Protein Binding Bank

PFAA Perfluoroalkyl acids

PFOA Perfluorooctanoic acid

Pharmacokinetics Process of the uptake of drugs by the body, the biotransformation they undergo, the distribution of the drugs and their metabolites in the tissues, and the elimination of the drugs and their metabolites from the body.

PHCP Personal and household care products

PHH Primary Human Hepatocytes: Primary cells that are freshly isolated from human sources (the liver in case of hepatocytes). Freshly isolated primary cells may rapidly dedifferentiate in culture, and they have a limited lifespan. Primary cell cultures commonly require complex nutrient media, supplemented with serum and other components. Consequently, primary cell culture systems are extremely difficult to standardise.

Pluripotent stem cell lines These cells are of embryonic origin (ES cells) or induced to pluripotency by genetic re-programming of somatic cells from donors (iPS cells). They share two main attributes, unlimited self-renewal –which makes them formally immortal- and pluripotency, the ability to differentiate into any cell type of the body at any stage of differentiation.

PNS Peripheral Nervous System

PoD The Point of Departure is the value on the dose-response curve that serves as the starting point for deriving corresponding health related outcomes (i.e., dose-response for low-dose extrapolation). The POD may be a NOAEL/LOAEL, but ideally is established from BMD modeling of the experimental data, and generally corresponds to a selected estimated low-level of response (e.g., 1 to 10% incidence for a quantal effect). Depending on the mode of action and other available data, some form of extrapolation below the POD may be employed for estimating low-dose risk or the POD may be divided by a series of uncertainty factors to arrive at a reference dose.

Polycomb changes Polycomb proteins are involved in setting and maintenance of epigenetic marks at developmentally regulated genes (such as HOX genes). Changes in the patterns of polycomb genes are indicative of changes in the epigenetic programs set across the genome.

PoT Pathway of Toxicity. See 'Toxicity Pathway'.

PSCs Pluripotent stem cells

QC Quality control

QIVIVE Quantitative *In Vitro* Concentration to *In Vivo* Dose Extrapolation

qRT-PCR Quantitative real-time polymerase chain reaction

QSAR Quantitative Structure-Activity Relationship: A QSAR is a theoretical model for making predictions of physicochemical properties, environmental fate parameters, or biological effects (including toxic effects in environmental and mammalian species). QSARs relate quantitative measures of chemical structure to continuous or categorical variables describing the property to be predicted.

QT interval: The duration of ventricular depolarization and subsequent repolarisation.

RCSB Research Collaboratory for Structural Bioinformatics

REACH Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals. The European Community Regulation on chemicals and their safe use (EC 1907/2006). The new law entered into force on June 1, 2007. The aim of REACH is to improve the protection of human health and the environment through the better and earlier identification of the intrinsic properties of chemical substances. At the same time, innovative capability and competitiveness of the EU chemicals industry should be enhanced.

Read-across A method for filling data gaps in either the analogue or category approaches. Endpoint information for one chemical is used to make a prediction of the endpoint for another chemical, which is considered to be similar in some way. In principle, read-across can be used to assess physicochemical properties, environmental fate and (eco)toxicity effects, and it may be performed in a qualitative or quantitative manner.

In qualitative read-across, the potential of a chemical to exhibit a property is inferred from the established potential of one or more analogues.

In quantitative read-across, the numerical value of a property (or potency of an endpoint) of a chemical is inferred from the quantitative data of one or more analogues.

RMCE Recombinase-mediated cassette exchange. RMCE is of increasing interest in the field of reverse genetics. The procedure permits the systematic, repeated modification of higher eukaryotic genomes by targeted integration. In case of RMCE, this is achieved by the clean exchange of a pre-existing 'gene cassette' for an analogous cassette carrying the 'gene of interest'.

RNA Ribonucleic acid

RO Reactive Oxygen Species

RPTEC/TERT1 Human renal proximal tubular cell line, immortalized by hTERT transfection

RT-CESTM Real-Time Cell Electronic Sensing

RTD Research and technical development

RXR Retinoid X Receptor

SAR Structure Activity Relationships: A theoretical model for making predictions of physicochemical properties, environmental fate parameters, or biological effects (including toxic effects in environmental and mammalian species). SARs are qualitative relationships in the form of structural alerts that incorporate molecular substructures or fragments related to the presence or absence of activity.

SAX Strong anion exchange fractionation technique

SCCS Scientific Committee on Consumer Safety. This EU Committee provides opinions on health and safety risks (chemical, biological, mechanical and other physical risks) of non-food consumer products (e.g. cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products) and services (e.g. tattooing, artificial sun tanning).

SEP Scientific Expert Panel of the **SEURAT-1** Research Initiative. The SEP provides scientific advice regarding the research work and future orientation of **SEURAT-1**.

shRNA Short hairpin RNA

siRNA Short interfering RNA

SMARTS A language in Computational Chemistry for describing molecular patterns.

SOP Standard Operating Procedure: A formal, written procedure that describes in detail how specific routine and test-specific laboratory operations should be performed. SOPs are required by Good Laboratory Practice.

SQL Often referred to as 'Structured Query Language' is a programming language designed for data management.

SREBP-1c: Sterol Regulatory Element-Binding Protein 1c

Structural alerts Atom-based fragments which, when present in a molecule, are an indication that a compound can be placed into a particular category.

STTF SEURAT-1 Training Task Force

Tanimoto criteria Molecular similarity criteria for chemicals based upon Tanimoto Coefficients.

TBBB The ToxBank BioBank (TBBB) will establish a banking information resource for access to qualified cells, cell lines (including stem cells and stem cell lines), tissues and reference materials to be used for *in vitro* predictive toxicology research and testing activities.

TBCR The ToxBank Chemical Repository will ensure the availability of test compounds to researchers of the **SEURAT-1** Research Initiative.

TBDW The ToxBank Data Warehouse will establish a centralised compilation of data for systemic toxicity.

TBGCD The ToxBank Gold Compound Database will provide a information resource servicing the selection and use of test compounds.

TD Toxicodynamics: Process of interaction of potentially toxic substances with target sites, and the biochemical and physiological consequences leading to adverse effects.

TG-Gates Data-base of the Japanese Toxicogenomics project - Genomics assisted toxicity evaluation system *(http://toxico.nibio.go.jp/english/index.html)*.

TK Toxicokinetics: Generally, the overall process of the absorption (uptake) of potentially toxic substances by the body, the distribution of the substances and their metabolites in tissues and organs, their metabolism (biotransformation), and the elimination of the substances and their metabolites from the body. In validating a toxicological study, the collection of toxicokinetic data, either as an integral component in the conduct of non-clinical toxicity studies or in specially designed supportive studies, in order to assess systemic exposure.

TOR Threshold of Regulation. A concept adopted by the US Food and Drug Administration

(FDA) to exempt from the requirement of a food additive listing regulation any substance used in food-contact substances (e.g., food-packaging or food-processing equipment) that migrates, or that may be expected to migrate, into food, if it becomes a component of food only at levels that are below the threshold of regulation. Specifically, an identified migrant of known chemical structure can be exempted if the incremental dietary concentration is below 0.5 μg/kg of diet and the substance has not been shown to be a carcinogen in humans or animals. If the FDA is satisfied that the conditions for exemption are met, the chemical does not ordinarily have to undergo toxicological testing, nor the formal pre-market safety evaluation by the agency.

Toxicity Pathway Cellular response pathways that, when sufficiently perturbed, are expected to result in adverse health effects.

Toxicological data Data relating to the harmful (toxicological) effects of chemicals. This may include information from animal, human or non-animal (*in vitro*) tests.

TTC Thresholds of Toxicological Concern: Human exposure threshold value for a group of chemicals below which there should be no appreciable risk to human health. The TTC may be used as a substitute for substance-specific information in situations where there is limited or no information on the toxicity of a compound, and where human exposure is so low, i.e. below the corresponding TTC, that adverse effects are not to be expected.

UPR Unfolded protein response pathway

US FDA United States Food and Drug Administration

US EPA United States Environmental Protection Agency

VE-cadherin Vascular endothelial cadherin

VPA Valproic acid

Web Service A method of communication between two electronic devices over a network.

WoE Weight of Evidence: The process of considering the strengths and weaknesses of various pieces of information in reaching and supporting a conclusion concerning the hazard potential of a substance.

ZFN-HR Zinc finger nuclease homologous recombination

This book is prepared by the Coordinating Action COACH team, consisting of the Scientific Secretariat and the Scientific Expert Panel (SEP)* within the SEURAT-1 Research Initiative

COACH: Coordination of projects on new approaches to replace current repeated dose systemic toxicity testing of cosmetics and chemicals (Grant agreement N° 267044)

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* A detailed description about the role of the Scientific Expert Panel including information about the members can be found in the Introduction

- On 11 March 2013 the full ban on animal testing for cosmetic products came into force. From this date the marketing of new cosmetic products tested on animals in the European Union is prohibited in accordance with the seventh amendment of the 'Council Directive on the approximation of the laws of the Member States relating to cosmetic products' (76/768/EEC). The European Commission, together with Cosmetics Europe, launched the Research Initiative 'Towards the replacement of *in vivo* repeated dose systemic toxicity testing' in order to develop a sound research strategy leading to the long-term target of 'Safety Evaluation Ultimately Replacing Animal Testing' (SEURAT). This Research Initiative is called **SEURAT-1** and comprises six research projects focusing on the development of new test methods in the field of repeated dose systemic toxicity.

- This is the last volume in a series of six annual reports that, step by step, have described how **SEURAT-1** has paved the way towards innovative safety evaluation of chemicals in various fields of application (for example, medicine, personal care, agriculture, food production, ingredients of everyday products).

- The specific goal of this Research Initiative was the development of *in vitro* test systems based on human cell lineages and related *in silico* methods, which is considered to be a first step towards the replacement of *in vivo* repeated dose systemic toxicity testing. **SEURAT-1** brought the long-term research target to the proof-of-concept stage.

The purpose of the book is:

to inform policymakers about scientific progress relevant to the implementation of European Directives and Regulations, fully respecting the 3Rs-principle;

to inform research policymakers about essential gaps in knowledge and corresponding research needs;

to open a dialogue with regulatory authorities to update current legislation in line with scientific progress;

to support industry in the implementation of the most advanced test methods, thereby increasing their competitiveness;

to encourage the extension of the Research Initiative activities at national, European and international levels.