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“Watching the Detectives” Report of the general assembly of the EU project DETECTIVE Brussels, 24-25 November, 2015

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Abstract

SEURAT-1 is a joint research initiative between the European Commission and Cosmetics Europe aiming to develop *in vitro* and *in silico* based methods to replace the *in vivo* repeated dose systemic toxicity test used for the assessment of human safety. As one of the building blocks of SEURAT-1, the DETECTIVE project focused on a key element on which *in vitro* toxicity testing

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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. The work conducted by the DETECTIVE consortium partners has established a screening pipeline of functional and “-omics” technologies, including high-content and high-throughput screening platforms, to develop and investigate human biomarkers for repeated dose toxicity in cellular *in vitro* models. Identification and statistical selection of highly predictive biomarkers in a pathway- and evidence-based approach constitutes a major step in an integrated approach towards the replacement of animal testing in human safety assessment. To discuss the final outcomes and achievements of the consortium, a meeting was organized in Brussels. This meeting brought together data-producing and supporting consortium partners. 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. Although some projects were still in progress and required continued data analysis, this report summarizes the presentations, discussions and the outcomes of the project.

Background

Alternative methods, replacing animal testing, are urgently needed in view of the European regulatory changes in the field of cosmetic products. On 11 March 2013 a full marketing ban on animal tested cosmetics and their ingredients, within the European Union, entered into force. From this date the marketing of cosmetic products, tested after the deadline, was prohibited in the European Union. The implementation of a marketing and testing ban followed the Seventh Amendment of Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products, which defined the step-wise phase-out of animal testing for cosmetic products as well as for cosmetic ingredients over the last 10 years. Accordingly, animal testing for finished cosmetic products has been prohibited since 2004, while testing for ingredients and ingredient combinations has been banned since 2009. A marketing ban has also been in place since 2009 for all human endpoints except for repeated dose toxicity, reproductive toxicity and toxicokinetics, for which the deadline was extended to 2013 (EU 1976, amended by EU 2003). This deadline was not further extended, despite an expert panel of scientists finding that the time required for establishing alternative methods for the full replacement of animal testing in the field of repeated dose systemic toxicity could not be estimated (Adler et al. 2011). The bans have been included in the most recent EU legislation on cosmetics, Regulation N° 1223/2009/EC.

These legislative changes have necessitated more concerted efforts in the scientific community towards the development and implementation of alternative methods, especially those that enable the replacement of animal studies for chronic or sub-chronic toxicity testing. Following a call for proposals under the HEALTH Theme of the 7th European RTD Framework, a joint Safety Evaluation Ultimately Replacing Animal Testing (SEURAT-1) research initiative was set up between the European Commission and Cosmetics Europe (formerly named COLIPA). SEURAT-1 commenced in January 2011 and aimed to develop *in vitro* strategies towards the replacement of repeated dose toxicity testing in experimental

animals, particularly as applicable to cosmetic ingredients (<http://www.seurat-1.eu/>). Even though SEURAT-1 was initially motivated by the urgent needs of the cosmetic industry, systemic toxicity testing is also relevant for a variety of applications in other industrial sectors, including the development of pharmaceuticals. The SEURAT-1 cluster consisted of 6 complementary research projects (Figure 1) that are considered as the “building blocks” to develop a novel “human safety assessment strategy” targeting repeated dose toxicity. The goal was the integration of the technologies developed and data generated in the different building blocks in order to understand how complementary novel approaches can be used to devise an overall strategy for reaching the ultimate long-term goal of replacing current repeated dose systemic toxicity testing in human safety assessment (Vanhaecke et al. 2011).

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

Within SEURAT-1, the DETECTIVE building block led innovations in the field of *in vitro* toxicity testing, moving toxicology beyond descriptive science towards mechanism-based predictions. The research conducted in the DETECTIVE project comprised a battery of established and innovative high-content and high-throughput functional and “-omics” technologies to establish new biomarkers for repeated dose toxicity. The term “-omics” refers to the high-content study of a family of biomolecules or modifications such as mRNA (transcriptomics), proteins (proteomics), metabolites (metabolomics) or epigenetic modifications of the DNA (epigenomics). The hypothesis-free investigations run with these methodologies represent a step forward from traditional investigations for biomarker discovery. Complementing the “-omics” strategy was the development of functional readouts for the identification of biomarkers of repeated dose toxicity *in vitro*. Functional parameters provided insights into the physiological effects of toxicants on specific cell functions. The technologies used for the functional readouts, were electrical activity, impedance measurements and high-throughput imaging. The data generated from the functional and “-omics” branches contributed towards the ultimate objective of DETECTIVE, the evaluation of the significance of putative *in vitro* biomarkers. An overview of the DETECTIVE project structure is presented in Figure 2.

Given the limited time frame of the consortium (2011 to 2016) the early selection of the most relevant target organs, on which DETECTIVE should focus, was imperative. Systematic screening of the opinions published by the Scientific Committee on Cosmetic products and Non-Food Products intended for consumers (SCC(NF)P, EU 1997) was carried out exploiting a previously established public database (Pauwels et al. 2009; Rogiers and Pauwels 2008). By analyzing the target organs involved in repeated dose toxicity studies for 154 cosmetic ingredients, it was found that the most frequently affected organs by cosmetic ingredients were the liver and the kidney (Vinken et al. 2012). Furthermore, as the mandate of SEURAT-1 was not restricted to cosmetics, it was also relevant to examine common toxicity effects relating, for example, to 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 (Kettenhofen and Bohlen 2008; Stummann et al. 2009). DETECTIVE, therefore,

concentrates on hepatotoxic, nephrotoxic and cardiotoxic effects, representing three target organs of repeated dose toxicity of cosmetics and pharmaceuticals in humans.

DETECTIVE Final General Assembly

To discuss the final outcomes and achievements of the consortium, a meeting was organized, in Brussels, prior to the preparation of the final reports and conclusion of the project. The meeting brought together data-producing and supporting consortium partners from Klinikum der Universität zu Köln (UKK), Joint Research Centre (JRC), Universiteit Maastricht (UM), Vrije Universiteit Brussel (VUB), Imperial College of Science, Technology and Medicine (IC), Deutsches Krebsforschungszentrum – Department of Biostatistics (DKFZ), ARTTIC, QURE Ltd (QUIRE), Medizinische Universität Innsbruck (IMU), Leibniz-Institut für Analytische Wissenschaften-ISAS-EV (ISAS), Fraunhofer-Gesellschaft zur Förderung der Angewandten Forschung E.V. (ITEM) and Universiteit Leiden (UL). The presentations focused on the current state of ongoing and concluding projects and the strategies employed by the data-producing groups on the identification of new relevant biomarkers of toxicity. The outcomes and deliverables, including the dissemination of results in data-rich “-omics” databases, were discussed as was the future perspectives of the work completed under the DETECTIVE project. Although some projects were still in progress and required continued data analysis, this report summarizes the presentations and discussions.

Integration of Biomarker Identification Strategies (Mathieu Vinken, VUB)

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 consortium (<http://toxbank.net/>). In addition, some DETECTIVE partners (IFADO, IMU, UL and VUB) 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 SEURAT-1 projects or even extend beyond the borders and time constraints of the SEURAT-1 consortium. 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, in collaboration with DKFZ. Groups were also required to provide data to QURE for data storage. From here a compendium of biomarkers

(from all partners) will be prepared in the form of the final report for DETECTIVE. The output of this work will be the publication of scientific papers and, where possible, the dissemination and sharing of this work with ToxBank.

Data storage and management (Raivo Kolde, Hedi Peterson, QURE)

Data storage solution for DETECTIVE consortium was provided by QURE who implemented it using Qure Data Management Platform. QURE's data storage solution provided 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.quiretec.com/>) holds data for more than 500 datasets across all the omics readouts and target organs. The database has been available for all the project partners for querying, comparing and receiving the conducted experiments throughout the DETECTIVE project. SEURAT-1 central warehouse ToxBank requires 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 DETECTIVE database QURE automatically formatted the metadata to ISA-Tab format and uploaded the data to the ToxBank for long-time archiving. This ensures that the valuable datasets produced in this consortium will be available in a structured manner to broader research community.

Statistics in DETECTIVE: Can we get by with t-tests? (Annette Kopp-Schneider, DKFZ)

Following the identification of a set of biomarkers, extensive statistical analysis was conducted to evaluate their inclusion in the compendium as a toxicity biomarker. DKFZ's objectives within DETECTIVE have been to perform statistical analysis of derived endpoints and to identify and analyse biomarkers for toxicity in humans. An overview of some of the different statistical techniques and methods employed to answer complex questions and extend the statistical strength of the identification of biomarkers, was presented. 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, Smyth 2004). A critical aspect in the identification of biomarkers for treatment effects was the joint evaluation of "-omics" technologies. iCluster+ (Mo et al. 2013) was used to find common patterns in data from multiple "-omics" technologies and discriminant features. Integrative visualizations 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 (Schäfer and Strimmer 2005) 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 (Ramsay et al. 2009) 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 visualization of statistical results was often the key to interpretation.

Liver toxicity

Challenging the predictive power and robustness of an adverse outcome pathway construct from bile salt export pump inhibition to cholestatic injury (Robim Marcelino Rodrigues, VUB)

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 HepaRG™ 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.

Assessment of repeated dose toxicity of valproic acid, aflatoxin B1 and cyclosporine A in the human liver using integrative “-omics” data analyses (Simone van Breda, UM)

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, a widely used drug in the treatment of epilepsy which is known to induce liver steatosis (Silva et al. 2008), 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 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, is produced by several *Aspergillus* fungi strains (Squire 1981) 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 (de Mattos et al. 2000). 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. Discussion highlighted that follow-up studies are 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 (Regina Stöber, IFADO)

The aim of this case study was to identify biomarkers which predict human hepatotoxic blood concentrations from publically available genome wide expression data of 150 compounds tested in 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.

VPA RAX case study: Detection and verification of biomarkers by using a read across (RAX) approach (Sylvia Escher, ITEM; Jan Hengstler, IFADO and Bob van de Water, UL)

A data-rich lead compound, VPA, was selected given 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 (ER) 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, RAX approach is a promising concept for biomarker detection and validation.

A High Content Imaging-based Toxicity Pathway Reporter Platform for Chemical Safety Assessment (Steven Wink, UL)

DILI remains a major concern for drug development and in clinical practice. At the moment PHH 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 (Wink et al. 2014). This system has enabled the establishment and characterization of reporters for oxidative stress, ER stress, and DNA damage, allowing single cell time-resolved and quantitative analysis of the toxicity pathway activation. 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 to cluster 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.

Renal toxicity

Transcriptomics, epigenomics, miRNA and metabolomic profiling to identify novel renal biomarkers (Alice Limonciel, IMU)

The mycotoxin ochratoxin A (OTA), a contaminant in foods and beverages, a renal carcinogen in rats (Mantle et al. 2005) 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 (Scholpa et al. 2014). This study revealed a strong impact of OTA on the transcriptome that has been previously described (Jennings et al. 2012). The contributions of epigenetic modifications and miRNA expression to the modulation of several protective stress responses, notably the Nrf2 response to oxidative stress (Limonciel and Jennings 2014) were also investigated. The alterations identified by metabolomics were particularly interesting, especially as they have high potential for translation as clinical urinary biomarkers.

Integrated ‘-omics’ analyses in the kidney model (Simone van Breda, UM)

This strategy integrated multiple “-omics” analysis for toxicological mechanisms of two renal carcinogens, KBrO₃ and OTA. Following three repeated doses over five days, cells were recovered after wash out and processed via multiple “-omics”. 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. In summary, 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.

Repeat dose after extended recovery (Paul Jennings, IMU)

Repeat dose investigations are complicated and not necessarily continuous. Few studies have investigated the effect of an extended recovery after an initial repeat dose exposure. In this study the effect of a second repeat dose exposure, to see whether cells truly recover or if there is a “cellular memory” of the first exposure, was investigated. RPTEC/TERT1 kidney cells were exposed to a high, but non cytotoxic concentration of CsA 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.

Cardiotoxicity

Identification of toxicity biomarkers for anthracyclines in human induced pluripotent stem cell-derived cardiomyocytes (Umesh Chaudhari, Harshal Nemade and Agapios Sachinidis, UKK)

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 (Mathur et al. 2015). 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 (DOX), a well-characterized cardiotoxicant (Albini et al. 2010), followed by washout of the test compound with further incubation in compound-free culture medium. 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 DOX, 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 DOX-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.

Effect of cosmetic ingredients and a cholestasis inducing compound on expression levels of proposed potential genomic cardiac specific and non-cardiac specific biomarkers (Umesh Chaudhari, UKK)

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: From Electrophysiological Perspective (Filomain Nguemo and Christoph Schäfer, UKK)

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.

Cross-organ strategies

Proteomics Progress (Laxmikanth Kollipara, René Zahedi, Albert Sickmann, ISAS)

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 (Titz et al. 2014). Usually the first response after stimulation of cells is the phosphorylation of heat shock proteins and other components of stress responses (Fulda et al. 2010). ISAS 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. Furthermore, ISAS developed 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 provided by IMU.

Metabolomic responses to toxicity *in vitro* – extracellular versus intracellular measurements (Hector Keun, IC)

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, HepaRG™, primary hepatocytes were developed. Firstly, in co-operation with UKK, the response of hiPS-CMs to DOX was analysed. Secondly, together with IMU, the metabolic response of RPTEC-TERT1 kidney epithelial cells to OTA and KBrO₃ was assessed. Finally, in collaboration with VUB, the exposure of cholestatic toxicant bosentan to HepaRG™ liver model cells indicated that low dose exposure to bosentan can induce mitochondrial dysfunction.

Common renal/hepatic strategy to identify cell-specific and generic transcriptomic signatures (Paul Jennings, IMU)

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 HepaRG™ 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 (Alice Limonciel, IMU)

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. This topic will be addressed in further EU level and international projects.

General Discussion

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

The output of the DETECTIVE project is considerable. The groups of the consortium have published over 50 peer reviewed scientific paper, related to DETECTIVE studies, since the inception of the consortium in 2011. This number will substantially increase once the final analyses are completed and results are collated for publication in the following year. Overall, the DETECTIVE consortium can list a number of achievements as contributing to the legacy of the consortium. 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, prioritization, test development and for chemical categorization (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. 2015). Technology including fluorescent cell sensors for pathway-specific toxicity screening was 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 hepatotoxicants 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.

Future perspectives

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. Such future activities are envisaged to address the limited scope of DETECTIVE/SEURAT-1 which focused on the use of a limited number of human primary cellular systems and test compounds. 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 (Jiang et al. 2015). 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. Furthermore, the knowledge generated through the detection of endpoints and biomarkers of repeated dose toxicity, by the DETECTIVE project, will contribute to the foundation of future research initiatives launched under the forthcoming research consortium EU-ToxRisk (<http://www.eu-toxrisk.eu/>). This program will focus 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. To this end, the progress already achieved by the DETECTIVE consortium in the development of robust, reliable, and specific *in vitro* biomarkers and surrogate endpoints for use in safety assessments of chronically acting toxicants, will directly carry over into these future research endeavours.

Abbreviations

AFB1	Aflatoxin B1
ANOVA	Analysis of Variance
AOP	Adverse outcome pathway
BSEP	Bile salt export pump
CsA	Cyclosporin A
DEG	Differentially expressed genes
DE-miRs	Differentially expressed miRNAs
DILI	Drug induced liver injury
DKFZ	Deutsches Krebsforschungszentrum – Department of Biostatistics
DMG	Differentially methylated genes
DOX	Doxorubicin
ER	Endoplasmic reticulum
hiPSC-CMs	Human induced pluripotent stem cell-derived cardiomyocytes
IC	Imperial College of Science, Technology and Medicine
IFADO	Leibniz Research Centre for Working Environment and Human Factors
IMU	Medizinische Universität Innsbruck
ISAS	Leibniz-Institut für Analytische Wissenschaften-ISAS-EV
ITEM	Fraunhofer-Gesellschaft zur Förderung der Angewandten Forschung E.V.

iTRAQ	Isobaric tags for relative and absolute quantification
JRC	Joint Research Centre
KBrO₃	Potassium bromate
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LIMMA	Linear Models for Microarray Data
LOEC	Lowest observed effect concentration
OTA	Ochratoxin A
PHH	Primary human hepatocytes
QURE	QURE Ltd
RTCA	Real-Time Cell Analyser
TF	Transcription factors
UKK	Klinikum der Universität zu Köln
UL	Universiteit Leiden
UM	Universiteit Maastricht
VPA	Valproic acid
VUB	Vrije Universiteit Brussel

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Figure 1.

SEURAT-1 aimed at the development of strategies to replace repeated dose systemic toxicity testing. This research initiative was based on the funding of six large-scale collaborative research projects that encompassed key themes related to human stem cell technology (SCR&TOX), organ-simulating devices (HeMiBio), human-relevant biomarker detection (DETECTIVE), computational modelling (COSMOS), systems biology (NOTOX) and integrated data analysis (ToxBank), as well as an umbrella coordination and support action project (COACH) (Vanhaecke et al. 2011).

Figure 2. Overview of the DETECTIVE project structure.