Executive Summary:
The project has focused on three relevant target organs of toxicity, the liver, the kidney and the central nervous system. The overall aim of the project was to improve the predictivity of in vitro systems for unwanted effects of pharmaceutical entities by applying a combination of “omics” technology in optimized cellular systems along with biokinetic quantification.

For the liver, 11 compounds were tested in three different hepatic models: primary human hepatocytes (PHH), primary rat hepatocytes (PRH) and the hepatoma cell line HepaRG. The different cell models were
capable of metabolising the selected parent compounds, albeit with different sensitivities. For example, ibuprofen metabolism was highest in PHH, where cytotoxicity was also lowest. Bioinformatic analysis of the transcriptomic signatures also provided highly useful mechanistic information. For example analysis of chlorpromazine differentially regulated genes revealed inflammation/hepatitis, cholestasis and hyperplasia to be the major mechanisms, which is in line what is known in vivo. A number of other tissue specific and mechanistic biomarkers have been identified.

A single model was chosen for the kidney, i.e. the non-transformed normal human proximal tubular cell line, RPTEC/TERT1. Nine compounds were tested for in depth omic analysis where cells were cultured on microporous supports and differentiated for 10 days prior to treatment. Furthermore, since renal disease often has an ischemic component, we have investigated the effects of hypoxia on compound-induced toxicity a number of compounds and conducted deep omic analysis for two of these. We have focused our bioinformatics analysis to three areas; (1) mechanistic effects, (2) tissue specific effects and (3) potential clinical biomarkers. The results from the three kidney kinetic compounds show that the RPTEC/TERT1 cells coupled with pharmacokinetics and high content omic approaches gives extremely detailed and quantitative insights into both the pharmacological and toxicological effects of compounds.

As CNS models, we analysed two neuronal primary models including a 2D mouse model and a 3D aggregating rat model. Twelve compounds were tested using both target assays and omics analysis, and as with the other organs a smaller subgroup was used for biokinetic modelling. Aggregating 3D brain cultures developed features of higher cellular organization, including neurons, astrocytes and oligodendrocytes. A stochastic time-concentration activity model for in vitro cytotoxicity has been developed describing transitions from healthy to stressed cells and from stressed cells to death. The 2D mouse model allows the measurement of neuronal activity by measuring electrophysiological alteration when cells are cultured on micro electrode arrays. For the majority of the compounds tested the neuroactive effects did not correlate with toxicity. Pattern recognition of this data allowed for the development of „activity fingerprints“ for the compound classes. An in vitro blood-brain barrier (BBB) was also investigated for its potential integration into the in vitro testing strategy for drug-induced neurotoxicity testing. The effects of single and repeated exposure regimens on an in vitro BBB model showed the treatment regimens strongly influence the exposure levels achieved and demonstrated the importance of evaluating BBB toxicity in repeated-dose testing.

Taken together, we have generated a large data-set of mechanistically-rich information for three target organs in long-term repeat-dose investigations. The data-set represents several layers of biological information and includes transcriptomic, proteomic and metabolomic profiles. Moreover we have quantified parent compounds and their distribution in various in vitro compartments. This has allowed us to develop biokinetic models where we could derive the real exposure concentration in vitro, enabling the definition of human relevant free and toxic exposure levels. The knowledge developed within the project can be applied for screening purposes to filter highly toxic substances from being tested in animals in preclinical studies, and therefore contributes to the 3R principle. Furthermore this project demonstrates the applicability of in vitro test systems for human drug safety evaluation and will form the basis for the eventual replacement of animals in pre-clinical testing regimes.

Project Context and Objectives:
In the development of new pharmaceutical entities lead compounds are designed based on the desired pharmacological effect; often, many derivatives of these lead compounds are synthetized early in the development for optimized pharmacological response. Toxicity testing of the many possible compounds with the desired pharmacological effects represents one of the major bottlenecks in pharmaceutical
development since toxicity testing is time consuming, requires large numbers of animal experiments, time and large amounts of test compound. In addition, for hazard assessment (assessing toxicities of the compound at high doses) the pharmacokinetics of a compound are investigated in animals (and sometimes in humans) to obtain candidate compounds with desirable pharmacokinetics.

Toxicity testing in animals usually relies on the incidence of histopathological changes and clinical chemistry in rodents. Toxicity studies range in duration from two weeks to two years and use 5 to 50 animals/dose group with usually three dose groups and a vehicle control. From the toxicity studies, detailed information on adverse effects and their dose-response is obtained, but the generated data require extrapolation to the human situation. Regarding pharmaceuticals, this is done by using differences between doses or blood levels, which cause unwanted effects and the doses or blood levels resulting in desired pharmacological effects.

The inclusion of biomarkers for undesired effects is not routinely performed in many of the standard protocols for toxicity studies, but biomarkers to predict toxicity for relevant target organs are under development and are increasingly applied to predict possible toxicities in the early phase of toxicity studies.

In vitro toxicology using isolated cells or cultured cells mainly focus on the mode-of-action of a chemical on the cellular level to study toxicodynamics. The results are usually integrated as support for the in vivo studies, but such mechanistic studies are also often initiated after results from in vivo studies are available for confirmation of mode-of-action. Some specific in vitro studies are used for pre-screening to exclude specific unwanted effects.

Current animal toxicity testing has a fairly high predictivity for adverse effects, but the main causes of failure in drug development remain to be due to toxicity and lack of efficacy (see above). In fact, unpredicted toxicity in animals accounts for 25% and human adverse events account for 11% of development failures making toxicity/safety the one major cause of drug attrition. There are several causes of poor correlation between animal and human toxicities. One of the main reasons is that animal species may not predict human metabolism. Also, the diversity of human patients and the different life-style susceptibility factors do not reflect the well-controlled experimental animal settings. Therefore, it is crucial to understand why individuals respond differently to drug therapy and to what extent this individual variability in genetics and non-genetic factors is responsible for the observed differences in adverse reactions. In addition, drug withdrawal from the market due to toxicity is the “worst case” for a pharmaceutical company. Again, the major reason for withdrawal is unpredicted toxicity in humans, mostly of an idiosyncratic nature and with the liver being the predominant organ affected (Fung et al., 2001; Guengerich and MacDonald, 2007; Schuster et al., 2005).

The use of human and mammalian cell-based assays played a key role in this project. Expert knowledge is required to integrate the many potential mechanisms of toxicities into the safety assessment process and to develop useful non animal-based systems to mimic these events in vitro, preferably at the earliest stages of drug development.

Recent advances in tissue and bioreactor technologies, molecular biology, toxicity modelling, and bioinformatics were leading to new approaches in the integration of toxicity testing in vitro into the early stages of drug development, with the possibility of significantly increased predictability. The project approach integrated these new developments to improve and optimize cell culture models for toxicity testing and characterized the dynamics and kinetics of cellular responses to toxic effects in vitro. The target organs most frequently affected by drug toxicity were taken into account, namely liver and kidney. Moreover, predictive models for neurotoxicity are scarce despite the fact that the neuronal system is a
frequent target of unwanted effects of drugs. The CNS was therefore also included as a target tissue. For each target organ, the most appropriate cell model was used. The choice of these models was based on availability and biological relevance to toxicity. It was not envisaged to undertake development of new cellular models, rather to adopt and optimize the “best choice” of established cell systems. The overall aim of the project was to improve the predictivity of in vitro systems for unwanted effects of pharmaceutical entities by applying a combination of “omics” technology and biomarkers in optimized cellular systems. Combination of response biomarkers and their dose response with pharmacokinetic modelling integrating human specific parameters. The combined use of in silico and in vitro techniques to predict drug toxicity in animals and humans may replace some current in vivo tests and significantly reduce the use of animals by screening out drugs with undesirable properties prior to preclinical studies in animals. The major advantages of such an approach are:

- reduced costs,
- quicker turnaround times,
- low amount of compound needed for testing enabling toxicity screening early in drug discovery (e.g. during lead optimization),
- early decisions on exclusion of candidates with unacceptable toxicity profiles and
- knowledge-based selection of most promising lead candidates (late stage)

Optimized cellular systems. The project focused on three major target organs of toxicity, the liver, the kidney and the central nervous system.

Hepatotoxicity observed in animal studies or human clinical trials remains the major reason for failure during drug development. The liver is therefore a major focus of early screening efforts in the pharmaceutical industry. If a compound is hepatotoxic in animals, it is only administered to humans after the toxicity is assessed and an adequate safety margin is estimated. However, hepatotoxicity may only be identified in clinical drug development or during marketing partly due to the low incidences of adverse reactions. In some of these instances, hepatotoxicity may have been observed in non-clinical studies but was not judged as significant, or signals may not have been apparent as in the case of idiosyncratic effects.

Besides the liver, the kidney is also a major target for toxicity of xenobiotics. There are multiple factors, which play a role in the kidney’s vulnerability to toxic damage: (1) the large surface area of the glomeruli and tubules provides an increased opportunity for absorption of toxin into the cells; (2) a high rate of blood perfusion resulting in the delivery of a higher dose of potentially toxic compound; (3) the tubular transport systems which may facilitate cellular uptake of compounds; (4) the kidney possess high levels of activity of Phase I and Phase II metabolizing enzymes, which catalyse formation of potential toxic metabolites; (5) the kidney has a large capacity to recover water and thus concentrate the solutes to be excreted from the body thus increasing toxin concentration; (6) cortical renal tubular cells have a high demand for oxygen, which results in vulnerability to oxygen deprivation. Due to the large functional reserve of the kidney, nephrotoxicity due to pharmaceuticals is often only observed with chronic exposure. In contrast to the liver, several well-established (primary and cell line) models for nephrotoxicity testing in vitro existed, which well represent the nephron. More recently organotypic model systems utilizing continuous medium replenishment (perfusion systems) have been developed which not only increase the differentiated status of the cells but also better represent the mode of exposure as seen in vivo. The proximal tubular epithelium represents the major mass of renal parenchyma and is highly vulnerable to reduced oxygen tensions, which occurs in the majority of chronic renal diseases. The effect of hypoxic
preconditioning of renal systems to pharmaceutical toxicity is often over looked, but is potentially an important factor for influencing toxic effects.

CNS drugs had a low chance of success in drug development. This is due to the complexity of the brain, the probability of CNS drugs to cause CNS side effects, and the requirement of CNS drugs to cross the BBB. Therefore, the large number of hits identified from primary high throughput discovery screens requires early, rapid and robust preclinical screening assays to assess whether compounds with desirable characteristics may cause neurotoxicity. In vivo neurotoxicity is difficult to study due to the inability of full experimental control of factors such as neuronal, hormonal and immunological stimuli. Therefore, in vivo results are not always reliable. Drug-induced side effects on the nervous system are usually tested in behavioural studies monitoring rodent general activity or complex multidimensional functional assays of motor and sensory perturbations. The CNS represents a high level of anatomical and physiological complexity (multiple neuronal and glial cell types), we therefore applied a variety of cell culture models and functional endpoints (neuronal and glial specific biochemical and electrophysiological profiling assays) to distinguish between general cytotoxicity and possible specific drug-induced neurotoxicity and elucidate specific mechanisms of drug neurotoxicity. In addition, in the case of centrally acting drugs, to be effective as therapeutic agents they first must cross the BBB. Conversely, to avoid the unwanted CNS effects, peripherally acting drugs must show no or limited brain accessibility. A testing strategy for this class of drugs in vitro requires an in vitro BBB model to assess the blood-brain penetration or possible BBB damage using specific endpoints such as trans-endothelial electrical resistance (TEER) and permeability assays. Since more than 98% of CNS-targeting drug candidates have been withdrawn because of poor permeability across the BBB, a reliable BBB in vitro model is essential in early stage drug development.

Integration of bioavailability/pharmacokinetics. The bioavailability/pharmacokinetics of a drug candidate is a key element in drug development. Indeed, the pharmacokinetics characteristics of a candidate drug must be adequate for the intended use of the drug, and, in addition pharmacokinetic information is absolutely necessary to interpret and predict both pharmacological and toxicological in vivo findings in animals and in humans. Unfavourable pharmacokinetic characteristics (such as insufficient bioavailability, inadequate distribution, too large or too small clearance, or unacceptable pharmacokinetic variability) have been an important cause of failure in late stages of drug development, after considerable amounts of work and money had been invested. However, the increasing awareness of the importance of pharmacokinetics as a determinant of drug development success has led to more work being devoted to the early generation of pharmacokinetic information on candidate drugs. As a consequence and as described above, the rate of drug development failures directly due to unfavourable pharmacokinetics has decreased from 40% to 10% between 1991 and 2000. According to the same source, “preclinical pharmacokinetic science no longer is considered a major obstacle to drug development, although there is room for further improvement”.

Generating pharmacokinetic information during drug development rests upon in silico, in vitro and in vivo methods, depending on the stage of development and number of candidate molecules to be screened. At all stages, pharmacokinetic modelling, or at least the use of concepts related to pharmacokinetic modelling (e.g. linking solubility to intestinal absorption, or lipophilicity to distribution characteristics) plays a crucial role in integrating the information generated, contributing to decisions as to which compounds will or will not be further considered, for pharmacokinetic reasons, as potential drugs within a given project. Pharmacokinetic is also the link between the administered dose and the observed effects. Modelling these effects taking into account the time course of concentrations in blood or other relevant body fluids is at the root of time-based simulations of effects, i.e. pharmacokinetic/pharmacodynamic (PKPD) modelling. PKPD modelling is the conceptual framework that allows the integration of the pharmacokinetic (PK) and
pharmacodynamic (PD) information in order to make predictions of pharmacological or toxic effects across doses and species, in particular from animal to man, but also from in vitro to in vivo predicted effects. These predictions based on margins-of-safety are then used to make decisions in drug development, such as excluding a compound from further development at a relatively early stage due to a high risk of toxic effects at pharmaceutically active doses. This conceptual framework will be central in this project, to integrate the information obtained, generate predictions and evaluate them against reference data.

Integration of “omics”, biomarkers and high content imaging for early prediction of toxicities in vitro. When toxins interact with cells and tissues they disturb the concentrations and fluxes of endogenous metabolites in key intermediary cellular metabolic pathways. In an attempt to maintain homeostasis and metabolic control, cells vary and equilibrate the compositions of their intra- and extra-cellular fluids. In more severe toxicity states, cell death leads to more dramatic biochemical changes due to loss of homeostasis and metabolite leakage from damaged cells. Whatever the severity of the toxic event, the subsequent alteration(s) in cellular biofluid composition are specific of the toxicity type. The use of a combined NMR and MS expert system approach allows to systematically explore the relationships between biofluid composition and toxicity and to generate novel combination of safety biomarkers. The approach of characterizing the metabolic profile of a specific cell/tissue or biofluid has been termed “metabol(n)omics” by analogy with genomics and proteomics. 1HNMR and MS-based spectroscopy’s are well suited to the study of toxic events, as multi-component analyses on biological materials can be made simultaneously. The complementary role of NMR and MS spectroscopy in analytical toxicology is thus essentially one of biochemical exploration, i.e. determining the range of biochemical perturbations caused by exposure to a toxin and whether these are biologically significant.

Profiling methods mainly based on “omics” and high-content imaging as well as other endpoints capturing deregulation of essential cellular processes, will deliver biomarkers and cluster modelling data to be used for integration in the hazard assessment data sets for further risk assessment. Improved knowledge on the toxicity mechanisms was obtained for some model compounds.

Generation of pivotal points for risk assessment from in vitro data. The project also addressed one of the major problems of in vitro methods, the absence of data indicating the point of departure for extrapolations: from in vitro studies, no-observed-adverse-effect-levels (NOAELs) serving as a starting point for extrapolations cannot be easily derived since systemic doses cannot be directly calculated from the concentrations of a drug applied to cells. In cell systems which best represent in vivo target organs, the most predictive endpoints indicative of adverse effects were used to determine the no-observed-effect-concentrations (NOEC) in vitro. The NOECs were based on the estimation of measured in vitro intracellular concentrations of the drug and/or its relevant metabolites. These NOECs were then be transformed to doses received using appropriate modelling techniques, in particular advanced PBPK modelling including Monte-Carlo techniques. Since the model systems is based on human cells and the PBPK-models incorporate human parameters and potential interindividual differences in human, the need for extrapolations is reduced (Figure 1).

In summary, the complex events possibly contributing to toxicities of new pharmaceuticals illustrate the difficulties and the challenges of mimicking them in vitro.

Project Results:
1. Liver
The liver is the organ most frequently affected by drugs in repeat dose toxicity studies and during clinical
drug development. Moreover, bioactivation in the liver plays a major role in general drug toxicity as well as idiosyncratic effects; and species-specific effects are often related to differences in drug metabolism between animal and man. In sub-WP2.1 we therefore focused on models that are able to predict long-term liver toxicity in human and rodent cell models. In addition and importantly, we determined toxicokinetic parameters in these liver cell models to derive intracellular concentrations of the test compounds. For selection of proper cell models, we aimed to characterize a human cell system that provides a high stability and is easily accessible thereby overcoming the shortcomings of primary cell culture systems. One such candidate cell model is the human hepatoma cell line HepaRG. This cell line is derived from a hepatocellular carcinoma and exhibits several hepatocyte-like functions such as stable expression of drug metabolizing enzymes (DMEs) (Aninat et al., 2006). These immortalized human hepatic cells were compared with the well-established “gold” standard of long-term human (PHH) and rat (PRH) hepatocyte sandwich cultures (Richert et al., 2004; Tuschl et al., 2009). The latter models have been shown by us and others, in previously published work, to maintain typical liver-like characteristics in terms of morphology, drug metabolizing capacity and stable protein and gene expression over more than two weeks. Eleven well characterized hepatotoxic and control compounds were selected (see also WP1 and period reports) and based on technical requirements four compounds (amiodarone, chlorpromazine, ibuprofen and cyclosporine A) were chosen to be used for in-depth biokinetic and toxicodynamic analyses. In vitro kinetics in liver cell models. Several comparative studies were performed to define in vitro kinetics of the reference compounds (see also period reports). The selected in vitro long-term cultures of primary rat and human hepatocytes and the human HepaRG cell lines were used to evaluate the intracellular exposure of the various cell culture systems after single and repeated treatment of the analgesic drug ibuprofen that shows mild hepatotoxicity. We characterised the metabolic fate of ibuprofen (i) after acute (d0/1) and daily repeated (up to 14 days) dosing on day 13/14 and (ii) in the different culture systems to define their relevance for in vivo prediction. All three culture systems showed a high metabolic capacity, with human hepatocytes showing the highest capacity. In fact, cytotoxic effects - as defined by the concentration that was cytotoxic to 10% of the cells (TC10) - was tenfold higher in PHH than for the other culture systems PRH and HepaRG. Interestingly, interindividual human donor variability showed that a low CYP2C9 activity, the major CYP in ibuprofen transformation, lead to an increased cytotoxicity in PHH. This further supports that species and human donor-specific properties in drug metabolizing capacity have to be characterized when analysing drug toxicity in vitro. In another study, the in vitro biokinetics of the model hepatotoxicant chlorpromazine was evaluated in the three different liver cell systems after repeated exposure (14 daily treatments). Samples were taken from medium, cells and well plastic at specific time points after the first and last exposure. Overall, the mass balance of chlorpromazine decreased in the course of 24 h, indicating the metabolism of the compound within the cells. The largest decrease in parent compound was seen in the primary cultures; in the HepaRG cell cultures the mass balance only decreased to 50%. Chlorpromazine accumulated in the cells during the 14-day repeated exposure (see Figure 2). At the 15-20 µM chlorpromazine concentrations, formation of lamellar bodies, typical of phospholipidosis was observed. Possible explanations for the accumulation of chlorpromazine are a decrease in metabolism over time, inhibition of efflux transporters or binding to phospholipids. The biokinetics of chlorpromazine differed between the three liver cell models and were influenced by specific cell properties as well as culture conditions. Mechanistic insights of chlorpromazine hepatotoxicity. In a further study, we evaluated the mode of toxicity action of chlorpromazine in the primary cell culture models (Parmentier et al., 2013). We analysed
transcriptomic data derived from short- and long-term cultured PHH exposed to chlorpromazine. Samples were collected from five PHH cultures after short-term (1 and 3 days) and long-term (14 days) repeat daily treatment with 0.1-0.2 µM chlorpromazine, corresponding to the therapeutic human peak plasma concentration as well as with a low concentration of 0.02 µM and a high concentration of 1 µM chlorpromazine. Differences were seen in the total number of deregulated genes between human donors and over time of treatment (Figure 3).

Transcriptomic hepatotoxicity signatures of chlorpromazine consisted of inflammation/hepatitis, cholestasis and hyperplasia in all 5 human donors, as well as of necrosis, fibrosis and steatosis in 4/5 donors. A signature indicative of cirrhosis was observed only after long-term 14 day repeat treatment in 4/5 donors. The inter-donor variability in the inflammatory response of human hepatocyte cultures to chlorpromazine treatment was associated with variability in the strength of transcriptomic hepatotoxicity signatures suggesting that features of inflammation could be related to the idiosyncratic hepatotoxic effects of chlorpromazine in humans (Table 1). Similar effects were observed also in rat primary cultures (long-term sandwich configuration). However, these effects occurred only at higher concentrations indicating a lower sensitivity of rat hepatocytes to chlorpromazine’ toxicity compared to human hepatotoxicity. These results are perfectly in line with the known effects of chlorpromazine in human patients as chlorpromazine is hepatotoxic at lower concentrations in human compared to studies in rats.

Evaluation of liver specific cellular functions by High Content Imaging (HCl). For high content imaging investigations rat hepatocytes in the Collagen I-Matrigel™ sandwich culture were used during 14 days. Ten drugs associated with different types of specific preclinical and clinical liver injury were evaluated at non-cytotoxic concentrations. Mrp2-mediated transport, intracellular accumulation of neutral lipids and phospholipids were selected as functional endpoints by using Cellomics™ Arrayscan® technology and assessed at five timepoints (day 1, 3, 7, 10, 14). Liver specific functional impairments after drug treatment were enhanced over time and could be monitored by high content imaging already after few days and before cytotoxicity. Phospholipidosis-inducing drugs chlorpromazine and amiodarone displayed the same response as in vivo. Cyclosporine A, chlorpromazine, and troglitazone inhibited Mrp2-mediated biliary transport, correlating with in vivo findings. Steatosis remained difficult to be reproduced under the current in vitro testing conditions, resulting into false negative (valproic acid) and positive responses (cyclosporine A).

The present results suggest that the repeated long-term treatment of rat hepatocytes in the Collagen I-Matrigel™ sandwich configuration might be a suitable tool for safety profiling of the potential to induce phospholipidosis and impair Mrp2-mediated transport processes, but not to predict steatosis. In human hepatocytes cultured in the Collagen I-Geltrex™ sandwich configuration from 2 human donors, cyclosporine A treatment enhanced specific effects at low non-cytotoxic concentrations (Figure 4). Inhibition of Mrp2-mediated transport was confirmed in hepatocytes from both human donors. Similar to rat hepatocytes neutral lipids accumulated in hepatocytes from one donor, which is regarded as an unspecific in vitro artefact.

Overall, these results proved that hepatocytes are a reliable and stable cellular model that provided good predictive strength to detect hepatotoxic compounds. HepaRG showed to be a good alternative that is partly less sensitive. Nevertheless, similar mode of toxicity action as observed in human (and rat) hepatocytes could be observed in HepaRG. Importantly, integration of biokinetics and modelling (see also WP3 and 5) together with high content imaging profiling (see WP4) allowed (i) to derive real exposure concentration in vitro that could be correlated with the real in vivo human situation, and
(ii) provide better informative mechanisms of toxicity (and pharmacology).

Taken together, the complex data set enables quantitative measurement that proves the concept that we can define human relevant free and toxic exposure levels in vitro. Further compounds have to be analysed in a broader concentration range to fully exploit these promising results.

2. Kidney

The main function of the kidney is the maintenance of whole body homeostasis and the excretion of waste products and thus this organ is exposed to a wide variety of chemical entities, some of which can cause nephrotoxicity. The proximal tubule region, due to its main functions of reabsorption and secretion is one of the most susceptible nephron regions. This is the nephron region to which we have focused in the Predict-IV project.

We have established and characterized long term cultures of both human primary proximal tubule cells and the human proximal RPTEC/TERT1 cell line (Aschauer et al., 2013). The RPTEC/TERT1 cells have a large advantage over cancerous cell lines and lines developed with oncogene introduction, such as HK-2 cells, in that they form stable monolayers for up to several months. We have demonstrated that the RPTEC/TERT1 model exhibits an extremely stable transcriptome after contact inhibition and differentiation and expresses several proximal tubule specific characteristics after this process (Aschauer et al., 2013; Wilmes et al., 2013). Furthermore, the cells exhibit very low glycolysis rates, which are often greatly increased in chemical induced stress. An increase in glycolysis can be easily determined by measuring lactate in the supernatant medium, making it an ideal monitoring endpoint, as it is non-destructive and extremely sensitive (Limonciel et al., 2011).

In this project we have screened over 25 nephrotoxins and selected 9 of these compounds for in depth omic analysis (Figure 5). Cells were cultured on microporous supports and differentiated for up to 10 days before treatment. For “omic” experiments non-cytotoxic concentrations of compounds were used. Three of these compounds cyclosporine A, adefovir dipivoxil and cisplatin were also used to develop pharmaco-/toxicokinetic models. Furthermore, since renal disease often has an ischemic component, we have investigated the effects of hypoxia on compound-induced toxicity a number of compounds and conducted deep omic analysis for two of these (adefovir dipivoxil and zoledronate). We have focused our bioinformatics analysis to three areas; (1) mechanistic effects, (2) tissue specific effects and (3) potential clinical biomarkers.

(1) Mechanistic biomarkers

We have delineated several dominant stress response pathways, with distinct transcriptomic signatures. Chief amongst these are the Nrf2 oxidative pathway, the unfolded protein response, the Hif-1alpha hypoxia response, the p53 DNA damage response and the metal stress response (Jennings et al., 2013). Additionally, many of these pathways exhibited detectable signatures in the proteomic and metabolomic data sets, as exemplified for Nrf2 and ATF4 in Figure 6 (Wilmes et al., 2013). The activation of these pathways allows us to build a mechanistic picture of how chemicals are interacting with the biological systems (Table 2).

In addition, we have utilised siRNA and high content imaging to further interrogate these pathways. Here slightly different experimental procedures were used. Cyclosporine A treatment of RPTEC/TERT1 cells cultured on 96 well plates over 14 days caused increased levels of reactive oxygen species (ROS), depolarization of mitochondrial membrane potential (reflected by decreased TMRM fluorescent signal) activation of caspase 3/7 and cytotoxicity, as measured by intracellular ATP in a time- and dose-
dependent manner. Figure 7 shows the sequence of events occurring after cyclosporine A treatment. The initial increase of ROS formation suggests a triggering role of the apoptotic and necrotic process. Cyclosporine A treatment resulted in significantly increased of the PERK/ATF regulated TRIB3 (Trb3, Tribbles homolog 3) transcript levels on day 1, 3 and d 14. Since TRIB3 is a negative regulator of NFkB and a sensitizer of the TNF- and TRAIL induced apoptosis, we investigated the role of TRIB3 in cyclosporine A-induced apoptosis by specific gene silencing using the Thermo Scientific Dharmacon Accell™ siRNA reagents to transfect the RPTEC/TERT1 cells. TRIB3 silencing partially inhibited the cyclosporine A-induced apoptosis and necrosis (measured by activation of caspase 3/7 and percentage of TOTO-3 positive cells, respectively), whereas the cyclosporine A-induced ROS formation was not affected. These results suggest that TRIB3 induction by cyclosporine A treatment plays a role in the cyclosporine A-induced apoptosis and necrosis. They further suggest that the cyclosporine A-mediated ROS and superoxide formation are independent events from TRB3-induction.

(2) Tissue specific effects

We have characterised the processes involved in RPTEC/TERT1 maturation to a confluent monolayer in order to identify potential tissue specific biomarkers (Aschauer et al., 2013). We showed that the transcriptome stabilised over this maturation period and the cells underwent a cycle arrest in G0/G1 phase. We demonstrated dynamic alterations in expression and localisation of adherens junction (AJ) and tight junction (TJ) proteins. For example, CDH1, CLDN2 and CLDN10 expressions were increased in the matured monolayer. Furthermore, the matured epithelium exhibited low levels of glycolysis in contrary to proliferating cells and possessed a higher oxidative capacity. On the gene expression level, we observed up-regulation of various genes involved in β-oxidation of fatty acids (e.g. BBOX1), cilia biogenesis (e.g. BBS1) and transport (e.g. diverse subunits of Na+/K+-ATPase) during the monolayer maturation process. The activities of transcription factors HIF1A and c-MYC that promote glycolysis were high in proliferating cells, whereas p53 and FOXO1 were activated in the matured monolayer.

We hypothesised that chemically induced sub-lethal injury resulting in cell stress may lead to cellular dedifferentiation and thus overlap with cellular processes occurring in maturation, albeit in the opposite direction. Thus, transcriptional signatures of the maturation process were cross-referenced to transcriptomic alterations of RPTEC/TERT1 cells treated with clinical and chemical nephrotoxins over long-term repeated dose exposures. Indeed, we have demonstrated that the differentiation/maturation state was severely impacted in the chemical-induced stress response, as the majority of the genes identified during epithelial monolayer maturation were altered by several compounds towards expression of proliferating cells. In addition, there is a large overlap between the top 100 probes in the cross-compounds comparison and the top 100 probes of the epithelial monolayer maturation dataset (EMMD) cross-referenced to the compound datasets. This implies that genes involved in monolayer maturation are heavily affected by the various nephrotoxins resulting in the loss of differentiated functions. Such processes included (1) barrier function, (2) transport, (3) energy metabolism, (4) cell adhesion and (5) ciliogenesis. These included members of the Na/K ATPase family, BBOX-1 and claudin-2. The loss of these markers represents a stress induced dedifferentiation and can be considered a compound-induced cellular dysfunction. In addition they were extremely sensitive and were altered at concentrations well below detectable cytotoxicity (cell death). The combination of mechanistic biomarkers and tissue specific biomarkers allow the assessment of the impact of the compound on cellular function (Table 2).

(3) Potential clinical biomarkers
Finally we have investigated potential translational biomarkers from the in vitro RPTEC/TERT1 cells to the clinical situation in vivo. We have uncovered some biomarkers such as heme-oxygenase 1 and lipocalin 2, which were also detected as increased in the urine of patients with chronic kidney disease (CKD). In addition, we have uncovered IL-19 as a previously unidentified renal injury biomarker. IL-19 mRNA was increased when RPTEC/TERT1 cells were exposed to several nephrotoxins. Furthermore, the IL-19 protein was detected in the supernatant by ELISA indicated it is a secreted protein. We could detect a temporal increase in secreted IL-19 from RPTEC/TERT1 cells in response to exposure to several of the tested nephrotoxins. The IL-19 protein was also increased in CKD patient’s urine and correlated positively to lipocalin 2 levels and negatively to glomerular filtration rates.

Combination of integrated omics with in vitro toxico/pharmacokinetics

The results from the three kidney kinetic compounds show that the RPTEC/TERT1 cells coupled with pharmacokinetics and high content omic approaches give extremely detailed and quantitative insights into both the pharmacological and toxicological effects of compounds. For example in the cyclosporine A study renal cells exhibit mitochondrial perturbations, oxidative- and ER-stress, only at the high supratherapeutic cyclosporine A concentration. Proteomics and follow-up targeted studies demonstrated that cyclosporine A induced CyP-B secretion in both therapeutic and supratherapeutic concentrations. The pharmacokinetic modelling showed that cyclosporine A at the high dose massively accumulated within the cells over the 14 days of exposure, which would explain the high stress induction at this concentration. There was no such cyclosporine A accumulation at the therapeutic dose and evidence of cells stress, but there was a close to maximal pharmacological activity (CyP-B secretion). Thus, the use of the high content omic approaches together with pharmacokinetics, allow us to separate the pharmacology activity and toxicity of this compound.

The RPTEC/TERT1 cell line is an excellent tool for repeat dose exposures of nephrotoxins. Due to its stability and high differentiation status it is very well suited for transcriptomic, proteomic and metabolomic analysis. Furthermore, we have demonstrated RPTEC/TERT1 cells to be very sensitive to nephrotoxins and have established several new in vitro biomarkers based on the loss of a differentiated phenotype. The relevance of this system to the clinical situation is reinforced by the fact that some of the markers discovered were also detectable in the urine of CKD patients. Furthermore, these novel biomarkers correlated to established renal injury biomarkers and clinical renal functional parameters.

3. CNS

Neurotoxicity is the outcome of complex interactions of a drug (or chemical) at molecular, cellular and tissue level of the central and/or peripheral nervous system causing an adverse effect. An adverse effect can be caused by changes of neuronal and glial cells chemistry, structure and function. Therefore, in vitro testing strategy for drug-induced neurotoxicity evaluation has to be based on the combination of relevant in vitro models that possess necessary molecular mechanisms and pathways that can be evaluated by sensitive, neuronal and glial specific endpoints in a quantitative manner.

In this project we proposed two neuronal primary cultures (2D and 3D) to determine how these two models could be used in a complementary manner applying different assays and taking into consideration the advantages of each model. Additionally, the in vitro BBB model was applied to determine when and why a BBB model should be included in the in vitro integrated testing strategy for drug-induced neurotoxicity testing.
3.1. The main results obtained from the in vitro BBB studies

The BBB is the principal route for the entry of most molecules into the CNS as well as it is the major hurdle that prevents many drugs from eliciting a toxicological effect within the CNS. CNS exposure is a function of several factors such as BBB permeability, plasma protein binding and brain tissue binding. Consequently, it is important to know whether a compound reaches the brain and what is the unbound concentration of a drug in the brain as this is a critical factor for predicting the in vivo outcome based on in vitro results. This yields also a key information for selection of drug/chemical concentrations for in vitro studies that are relevant to the in vivo situation.

A. Extrapolation from in vitro BBB drug penetration to determine in vivo CNS exposure

The data obtained from in vitro BBB permeability studies of 12 drugs have been integrated into pharmacokinetic models to determine the selection of the relevant concentrations of drugs for in vitro neurotoxicity studies using neuronal models.

For repeated dose neurotoxicity testing it is important to predict the free concentration of the drug in the brain (unbound in brain) reaching the target cells at steady state as this is a critical factor for predicting in vivo outcome based on in vitro results. The determination of the ratio between the concentration unbound in brain (Cu,br) and unbound in plasma (Cu,pl) represent a convenient approach to determine whether certain plasma exposures are likely to yield concentration unbound in brain which would result in a neurotoxicological response based on the findings of in vitro neurotoxicity studies.

In PREDICT-IV a new method was proposed to predict the concentration based on unbound brain concentration and unbound plasma ratio (Cu,br./Cu,pl.) under steady-state conditions using a co-culture in vitro BBB model. This new methodology, has been used to predict the Cu,br./Cu,pl ratio at steady-state of the 12 CNS compounds. These Cu,br./Cu,pl ratios generated in vitro could be useful as from the known in vitro neurotoxic concentrations and estimated plasma exposure it is possible to make an early prediction of the risk of a toxicological effect in the CNS.

B. Successful optimization of the in vitro BBB model for repeated dose toxicity studies

An important achievement of the Predict-IV project was that for the first time an in vitro BBB model was successfully adapted for studying the long-term exposure effects induced by low concentrations of drugs. Such approach mimics closer the realistic human exposure. The ability to maintain the characteristics of the BBB (complex tight junctions, low permeability and active efflux pumps) in optimized in vitro BBB model makes it possible to investigate the effects of repeated-dose treatment with 12 selected drugs over a two-week period. Only cisplatin and propofol were found to exert a toxic effect on the in vitro BBB following repeated-dose treatment. There is no published evidence to suggest that propofol is toxic for the BBB whereas the neurological adverse drug reactions reported in human with cisplatin have been associated with BBB disruption on the basis of in vivo studies in rats and rabbits (Namikawa et al., 2000; Sugimoto et al., 1995).

The absence of toxicity for other 10 tested drugs in our BBB model, confirmed in vivo studies where no specific BBB toxicity have been reported for these drugs. Therefore, these results emphasize the reliability of our in vitro BBB model that is suitable not only for acute but also for repeat-dose toxicity assessment. Furthermore, the effects of single and repeated exposure regimens on BBB integrity were compared for several drugs. The results obtained for colchicine, a potent anti-inflammatory agent and known to induce neuropathy when used chronically were further explored. Compared to single exposure, a 100-fold lower concentration of colchicine in 14 days repeated-dose treatment was toxic. This finding demonstrates the
importance to evaluate the BBB toxicity in repeated-dose testing as in some cases it differs dramatically (as in the case of colchicine) when compared with acute treatment.

C. Development of a miniaturized BBB model and replacement of glial cell culture by the glial conditioned medium
The next important step forward was achieved by miniaturizing the classical BBB model (co-culture endothelial cells with astrocytes) that in the classical model is established in 6 well plates. For the purposes of this project instead of 6 well plates the BBB model was successfully carried out in 24 well plates and the culture of glial cells was replaced by glial conditioned medium. These optimizations make the in vitro BBB model suitable for robot screening platforms (HTS) and further experiments demonstrated that the miniaturized BBB model (and without glial cells) responded in a similar way as the classical model to the repeated dose drugs treatment.

D. Evaluation of the possibility to study the effects of drug drug interaction (DDI) at the BBB level
It was recently shown that co-administration of cyclosporine A and colchicine in healthy human subjects results in substantially higher colchicine exposure (compared with the absence of cyclosporine A) and contributes to the associated adverse effects (Wason et al., 2012). We therefore evaluated the effects of repeated-dose colchicine treatment in the presence or absence of cyclosporine A and found that the presence of 1 µM cyclosporine A potentiates the toxic effects of colchicine on ECs. This finding suggests that BBB dysfunction may be involved in neurotoxicity related to drug–drug interactions.

Summing up, BBB plays an important role in in vitro neurotoxicity evaluation, as it allows a better estimation of the actual drug concentration that is reaching the target site (brain cells). This knowledge is critical for the selection of the relevant concentrations for in vitro experiments. The unbound brain/plasma ratio generated in vitro is important as from the known in vitro neurotoxic concentrations and estimated plasma exposure it is possible to make an early prediction of the risk of a toxicological effect in the CNS.

3.2. The main results obtained from aggregating brain cell cultures (3D model)
Aggregating brain cell cultures were proposed as a 3D model for evaluation of drug induced toxicity as this test system was the most sensitive and reliable for in vitro neurotoxicity evaluation based on the results obtained in the previous IP project ACuteTox, where toxicity induced by chemicals was tested after acute exposure. These cultures contain all types of brain cells (neuronal and glial). The 3D cellular architecture of the spontaneously forming aggregates resembles organotypic structures and functions (Honegger et al., 1979; Honegger and Zurich, 2011). The cultures develop features of higher cellular organization such as functional synapses, myelinated axons, spontaneous electrical activity, and drug-induced glial reactivity, and therefore express many possible targets for drugs.

A. Mathematic modelling of toxic action in aggregating brain cell cultures
A stochastic time-concentration activity model for in vitro cytotoxicity has been developed from LDH results obtained in brain aggregates. This model describes transitions from healthy to stressed cells and from stressed cells to death, and is now published (Renner et al., 2013). The time-concentration activity modelling was applied not only to predict the cytotoxicity of compounds but also specific enzymatic activity of the different brain cell types.

B. Aggregating brain cell cultures express metabolic activity
The metabolic capacity of in vitro models is a critical issue since the toxicity of many drugs/chemicals is induced by generated metabolites. It has been shown in this project that indeed aggregating brain cell cultures exhibit metabolic properties. The constitutive expression of several cytochromes P450 was shown in neurons and astrocytes and in a very few oligodendrocytes. Furthermore, the presence of amiodarone metabolites was detected in the cells confirming again the metabolic capabilities of this 3D model.

C. Identification of biomarkers and neurotoxicity pathways that could be used for evaluation of drug-induced neurotoxicity

The most promising endpoints found in ACuteTox project comprise the gene expression of neurofilament proteins, glial fibrillary acidic protein, myelin basic protein and heat shock protein-32. In Predict-IV, again these four genes proved to be very powerful endpoints for neurotoxicity testing. However, eight new candidates were identified by transcriptomics analyses. These genes were evaluated in the bank of mRNA issued from the ACuteTox project, to determine whether they may improve the performance of the four above cited genes. Two canonical pathways of importance for CNS were enriched: synaptic long-term potentiation and axonal guidance, but no enrichment of protein ubiquitination and Nrf2-mediated-oxidative-stress response, the major pathways identified in kidney cells, was observed. Interestingly these results pointed out that biomarkers for neurotoxicity testing are related mainly to brain specific mechanism of toxicity and not to general cytotoxic pathways. About forty intracellular metabolites were identified by NMR. Among them, metabolites related to neurotransmitters and linked to energy substrates were decreased after exposure to several drugs. Neurotransmitters are highly specific markers for brain function and may prove to be sensitive biomarkers for neurotoxicity. Proteomics showed the deregulation of about 100 proteins, such as synaptotagmin, neuromodulin and protein S100 after exposure to several drugs. The results obtained are in line with the view that the predictive capacity of a model for neurotoxicity detection is directly related with its capacity to reproduce the structural and functional specificities of the in vivo nervous system, which is most closely achieved by 3D cultures.

3.3. The main results obtained from murine 2D cortical network cultures

The main rationale for applying the 2D cortical network model was to evaluate whether it is possible to predict neurotoxicity based on electrophysiological changes in the cortical network activity (Figure 8) in addition to the omics approach. For this purpose a phenotypic technology for the recording and mathematical analysis of neuronal network action potentials using microelectrode arrays has been developed. The activity pattern of the cell cultures can be assessed from an embryonic stage up to several weeks after initial 4 weeks of maturation. The network activity is described by 200 parameters and is analysed by sophisticated pattern recognition methods.

A. Evaluation of neuronal electrical activity

To develop experiment designs for clear definition of neurotoxicity endpoints, we conducted several approaches: (i) an acute pattern recognition approach, (ii) an acute isobolographic approach by co-exposure of 2 compounds, and (iii) a chronic repeated dose treatment approach.

(i) The aim of the acute electrical pattern analysis approach was to develop a model for prediction of neurotoxicity based on acute concentration-response curves for the 12 test compounds in four classes: (I) neurotherapeutic and neurotoxic (amiodarone, bufomedil, chlorpromazine), (II) neurotherapeutic and non-neurotoxic (carbamazepine, diazepam, propofol), (III) non- neurotherapeutic but neurotoxic (cisplatinum, ciprofloxacin, cyclosporine A) and (IV) non- neurotherapeutic and non-neurotoxic
(loperamide, nadolol, ondansetron).
The concentration-response curves for the activity changes induced by the 12 compounds revealed that except for cyclosporine A, specific acute neuroactive effects induced by the compounds do NOT correlate with cytotoxicity. The analysis showed, that with a small dataset of acute concentration-response curves divided into the four groups using pattern recognition methods, the compounds could (with one exception at the low concentration) already be correctly assigned by their „activity fingerprint" to their respective groups. The obtained results further demonstrate that it seems very promising to intensify in the future the analysis of activity data by pattern recognition towards specific conditions correlating with neurological psychopathologies, such as depression, dementia, insomnia, anxiety etc. that could be side effects of certain drugs.

(ii) Combination therapy is an important treatment modality for many diseases in part due to its sometimes superior efficacy, as compared to monotherapies. We performed an isobolographic study to identify the effects of co-application of the sedative, anxiolytic drug diazepam and the antiarrhythmic amiodarone because of their adjunctive application for treatment of atrial fibrillation and other arrhythmias. The study revealed that the two compounds influence each other sub-additively. Diazepam becomes less effective and the effective ranges of both drugs were found to be narrowed with increasing amounts of amiodarone, leading to a limited therapeutic value. Therefore, the isobolographic approach using functional compound screening endpoint can deliver information on wanted and unwanted actions of drugs in combination.

(iii) Regarding the chronic repeated-dose approach (14 DIV) a detailed analysis of the electrical activity changes caused by cyclosporine A was performed. The data revealed that cyclosporine A at 0.1 µM and 2 µM causes an increase in spike and burst rate and an increase of network synchronicity already after 24 h of treatment as well as after 14 days. We conclude that our approach using electrical activity pattern changes as physiological endpoint is suitable for searching the differences in the effects caused by acute versus repeated dose treatments.

B. Omics analysis (2D cultures)
The metabolomics analysis revealed that the four compounds (chlorpromazine, amiodarone, diazepam, cyclosporine A) investigated, below cytotoxic concentrations, elicited after repeated dose treatment significant up and down regulation of 9 and 19 metabolites, respectively. The compounds mostly affected amino acid and transmitter or energy metabolism with minor contributions to redox and signalling pathways. Metabolomics could deliver a highly predictive endpoint, if neurotherapeutic relevant changes can be separated from neurotoxic ones, with well-defined compounds and concentrations as references for calibration.

The proteomics analysis showed after 14 days of repeated dose treatment (below cytotoxicity concentrations) up and down regulation of 20 and 22 proteins, respectively. The identified proteins could serve as predictive biomarkers, if neurotherapeutic relevant changes could be separated from neurotoxic ones with well-defined compounds as references for calibration.

Thus, O changes were different for the four compounds although effects on some metabolites and proteins were common.

3.4. General conclusion of the WP2.3
The results obtained from two neuronal models suggest that applied 2D and 3D model are complementary as they are suitable for measuring different endpoints that are necessary to build an in vitro integrated testing strategy for neurotoxicity testing.
Aggregating 3D brain cultures develop features of higher cellular organization described above. Therefore, the drug-induced toxicity was examined by applying a series of different endpoints, including enzymatic activities, glucose consumption, mRNA levels and omics analysis. A very high number of samples can be generated, each containing a large amount of genes, proteins, and metabolites, which are reliably analysed by all applied endpoints including omics techniques. Based on omics studies, potential biomarkers and toxicity pathways specific for neurotoxicity were identified. Further analyses are required to test whether the biomarkers identified after long term exposure are also regulated in cultures acutely exposed to chemicals. These studies will determine whether the same biomarkers could be relevant to drugs and chemicals independently from the duration of exposure (acute or long term). Two canonical pathways of high importance for the CNS were also identified, synaptic long-term potentiation and axonal guidance, as potential drug targets. Furthermore, the previously suggested metabolic competence of 3D aggregating brain cell culture has been confirmed by showing the expression of different cytochromes P450 and the intracellular presence of amiodarone metabolites. The above results indicate that this 3D system is a valuable and robust model for repeated dose neurotoxicity studies.

Complementary to the endpoints evaluated in the 3D model, neuronal electrical activity recording was performed using 2D model. It is a very sensitive, neuronal specific phenotypic endpoint that permits to directly evaluate the function of neuronal networks, fundamental for neuronal communication. With a small dataset of acute concentration-response curves of four groups of neuro-therapeutic and neurotoxic compounds, using pattern recognition methods, the compounds could be assigned by their „activity fingerprint“ to these groups. These are important results suggesting that by extraction of “neurotoxic activity” parameters, the prediction of neurotoxicity could be significantly improved in preclinical drug development. Furthermore, the model allows a prediction of neurotoxicity of new compounds with unknown mode of action by referring to the data base of NeuroProof of more than 100 well-characterized compounds (mainly agonists and antagonists of neuronal receptors).

In neurotoxicity evaluation, the first question to be answered is whether a compound is reaching the brain, and if so at what concentration. In vitro BBB studies allow the estimation of the actual drug concentration that is reaching the target site (brain cells). This knowledge is critical for the selection of the relevant concentrations for in vitro experiments. The proposed approach based on the calculation of the unbound brain/plasma ratio generated in vitro and estimated plasma exposure permits to make an early prediction of the risk of a toxicological effect in the CNS.

Taking into consideration all obtained results it is concluded that an integrated testing strategy (ITS) for in vitro neurotoxicity testing should be based on the complementary neuronal models (2D and 3D) as they permit to apply different and complementary assays to cover the broad range of possible cellular targets of drug/chemical induced toxicity. The in vitro BBB model should be included in such ITS especially when a new compound is tested and data on the BBB transport and brain concentration have to be defined.

4. Kinetics and modelling

Results obtained in vitro have to be extrapolated one way or another to make predictions of in vivo effects in humans. It is impossible to evaluate quantitatively the risk of toxic side effects for a drug simply on the basis on in vitro data, even if perfectly accurate. Indeed, the qualitative nature of the risk is then known, but not its magnitude. For example, in vitro, brain cells may be more sensitive than liver cells. But the reverse may be true in vivo. Why? Simply because in reality the drug may not be able to enter the brain at all, leading to no exposure of the brain cells in vivo. No exposure means no risk, even if the cells are potentially sensitive. Understanding these complex matters of exposure within the body is the object of a
well-developed science: Pharmacokinetics. Therefore, the kinetics and modeling approach taken by Predict-IV can be subdivided into four components which have each a firm justification and a logical articulation in the context of quantitative in vitro to in vivo extrapolation (QIVIVE):

- In vitro kinetic experiments, data analysis and modeling: This part of the work is devoted to linking the quantities introduced daily in repeated in vitro tests to the quantities actually seen by the cells in those tests. It is a fact that the molecules tested may for example accumulate within the cells over 14 days, therefore causing near the end of the experiments effects not seen earlier. Some cells, obtained from particular human donors, for example, may respond differently to the exposure: is that simply due to a poor replicability of the test, or is it due to a different metabolism or transport in and out those cells, due to different phenotypes? In the first case, we would question strongly the quality of the results obtained. In the second case, we explain away the variability, and have a way to correct for it through modeling, and we reinforce the confidence we have in the test results. Understanding in vitro pharmacokinetics is therefore a key step in QIVIVE.

- Physiologically based pharmacokinetic (PBPK) modeling has been further developed within the framework of the project. Its aim is to simulate and predict the fate of a drug inside the body or an animal or human. We now have tools and methods powerful enough to be able to predict (in totality or in part) the variability of drug effects across human individuals. PBPK modeling is an essential link in the QIVIVE chain, essential for transferring to the whole body the results obtained in vitro.

- Large-scale omics dose-response analyses: The data streams generated by the state of the art in vitro tests, developed and performed in Predict-IV, included transcriptomic, proteomic and metabolomic data. Such data can provide a comprehensive picture of all the alterations of cells functions induced by a drug after exposure. Those alterations may be beneficial if the cell is sick and the drug in fact helps restore proper function. But in some cases, typically at too high doses, toxicity to the cell may develop, because the drug may end up interfering negatively with some of its important functions. The problem with omics data is that they come to us rather as a tens of thousands piece puzzle than as a nice and clear picture. Reconstructing the picture from the pieces is a huge problem and the focus of much bioinformatics research in the world today. PREDICT-IV bioinformatics researchers have advanced the state of the art in this field by developing software and methods able to sift the most significant features of the puzzle, therefore reducing the size of the interpretation problem.

- Mechanism based modeling of cellular toxicity: We also developed mechanistic dose-response models focused on the important features detected by the above methods. The first model was set up to describe the dynamics of brain cell populations exposed to potentially neurotoxic compounds. The second mechanistic model we developed is a general model of cellular response to the oxidative stress. Cells are able to manage such a stress, up to a certain point at which the cell’s defenses are overwhelmed. The model is able to relate quantitatively the drug levels inside cells (as estimated by a pharmacokinetic model, for example) to the intracellular level of oxidative stress. We actually linked the various models developed in the framework of Predict-IV, in the case of cyclosporine A, as a demonstration of an integrated approach to QIVIVE (see Figure 9).

Each of the above endeavors has yielded its own results, which are summarized in the following.

In vitro kinetic experiments, data analysis and modeling
An experimental strategy has been proposed and used to measure biokinetics (i.e. the in vitro fate of the tested compounds). The critical parameters actually measured were:

- Chemical stability of the compound over time under the experimental conditions used during the single/repeated dose test.
In case of significant hydrolysis (>10% of the nominal concentrations) of the test chemical, measures to reduce it has been considered on the basis of the causative factors (i.e. change of the solvent used for the stock solution, as in the case of amiodarone, hydrolysed in aqueous solution) or a correct estimation of the loss has been taken into account.

- Adsorption to physical component(s)
  Depending on the liposolubility of the tested chemical, the possibility exists that it is adsorbed to the plastic devices and/or membrane filters on which cells are seeded. Plastic adsorption and physical sequestration in collagen/matrigel (possibly misinterpreted as intracellular bio-accumulation) when sandwich hepatocyte cultures are used, have been measured.

- Binding to medium macromolecules, essentially proteins.
  Whenever possible in the setting up of methods serum-free media were used, and in the case of HepaRG serum % has been lowered as much as possible (around 2%). The same serum batches were used for the replicates.

- Free vs bound concentrations over time
  Methods were set up and applied as in the case of chlorpromazine.

- Intracellular concentration
  The number of cells strongly affects the outcome of toxicity testing, by changing the levels of internal dose. Within the project the choice to use TC10 (expressed as toxic concentration for 10% of the population) and 1/10 TC10 as testing concentration, implies that the number of cells was nearly constant during the treatment. The intracellular concentrations (as well the one in the medium) was measured by serial sampling of medium and cells over time during the first (D0; 5 time points) and the last (D13; 5 time points) day of treatment.

- Metabolic competence
  A decrease in the parent compound concentration recovered from all the compartments (cells, medium, plastic/collagen adsorption), equivalent to a decrease in the Mass Balance suggests the occurrence of a biotransformation reaction. The kinetics of the major metabolite over time has been measured in the case of amiodarone. The characterization of the model in this respect has been made available for the liver and the kidney models used in the project and was carried out for the 3D CNS model. Results also pointed out the need to know and characterise also the expression/activity of transporters and the usefulness of having samples from single human donors. These indeed allow to account for the interindividual variability, depending on kinetic parameters, such as the metabolic competence. To this aim it has been proven the possibility to use cryopreserved human hepatocytes in biokinetic studies, selecting them on the basis of their specific content/activity of the enzymes involved in the test item biotransformation.

The sampling strategy used was an attempt to optimise the balance between the number of samples to be generated and the amount of information obtained: once the results were obtained and analysed it was clear that for some compounds the availability of additional intermediate sampling time between D0 and D13 would be beneficial, also for the robustness of model built to describe in vitro kinetics, allowing the prediction of test item concentrations in different compartments during the whole treatment period. Kinetic analysis was carried out only on a selected number of chemicals among those tested within the Project, selected on the basis of the availability of relatively simple detection methods with sufficient sensitivity to quantitate fractions of the nominal concentrations. Sample preparation, extraction methods
and corresponding recoveries were checked and optimized for every single model/matrix. For each chemical and in each model two concentrations were tested (each sample in duplicate) in at least three biological replicates: around 13000 samples were collected and analysed. The detailed methods and analysis of biokinetic data on single chemicals in each organ/model have been already reported in SOPs and in the DL-D3.6 and DL D3.12. In the following only some specific aspects will be described to support the usefulness of applying the above described strategy in the definition of the in vitro biokinetic profile of chemicals.

The importance of measuring the concentrations of the stock/working solution as well as the chemical stability of the test item.

In most cases a difference up to 20% was measured between the theoretical and the actual nominal concentrations in the stock and/or working solutions, and such a difference was considered in the calculations. When the difference was marked (>50%) it was decided to ignore the results from that biological replicate (not just for the kinetic experiments, since also effects were affected) that was eventually repeated. With adefovir dipivoxil in kidney cells, a time dependent hydrolysis was evidenced at 37°C: the reaction was quite fast (<15% of the parent compound after 3h hour). It was then to measure also adefovir, the hydrolysis product, and to consider the different ability to enter the cells: only adefovir dipivoxil could be recovered in kidney cells.

The importance of measuring free concentration

By using chlorpromazine, characterised by a high degree of protein binding, it was shown that at a fixed time in Balb3T3 most chlorpromazine was detectable in the medium (green part in Figure 10) independently on the initial nominal concentration; in Caco-2 some chlorpromazine was taken up by the cells (proportionally decreasing with increasing nominal concentrations), similarly to HepaRG. Once looking at cell death, by taking into account the nominal concentrations, the ranking of sensitivity was Balb3T3 > HepaRG > Caco-2, whereas, when the free concentration of chlorpromazine in the medium was used, sensitivity in all three cell systems was comparable, demonstrating that susceptibility to cytotoxicity is highly dependent on the dose metric used (Broeders et al., 2013).

The importance of measuring intracellular concentrations

Measurements of the chemical limited to the medium are not sufficient to define in vitro kinetic profile. This was quite evident looking at data obtained with ibuprofen in the three hepatic models: the intracellular concentration was quantitatively similar at steady state in human and rat hepatocytes, despite a 10-fold higher ibuprofen concentrations used for treatment. This feature was attributed to different metabolic rate, with PHH metabolising ibuprofen much more efficiently than PRH (kinetic-dependent species differences in toxicity. Ibuprofen levels in the supernatant do not allow to understand the different kinetic behaviour within the cells. By measuring the intracellular concentration as well as the Mass Balance, it is possible also to evidence bio-accumulation of the test item, as it was observed by testing cyclosporine A in the kidney cells at the high concentration, as shown in Figure 12. The intracellular content increased with time with an absolute fold change of 5 from D0 to D13, which can be explained with a metabolic system and/or a Pg-P activity unable to cope with the high concentration used. Indeed, proximal tubule cells do express CYP3A4, which is the main enzyme responsible for cyclosporine A metabolism. Additionally, these cells express high levels of Pg-P and cyclosporine A is one of its most potent substrates. At the lower concentration, RPTEC/TERT1 cells could maintain low cytosolic concentrations, but not at the high concentration. The
building of the model allows the prediction of the intracellular concentrations also in between D0 and D13. The kinetic behaviour of cyclosporine A in hepatic models was different with respect to the kidney cells, but difference between primary cell culture and the cell line was also observed. No bioaccumulation potential, due to an efficient metabolism, was shown by HepaRG; the PHH enriched intracellular cyclosporine A levels after high dose treatment for 14 days was the result of cyclosporine A physical sequestration in the matrigel layer. At variance, PRH treated with cyclosporine A were able to take up the compound, but not to metabolize it efficiently. Resulting bioaccumulation was observed after low and high dose treatment, not related to bioaccumulation of cyclosporine A in the collagen layer (absent in the blank study, representing the ‘worst case’). The higher metabolism in HepaRG cells with respect to PHH might be an effect of basal higher CYP3A4/5 activity in the cell line or on the other hand induced CYP3A4/5 activity by DMSO. Again the use of kinetic data could account for the difference between human and rat cyclosporine A-induced toxicity related to the metabolism pathway via CYP3A4.

In cells exposed to amiodarone we could also detect its major toxic metabolite mono-N-desethylamiodarone (MDEA). Figure 11 shows the intracellular kinetic behaviour of amiodarone and MDEA in HepaRG cells: the parent compound was rapidly taken up by cells and metabolised. Indeed, at D0 after 1 hour MDEA was already detectable and its concentration progressively increased with time; after 2h the parent compound concentration reached the steady state, remained almost constant along 24h. At D13 the amount of intracellular MDEA was 10 fold higher than the parent, indicating a marked bioaccumulation, as also supported by the Mass balance value. MDEA was never detectable in the extracellular space. The sum of amiodarone and MDEA, plus amiodarone adsorbed to the plastic (around 10-20%) at 24 h in D0 was around 70%, suggesting that other amiodarone metabolites could be formed, or alternatively, a small MDEA % was further metabolized e.g. by conjugating enzymes.

When amiodarone was used to treat the two CNS models, at D0 amiodarone entered the cells progressively over the 24 h and correspondingly its concentration decreased in the medium. During the first day of treatment MDEA was not detectable and this could be due either to formation below the LOD of the analytical method (1.9 nM) or to inability for constitutive enzymes to work. At D13 the intracellular amiodarone concentration was almost doubled, likely due to some bioaccumulation of the parent compound: the detection of a small MDEA amount at D13 indicate that the models are endowed with some metabolic competence either constitutive (with MDEA levels accumulating over time reaching concentration above the LOD) or induced by the treatment.

We modeled the in vitro pharmacokinetics of cyclosporine A, ibuprofen, chlorpromazine, cisplatin and amiodarone in rat primary hepatocytes, human primary hepatocytes, HepaRG (hepatocyte-like) cells or kidney cells (RPTEC/TERT1). To that effect we developed several variants (as required by the substance studied) of a three-compartment model we published (Wilmes et al., 2013). The model describes the change in time of the total quantity of the substance of interest in the assay medium, in cells lysate and bound on medium proteins and/or plastic vial walls of the assay system.

To estimate the model parameters, statistical distributions of their values were obtained by Bayesian numerical calibration (Bois, 2009) with in vitro experimental data. For each parameter, a non-informative (vague) prior distribution was used “to let the data speak”. To model variability between individuals or experiments, a multilevel framework was used.

Our analyses clearly show inter-individual differences in the concentration versus time profiles obtained. Importantly, the concentration vs. time profiles reconstructed give a firmer ground for relating dose to effects in vitro. For example, we showed that cyclosporine A (Figure 12) accumulates disproportionately in cells with increasing dose. That explains why the effects observed at the highest dose were much larger.
Physiologically based pharmacokinetic (PBPK) modeling

Transferring in vitro generated toxicity knowledge to in vivo requires mechanistic PBPK models to translate dose to the concentration at the site of action. PredictIV has greatly contributed to the advancement of the state of the art in PBPK modeling. We used the PBPK models implemented in the Simcyp Simulator. The Simcyp software is commercialized internationally and used by a broad group of users in pharmaceutical industry, academia and regulatory agencies.

First, the original Simcyp model, developed for humans (Figure 13) and animal species (rat, mouse and dog), was further expanded for those species. The structure of the model did not need to be significantly changed (a great advantage of PBPK models), but just mainly its parameter values. That represents, however, a massive amount of work. In return, we now have sophisticated animal PBPK models that allow calibration and validation with animal data. Such data should be progressively phased out in the future, but historical data will remain, and in the meantime in vitro methods will certainly need validation with animal data.

Second, obtaining accurate prediction of the concentration at the site of action plays a crucial role in connecting the driving concentration to the relevant toxicity biomarkers or endpoints. To this end, some of the tissues (liver, brain and kidney) in the PBPK model which were initially characterized assuming homogenous well-stirred models were expanded to more complex models. The original model was complemented with a better algorithm to predict equilibrium blood over plasma concentration ratios (B/P) (Figure 14). B/P is a widely used parameter for predicting the rate of drug metabolism, for example. Also, the liver well-stirred model was expanded to allow incorporation of uptake and efflux transporters. Passive and active (saturable) transport at both liver sinusoidal and canalicular membranes are accounted for and the impact of binding and ionization was considered. The enterohepatic recirculation of drugs is taken into account in the new extension of the Advanced Dissolution, Absorption and Metabolism (ADAM) module. Finally, a new sophisticated mechanistic kidney ("Mech KiM") model was developed that links drug characteristics to knowledge of renal physiology (Figure 15). The model facilitates the prediction of drug concentrations in different part of kidney including proximal tubule cells.

Third, a considerable effort has gone into preparation of new and improved "compound files", which contain the data needed to simulate specific drugs. Metformin was one of the compounds investigated in this project. A full PBPK model and corresponding compound file has been successfully developed for metformin within the Simcyp Simulator (V12, release 2).

Finally, given the limitation of available fixed pharmacodynamic models in the Simcyp Simulator and the need to be able to incorporate user-defined efficacy and toxicity models a new scripting feature was added to the Simulator. The custom scripting module also facilitates handling of user defined differential equations to adequately covers the pharmacodynamic models described for example in the following sections.

Large-scale omics dose-response analyses

That part of our activity brought its own methodological developments, which will be applicable much beyond the activities of Predict-IV. It also supported the omics data analyses which have been presented above on specific toxicity mechanisms and markers. We focus here on the results obtained in two general areas: Statistical tools for meta-analysis of concentration-response experiments and multi-marker panels
able to detect the toxicity triggered by different drugs.

Summarizing EC50 estimates from multiple concentration-response assays: Concentration-response studies are routinely performed to investigate the safety and efficacy of candidate drugs. The EC50 is the concentration of a particular compound that reduces the maximal response by 50%. The EC50 is usually estimated by statistical modeling. Often, more than one concentration-response experiment is carried out to, requiring summarization of EC50 estimates from multiple experiments. The individual EC50 estimates vary to some extent from experiment to experiment due to unknown biological reasons, even under the same experimental design and in the same laboratory. In this context, so-called "mixed-effects models" are often used to estimate the 'average' EC50. However, such models are quite complicated. An alternative, we proposed the application of a meta-analysis approach, as used in epidemiology, for example. As shown in Figure 16, for a real world dataset, our approach gives almost the same results as the mixed-effects modeling approach, while being much simpler. The meta-analysis strategy is a robust and promising method for summarizing EC50 in drug development and chemical safety assessment.

Multi-marker panels: We essentially argue for the need of a multi-marker panel for coping with the diverse underlying toxicity mechanisms triggered by different chemical compounds. Only such panels may allow generalization to different drugs. The marker panels we developed probe the full spectrum of perturbed relevant toxicity molecular mechanisms. Molecular phenotype models reflecting compound induced cell stress were developed separately where for the RPTEC/TERT human kidney in vitro system as well as for the primary rat liver cells in vitro system (Figure 17). For the liver, the marker panels derived with and without the help of molecular phenotype models perform equally well in terms of differentiating toxic and non toxic condition in vivo. For the kidney, however, the marker panels derived when applying the molecular phenotype model shows significantly higher performance. Finally we found biomarker panels which have the potential of bridging in vitro and in vivo findings. The markers selected are good candidates for further evaluation and refinement.

Mechanism based modeling of cellular toxicity

We focused our efforts here on the modeling of cell stress in cultured brain cell aggregates and on the modeling of oxidative stress in kidney cells.

In vitro aggregating brain cell cultures containing all the different types of brain cells have been shown to be useful for assessment of neurotoxicity. These three-dimensional cultures have been used for the detection and study of compound effects on the central nervous system. Concentration-dependent neurotoxicity of selected compounds has been determined at several time points during compound exposure through enzyme activity and gene expression measurements. We developed a biologically-based mathematical model of the impact of drugs on the different brain cell populations. The model can be used to quantify the neurotoxicity of compounds (Figure 18).

The sophisticated mathematical framework of "Markov processes" was used to model the survival of brain cells undergoing a chemical attack. Brain cells are assumed to be either in a healthy or in a stressed state. Cells may switch between these states or die because of too high concentrations of drugs. Since cell numbers are not directly measurable, enzyme activity and gene expression measurements can be used as surrogate measures. Using this model, we were able to propose new experimental assay designs. For example, for propofol and buflomedil we found that measurements taken at early time points can be used to correctly predict effects later in time, so that long-term experiments can potentially be shortened,
therefore increasing the efficiency of safety testing for drugs and general chemicals.

Modeling and predicting oxidative stress in human kidney cells was our last challenge. The model we developed is fairly complex because it tracks and simulates the behavior or an important cellular defense pathway against stress. In a nutshell, reactive oxygen species (for example hydrogen peroxide) can be formed by various cellular reactions and increased dramatically by some toxic chemicals or drugs when over-dosed. Those oxidants are toxic to cells, but cells have developed a complex detection and control mechanism against them, through the so-called nrf2 pathway. However, if the chemical stress is too intense, the nrf2 defense mechanism can be overwhelmed. To test the prediction capacities of the model we applied it to the case of exposure of human renal cells (RPTECs) to cyclosporine A. The model was parameterized using Bayesian statistical methods, with a subset of the transcriptomic, proteomic and metabolomic data generated by the other partners of the Predict-IV.

To get a full picture of the impact of cyclosporine A administration on human kidney cells in vivo, we coupled Simcyp's PBPK predictions of cyclosporine A distribution in the human body during therapeutic dosing with the oxidative stress model. Figure 19 shows the PBPK simulated time course of cyclosporine A in the cells and blood plasma of humans assuming that each kidney cell has an identical exposure (a reasonable assumption) and the calculated resulting oxidative stress at the cell level based on the nrf2 model (Figure 20).

Figure 20 therefore shows predictions of a long-term in vivo human response on the basis of repeated exposure in vitro data and mechanistic mathematical modeling of those data. The variability of expected response in different subjects is also shown in Figure 20: The range of variability for ROS levels in kidney cells spans about two orders of magnitude, implying that some subjects may be much more susceptible than others to cyclosporine A side-effects.

The PBPK and oxidative stress models we have developed are fairly generic and could be applied, with modest changes, to many chemicals, diverse human populations, and various organ types. The changes would be mostly parameter values, which could be obtained in vitro on human cells, with short term assays. No animal experiments would be needed. Indeed, this is just a proof of concept of the feasibility of an integrated animal-free approach, and it should be validated before use in actual drug development. But the basic ingredients of its success have been exercised in Predict-IV and presented here.

Potential Impact:
Potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results

Contribution to the State-of-the-Art
The complex events possibly contributing to toxicities of new pharmaceuticals illustrate the difficulties and the challenge to mimic them in vitro. The use of in silico and in vitro techniques to predict drug toxicity in animals and humans could replace some current in vivo tests and significantly reduce the use of animals by screening out drugs with undesirable properties prior to preclinical studies in animals. Moreover, a survey to examine the strengths and weaknesses of animal studies to predict human toxicity indicated that current safety studies in animal models do not adequately predict human toxicities. There are several causes of poor correlation between animal and human toxicities. One of the main causes is that animal species do not always predict human metabolism. Also, the diversity of human patients due to environmental or life-style susceptibility factors do not reflect the well-controlled experimental animal
settings. Therefore, it is crucial to understand why individuals respond differently to drug therapy and to what extent this individual variability in genetics and non-genetic factors is responsible for the observed differences in adverse reactions. The use of human and mammalian cell-based assays plays a key role in this endeavour. A broad expert knowledge is required to integrate the many potential mechanisms of toxicities into the safety assessment process and to develop useful non animal-based systems to mimic these events in vitro possibly at the earliest stages of drug development. It is therefore obvious that a large scale European effort is necessary to accomplish the ambitious goals of Predict-IV.

The overall aim of Predict-IV was to develop strategies to improve the assessment of drug safety in the early stage of development and late discovery phase by an intelligent combination of non animal-based test systems, cell biology, mechanistic toxicology and in-silico modelling, in a rapid and cost effective manner. A better prediction of the safety of an investigational compound in early development can be delivered by applying advances in predictive toxicology (toxicogenomics and metabolomics, prediction of pharmacokinetics and high content imaging) and modelling.

The potential implications for the pharmaceutical industry are enormous since well evaluated and predictive methods and testing strategies could accelerate development of safe and efficacious novel medicines in two ways:

(i) by the elimination of drug candidates with non-acceptable adverse effects early in the process of drug discovery & development (“fail early – fail cheap”) and

(ii) by the identification of drugs with a favourable and acceptable toxicity profile that reduces the risk of late-stage, i.e. costly failures in drug development.

The approaches developed within the project are also very relevant in the context of several pieces of EU legislation (e.g. REACH, Biocides, Cosmetics) that require the assessment of systemic toxicities for the safety evaluation of chemicals. In this regard, non-animal based integrated approaches and testing strategies are sought.

Socio-economic impact

• Large project with many partners and complex structure can be effectively managed using electronic tools.
• Significant contribution to education of young scientists in the important area of toxicity assessment and integration of results from such studies into safety assessment
• Significant progress in development of strategies to reduce animal testing by integration of molecular endpoints analysed by modern technologies and by integration of basic principles of toxicology into endpoints assessed.
• Significant contribution to the development of integrated testing strategy. Predict-IV has demonstrated, using well-defined model compounds with known effects in humans, that combination of prediction of toxicokinetics in vivo and molecular mechanisms can predict doses required and modes of action for specific target organs.
• While more examples are required to further strengthen database, the obtained results show that significant societal concerns, i.e. use of animals in toxicity testing and new approaches to risk assessment for chemicals in general, can be addressed in part by targeted non-animal testing and in silico tools. An integrated testing approach, however, will need further expansion of the model compounds and will also need to include QSAR and other non-testing approaches such as the concept of the “threshold of toxicological concern” (TTC) and read-across.
In summary, the results from Predict-IV have significant potential to contribute to a reduction of animal testing in toxicity assessment when further validated used chemicals with different structures and physico-chemical properties. In addition, integration of the developed approach may reduce the cost associated with development of new drugs and thus may speed up drug development.

Scientific impact
The project has generated a large number of publications (43) in high impact international journals and has resulted in 17 completed/pending PhD thesis and 4 Master thesis. These publications describe new model systems and novel endpoints to be integrated for toxicity testing regarding cellular systems with relation to relevant target organs. In addition, the concept of integration of the bioavailability and the kinetic behaviour of chemicals in the relevant in vitro system has been successfully introduced to optimize outcome of toxicity testing strategies in vitro. This will give improved information on the time-concentration-response curves and is essential for comparing kinetic parameters across different systems. The well defined toxicokinetic aspects considered in vitro were then integrated into available toxicokinetic models and permitted prediction regarding well characterized model compounds on their toxicokinetic behaviour and expected toxicities in humans. Therefore, the general approach of the project has been demonstrated to be feasible and has generated a template for further evaluations of chemicals with different physico-chemical properties to significantly reduce needs for toxicity testing in animals. In addition, the project has generated optimized models for neurotoxicity testing and an optimized blood-brain-barrier model for integration into neurotoxicity assessment.

In conclusion, an integrated omic approach combined with stable human-derived in vitro cell culture models has the potential to considerably advance our understanding of chemical induced cellular perturbations and will be an extremely useful approach to drug and chemicals safety paradigms.

Main dissemination activities and exploitation of results
One of the main dissemination activities was the organisation of a symposium at the 49th congress of the European Societies of Toxicology (EUROTOX 2013) held on September 1 – 4, 2013 in Interlaken, Switzerland with presentation of Predict-IV results to a large European and international toxicology audience.

• Oral presentation: Armin Wolf (P8), “Predict-IV Project Overview - Profiling the toxicity of new drugs: a non animal-based approach integrating toxicodynamics and biokinetics“
• Oral presentation: Paul Jennings (P3) “An integrated omic approach to characterise nephrotoxin induced stress responses in renal epithelial cells”
• Oral presentation: Emanuela Testai (P4) „The relevance of toxicokinetics in in vitro studies”
• Oral presentation: Frederic Bois (P5) “Integrating toxicokinetics and toxicodynamics to predict in vivotoxicity“
• Oral presentation: Davide Germano (P8) „Determination of liver specific toxicities in rat hepatocytes by High Content Imaging during 2-week multiple treatment“
• Poster: Jérémy Hamon (P5), Paul Jennings (P3), Frédéric Y. Bois (P5). „Integration of omics data and systems biology modeling: Response of the NFE2L2 pathway in human renal kidneys cells exposed to cyclosporine A in vitro“
• Poster: Davide Germano, Marianne Uteng, Philippe Couttet, Olivier Grenet, Salah-Dine Chibout,
Francois Pognan, Armin Wolf (P8) “Determination of liver specific toxicities in rat hepatocytes by High Content Imaging during 2-week multiple treatment”


“Pharmacodynamic profiling of EGFR inhibitors in human keratinocytes”

• Poster: Lysiane Richert (P9), Phil G. Hewitt (P2), Bas Blaauuboer (P12), Frederic Y. Bois (P5), Stefan O. Mueller (P2) and P. Annaert ,Insights into mechanisms underlying inter-individual susceptibility to Drug-Induced-Liver-Injury (DILI) from data on in vitroexposure, transcriptomics and functionality of cryopreserved human primary hepatocytes – The example of Chlorpromazine”

• Poster: Anna Price, Marco Fabbri, Maria Grazia Sacco, Laura Gribaldo, David Pamies, Giorgica Pallocca (P6) „miRNA profiling as a tool for developmental neurotoxicity pathway analysis in human in vitro model”

• Poster: Germaine L. Trusi (P2), Celine Parmentier C (P9), Lysiane Richert (P9) Stefan O. Mueller (P2), Phil G. Hewitt (P9) “Multiple endpoint approach improves prediction of hepatotoxicity”

A complete list of the dissemination activities can be found in the second part of this report. A complete list of scientific publications resulting from the project can also be found in the second part of the project report. In addition, the consortium has arranged a special issue with the scientific journal “Toxicology in vitro” which will present overview articles on the project results and their implications regarding toxicity testing.

List of Websites:
All relevant contact details can be found on the project’s public website located at [http://www.predict-iv.toxi.uni-wuerzburg.de/](http://www.predict-iv.toxi.uni-wuerzburg.de/)

Participants with complementary expertise to address the widely different issues contributed to the project.
Established and optimized cell culture systems with the ability to indicate deregulation of specific cellular processes within a short time after exposure (early markers of toxicity) were used. The combination of profiling methods such as “omics” promises further insights into cellular responses to xenobiotic insults. In this area, the project relied on the knowledge gathered from other large research efforts.

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