The safety assessment of potential drug candidates remains a challenge for the pharmaceutical industry. Hepatotoxicity is one of the major reasons why candidate drugs fail during preclinical or clinical trials. The liver is responsible for the metabolism of drugs and toxic compounds, thus it is the most vulnerable organ for drug-induced toxicity. Drug-induced hepatotoxicity can be intrinsic (dose-dependent) or idiosyncratic (low incidence and largely dose-independent). Preclinical in vivo testing of drug toxicity is accompanied by severe animal suffering and discomfort and is only partly predictive for human toxicity because of species differences. This emphasizes the need for the development of new screening methods that address the toxicological hazards early in the drug discovery process. Therefore, great effort is put in developing new screening methods in order to find novel and more accurate preclinical and clinical biomarkers. This will lead to a safer and more efficient drug discovery and development process. With the traditional biomarkers for liver injury, the differentiation between different classes of hepatotoxicity is difficult. Moreover there is a need for reliable in vitro toxicity screening tests, which improve the prediction, characterization and understanding of drug-induced hepatotoxicity. A prerequisite for a proper prediction is that the model used has adequate drug metabolizing capacity, as drug-related liver toxicity often results from toxic metabolites formed in the liver. In addition, such methods are developed to be applicable to human tissue in order to better predict liver toxicity in man by avoiding interspecies extrapolation. The Precision-Cut Liver Slice (PCLS) model has been shown to be adequate to study drug metabolism and toxicity [34]. This model can be positioned between in vivo experiments and the currently used cell culture models and can be regarded as an ex vivo model, with all the different cell types of the liver present in their natural architecture and with intact cell-cell and cell-matrix contacts. For toxicity studies this is of great importance because drug-induced toxicity is currently recognized as a multi-cellular process, where in addition to hepatocyte functions, also cell-cell interactions and non-parenchymal cell functions are considered to be important contributors to the toxicity process [31].

The application of transcriptomics enables investigating the changes in gene expression of the complete genome induced by drug exposure. Through the measurements of the global gene expression it is possible to identify phenotype specific hepatotoxic pathways and mechanisms. In addition, it is possible to select similar endpoints in vivo and in vitro, facilitating to perform in vitro to in vivo comparisons. Even if the endpoints are not similar they could be predictive if a good correlation between the in vivo and in vitro endpoints can be established. Thus, the application of transcriptomics facilitates the development and use of ex vivo or in vitro models for the prediction of hepatotoxic responses in humans. Also, a correct classification of the hepatotoxicants based on their hepatotoxic phenotype is instrumental for the safety assessment of drugs. Drug-induced liver injury may result in different toxicity phenotypes such as hepatic cholestasis, i.e. impairment of bile flow and increase in intracellular accumulation of bile salts, necrosis, i.e. a form of premature cell
death due to damage by for instance free radicals and/or toxic metabolites, or fibrosis, i.e. the accumulation of collagen.

The aim of this thesis was to obtain insight in the use of PCLS as ex vivo model in combination with transcriptomics for the identification and classification of hepatotoxic compounds, and in the elucidation of the mechanisms of the hepatotoxic effects of those compounds at the gene and pathway level. For this aim we analyzed gene expression profiles of PCLS exposed to compounds inducing fibrosis, necrosis, cholestasis and idiosyncratic liver injury.

Validation of rat PCLS as model to study fibrosis

In chapter 2, we aimed to further characterize PCLS as a suitable model to identify whether early changes in gene expression could give an indication of the phenotype of long-term toxicity induced by hepatotoxicants inducing necrosis or fibrosis. In this study, we performed the comparative analysis of the gene expression profiles of rat PCLS induced by paracetamol (APAP) and carbon tetrachloride (CCl₄), which are known to induce toxicity by different mechanisms, being necrosis and fibrosis respectively. The comparison was performed using gene expression patterns, regulated genes, and pathway and upstream regulator analysis of the regulated genes. Gene expression pattern analysis revealed characteristic changes in expression patterns due to exposure to a toxic concentration of each of the compounds compared to the corresponding control. Comparison of the regulated genes showed that there is considerable overlap among the genes regulated by both toxins but there is also a significant number of genes uniquely regulated due to either APAP or CCl₄. Some of those genes uniquely regulated due to CCl₄ treatment include genes related to fibrogenesis. Genes involved in the hepatic stellate cell activation and the onset of fibrogenesis such as CRYAB (alpha-B crystallin), KLF6 (Kruppel-Like Factor 6) and HSP47 (Heat shock protein 47) were upregulated indicating initiation of the fibrotic processes in CCl₄ treated slices, as was shown before [72, 74, 73, 115, 116, 85]. The growth factor TGF-β1 (Transforming growth factor beta 1) plays a key role in fibrosis via hepatic stellate cell activation [214]. From the TGF-β1 gene network resulting from the analysis of the regulated genes due to APAP or CCl₄ treatment, it can be seen that genes that are causally linked to TGF-β1 and have a clear role in fibrosis such as JUN (Jun Proto-Oncogene), LITAF (Lipopolysaccharide-induced tumor necrosis factor) and SERPINE1 (Serpin Peptidase Inhibitor, Clade E, Member 1), were upregulated only in the case of exposure to CCl₄ but not to APAP. This observation indicates a substantial involvement of TGF-β1 in the toxicity process initiated by CCl₄ but not by APAP, and gives an indication that early fibrotic processes are activated within 16 h due to exposure to a toxic concentration of CCl₄. Upstream regulator analysis revealed several regulators that are known to control the expression of the regulated genes and that are known to be related to hepatic fibrosis. In conclusion, the early gene expression changes after short-term exposure to CCl₄ and APAP reflect the characteristic difference between these compounds in their ability to induce liver fibrosis after chronic dosing in vivo. This study indicates that transcriptomic analysis of PCLS can be used to identify the early events in PCLS that are indicative of a pathology (fibrosis) that develops after chronic injury. Further studies with more fibrotic and non-fibrotic compounds are needed to verify this finding and to identify a set of biomarkers that can be used in the future in drug-induced toxicity screening.
In chapter 3, we aimed to validate human PCLS as an ex vivo model that reflects the drug-induced cholestasis processes using transcriptomic analysis. To date, human PCLS were not used for studies on cholestasis. Hepatotoxins that are known to induce cholestasis in humans, such as cyclosporine, chlorpromazine, ethinyl estradiol and methyl testosterone were tested. In addition, ANIT (alpha-naphthyl isothiocyanate), a well-known cholestatic compound in rats, was included in the study. For many cholestatic drugs, the primary causative event involved in cholestasis is the inhibition of BSEP (Bile salt export pump) resulting in the intracellular accumulation of bile acids. It was hypothesized that incubation of PCLS in conventional culture medium would not be very sensitive to the toxic effects of these BSEP inhibition, as they would only be exposed to the newly synthesized bile acids. Therefore, a non-toxic bile acid mixture (60µM) was added to the incubation medium in order to create an environment similar to the physiological concentration in the portal vein of man in vivo [119]. Pilot experiments showed that indeed the bile acid concentration in the slices is maintained during incubation with bile acids whereas it is strongly decreased during incubation in conventional medium. Transcriptomic analysis revealed that cholestatic drugs clearly induced the regulation of genes and pathways associated with cholestasis in human PCLS when incubated in the presence of bile acids (60µM of bile acid mix). Also, the observed gene expression pattern of cholestatic injury was concentration dependent for all drugs. Hepatic cholestasis was among the top 5 regulated pathways. The majority of the pathways regulated in the human PCLS are represented in the Adverse Outcome Pathway (AOP) for cholestasis as proposed by Vinken et al., including the primary direct cellular responses and secondary adaptive responses involved in bile acid induced cholestatic injury [16, 17, 18], such as NRF2 (Nuclear factor (erythroid-derived 2)-like 2) mediated oxidative stress response, inflammation mediated hepatic fibrosis, endoplasmic reticulum stress, and activation of the coagulation and complement system. It is well known that adaptive responses to intracellular bile acid accumulation are mediated via FXR (Farnesoid X receptor), LXR (Liver X receptor), PXR (Pregnane X receptor), and VDR (Vitamin D receptor) nuclear receptors. As expected, in the PCLS exposed to the cholestatic drugs, signaling pathways such as FXR, LXR, PXR and VDR as well as the related cholesterol biosynthesis pathways were affected. Activation of nuclear receptors such as FXR, LXR, PXR and VDR, triggers cellular adaption to counteract bile acid accumulation and thus cholestatic liver injury [19]. In contrast with the expected activation of FXR as indicated in the AOP, the target genes in the FXR pathway were downregulated in the human PCLS including genes known to play a role in cholestasis such as MDR3 (Multiple Drug Resistance 3), BSEP (ABCB11) and SHP (Small Heterodimer Partner). Downregulation of BSEP may indicate a direct effect of the tested cholestatic drugs, as potent BSEP inhibitors have been shown to downregulate BSEP expression in primary human hepatocytes [128]. Moreover the decreased expression of FXR can at least partly explain this reduced FXR signalling. This is in line with the finding that both FXR and SHP expression was reduced by 90% or more in cholestatic patients [15]. Thus, based on our findings it can be postulated that exposure to cholestatic compounds could lead to compromised FXR mediated adaptive responses, causing cholestatic injury. Also, downregulation of genes involved in cholesterol transport such as ABCG5 and ABCG8 indicate a loss of the protective action of LXR. In addition, also several genes in the PXR and VDR pathways were mostly downregulated. Together, the reduced activation of FXR,
LXR, PXR and VDR could be responsible for reduced adaptive responses to the effects of the cholestatic drugs and lead to development of cholestatic injury.

Compromised adaptive responses could lead to deleterious cellular effects via toxicity processes such as oxidative stress and endoplasmic reticulum (ER) stress. Oxidative stress is implicated to play a role in the pathogenesis of drug-induced cholestasis as a result of bile acid accumulation. We observed the activation of NRF2 mediated oxidative stress response in the human PCLS treated with cholestatic drugs. This indicates that detoxifying mechanisms are activated in the PCLS to alleviate the oxidative stress probably due to accumulating bile acids. Whether indeed the bile acids accumulate in the slices after exposure to a cholestatic drug remains to be established and is currently under investigation in our lab.

A recent study showed that ER stress is involved in the bile acid induced hepatocellular injury [124]. In line with this, we also observed that ER stress, unfolded protein response (UPR) and protein ubiquitination pathways were among the most affected pathways. The UPR signaling pathway is activated in response to ER stress and promotes cell survival and adaptation. There is increasing evidence for the involvement of ER stress in cholestasis [215, 176, 177, 149, 124]. Our results suggest that ER stress, protein ubiquitination and UPR may be early cellular effects in drug-induced cholestasis. Further studies will be necessary to elucidate the exact role of these processes in bile acid mediated cholestasis.

Hepatic fibrosis and hepatic stellate cell activation was also observed in human PCLS due to exposure to the cholestatic drugs. Indeed, accumulation of bile acids by obstructive cholestasis [134], was shown to lead to an inflammatory response in vivo which in turn leads to activation of hepatic stellate cells and liver fibrosis.

The genes involved in cholesterol biosynthesis, the starting material for the synthesis of bile acids in the liver were downregulated in human PCLS indicating the adaptive response of hepatocytes to decrease cholesterol synthesis as a response to cholestatic drugs. Interestingly, this was also observed in mouse PCLS exposed to cholestatic drugs [112, 113].

We also compared our findings with gene expression data obtained from liver samples of patients with cholestasis due to biliary atresia and intrahepatic not drug-induced cholestasis [123]. Comparison of the affected pathways between human PCLS and the patient samples revealed that there was good overlap with respect to the processes involved in cholestasis, although more pathways were affected in vivo. For instance, tight junction signalling was affected in patient samples but not in human PCLS. An explanation for the observed differences between in vivo data and the ex vivo data could be due to the different causes of cholestasis or the large difference in time frame as the patient samples represent fully developed cholestatic disease in infants. Human PCLS should therefore preferably be validated by comparing with human liver tissue of patients suffering from drug-induced cholestasis, but to our knowledge such data has not been published to date.

In conclusion, the transcriptomic analysis of human PCLS exposed to cholestatic drugs in the presence of bile acids revealed that this model reflects the primary toxicity and adaptive processes associated with hepatic cholestasis. The results suggest that decreased adaptive responses mediated via nuclear receptors are associated with these cholestatic effects and lead to the subsequent toxicity processes such as oxidative stress, ER stress and UPR response. Our study demonstrates that human PCLS is a suitable model for future application in drug screening for cholestasis and to identify possible mechanisms of toxicity of cholestatic compounds, when incubated in the presence of a physiological concentration of bile acids. Further studies may reveal biomarkers for DICI. Insights gained from the pathway
analysis such as decreased activation of the FXR pathway, downregulation of cholesterol biosynthesis, increased ER stress response and NRF2 mediated oxidative stress response, could be included in the adverse outcome pathway of cholestasis.

Classification of cholestasis and necrosis inducing drugs

In chapter 4, we aimed to classify hepatotoxins according to their known phenotype of toxicity, cholestasis or necrosis, based on the gene expression profiles after exposure of human precision-cut liver slices and to identify possible classifier or marker genes. In addition to the five cholestatic compounds studied in chapter 3, the human PCLS were exposed to five hepatotoxins: acetaminophen, benziodarone, chloramphenicol, colchicine, and nitroso-diethylamine known to induce hepatic necrosis. In all these experiments the PCLS were exposed to the toxic compounds in the presence of the physiological bile acid mix. Machine learning analysis on gene expression data of PCLS exposed to these five cholestatic and five necrotic compounds resulted in four classification models based on two different algorithms namely SVM (Support vector machine) and RF (Random forest) and two different tested concentrations (low and medium), which were 70-80% accurate in predicting the phenotype of the hepatotoxins. Interestingly, chloramphenicol was consistently classified as cholestatic compound despite the fact that it is generally considered a direct acting necrotic compound. However, some studies indicated that chloramphenicol can also cause cholestasis [157]. In spite of the fact that the compounds were chosen based on their literature reported phenotype of liver toxic phenotype, it is well known that cholestasis often presents as mixed cholestatic and hepatocellular injury [141]. Further, recent evidence suggests that inflammatory cell-mediated necrosis might also accompany cholestasis. Steiner et al., reported that this overlap of mechanisms involved in the toxicity of necrotic and cholestatic compounds further complicates the classification of hepatotoxins into the correct phenotype of toxicity [150]. However, despite the complexity owing to overlap of mechanisms in toxicity for the classification of necrosis and cholestasis, the developed classification models were able to classify the hepatotoxins with relatively good accuracy. The low concentration gene expression profiles gave better prediction accuracy than the medium concentration, which can probably be due to the accompanying necrosis at higher concentrations of the cholestatic compounds. In conclusion, although all four models gave a reasonably comparable overall performance in compound class prediction accuracy, the SVM low concentration model shows the highest prediction accuracy in correctly classifying all 5 cholestatic compounds and the classifier genes identified by this model are consistent across concentrations and not too sensitive for inter-individual variation, which supports the reliability of this model in future settings. Further analysis of the function of the classifier genes identified by the SVM low model showed that they are involved in ER stress, oxidative stress and unfolded protein response (UPR), and lipid and cholesterol metabolism including a Sodium/Bile Acid Cotransporter, which is well in line with the findings of chapter 3. Classifier genes identified in our human PCLS model were compared with cholestasis-specific classifier genes reported in different rat in vivo studies [150, 147, 52, 29, 30]. No overlap was observed among the classifiers genes between rat in vivo and human ex vivo. This lack of concordance could be partly due to species differences and underlines the importance of the human cells or tissues to identify human specific biomarkers. It should also be mentioned that there was also little or no overlap among the classifier genes found for
the cholestasis phenotype between different rat in vivo studies [150, 147, 52, 29, 30]. The lack of concordance in these studies further questions the applicability of the identified markers and could be partly due to overfitting of the data.

In conclusion, gene expression profiling after ex vivo exposure of precision-cut human liver slices to hepatotoxicansts known to induce either cholestasis or necrosis resulted in a classification model that showed good accuracy in distinguishing cholestasis from necrosis. Despite the limitation of the low number of compounds studied at a single time point (24 h), the developed models were able to classify the hepatotoxicants based on their phenotype or mechanism of toxicity with a good accuracy and the identified classifier genes are associated with the phenotype of toxicity. The identified classifiers were mechanistically involved in endoplasmic reticulum stress, unfolded protein response and other stress response pathways, phenomena shown to play a role in cholestasis (chapter 3). They appeared consistent across different concentration levels, different predictive algorithms and inter-individual variation in response. Hence, the human PCLS model is a useful model to study the mechanisms of drug-induced toxicity and to classify toxins based on their mechanism of toxicity and to identify and validate classifiers responsible for drug-induced liver toxicity in humans. A limitation of our study is the low sample size and further validation of the identified classifiers by incorporating additional compounds will be necessary.

Human PCLS to study mechanisms involved in drug induced in idiosyncratic toxicity

In chapter 5, we applied transcriptomic analysis to understand the possible mechanisms or pathways that might be involved in idiosyncratic drug induced liver injury (IDILI). Several hypotheses have been tested in animal or human models to study IDILI and associated mechanisms. Among the hypothesis tested the inflammatory stress hypothesis is of particular interest due to its inter-relation to other hypothesis such as mitochondrial stress hypothesis [26] as it was reported that inflammatory mediators induced during inflammation could induce mitochondrial dysfunction [216, 217, 218]. Recently, Hadi et al. were the first to study the inflammatory stress hypothesis in human and mouse precision-cut liver slices ex vivo [195] and found that co-incubation of LPS (Lipopolysaccharide) with several IDILI drugs, among which clozapine, resulted in synergistic toxicity. To further understand the possible mechanisms involved in clozapine induced IDILI, we compared the gene expression profiles of clozapine with its non-IDILI analogue olanzapine in the presence and absence of LPS. Pathway analysis using immune-mediated and cellular stress response signalling pathways to compare the effects in the different treatment groups revealed the enhanced activation of toll-like receptor signalling, HMGB1 (High-mobility group box 1) signalling, iNOS (Inducible nitric oxide synthase) signalling, p38-MAPK (Mitogen-activated protein kinase) and NRF2 oxidative stress response in LPS+clozapine. Several inflammatory mediators involved in the different inflammatory signalling pathways such as IL1A (Interleukin-1 alpha), IL1B (Interleukin-1 beta), ICAM1 (Intercellular adhesion molecule 1), GM-CSF (Granulocyte-macrophage colony-stimulating factor), MAPKAPK-2 (MAPK-activated protein kinase 2) and PAI-1 (Plasminogen activator inhibitor-1) were significantly regulated with enhanced expression in LPS+clozapine co-treated human PCLS compared to the human PCLS exposed to LPS or clozapine alone, or to LPS+olanzapine. This enhanced production of inflammatory mediators might contribute to the liver injury represented by necrotic areas in LPS+clozapine-treated human PCLS. Also, the enhanced
expression of NFkB, iNOS, AP-1 and IFN, leading to enhanced activation of iNOS in LPS+clozapine, might contribute to the IDILI by enhanced production of reactive nitrogen species causing mitochondrial damage [212]. Drugs that are more often associated with IDILI are shown to cause strong activation of NRF2 mediated stress response [197]. In our results, clozapine showed activation of NRF2 stress response but not LPS or olanzapine and the clozapine-induced NRF2 response was further enhanced in the presence of an inflammatory stress condition (LPS). There is also increasing evidence that numerous drugs associated with idiosyncratic drug reactions cause mitochondrial dysfunction [182, 184]. In accordance, pathway analysis of the 719 genes uniquely regulated due to LPS+clozapine treatment also revealed that oxidative phosphorylation was the predominantly affected pathway and many genes involved in complex I-V of the electron transport chain were downregulated. This further strengthens the role of the mitochondrial damage in inflammation-associated IDILI. In addition, hepatic gene expression analysis suggested the activation of HMGB1, p38 MAPK, NFkB signalling pathways to be possibly involved in the LPS+clozapine induced IDILI.

In conclusion, the human PCLS seems to be a promising ex vivo model for characterizing IDILI and toxic mechanisms associated with it. Inflammatory-associated mitochondrial dysfunction was identified as a potential mechanism of inflammation-associated IDILI. Further research including more IDILI-related drugs together with their non-IDILI-related comparator drugs would be necessary to confirm the findings.

**Limitations and future perspectives**

In the studies described in this thesis, the precision-cut liver slice model is validated as a model to study compound or drug induced toxicity mechanisms. Moreover, the transcriptomic analysis revealed that the PCLS can serve as a useful model to identify intrinsic drug induced toxicity phenotypes such as necrosis, cholestasis, and fibrosis as well as IDILI.

One of the main limitations in these studies is that a limited number of compounds were studied, in a limited number of human liver samples, mainly due to practical reasons such as limited availability of human tissue, and the costs for the microarray measurements. Further studies with an additional set of compounds are necessary to confirm the findings reported in this thesis. The limited availability of human donors for human liver slices in turn limits the use of PCLS for toxicogenomics research. However, the results of our studies confirm that even with 5 different liver samples a fairly good characterization of hepatotoxicity can be obtained. Although the human livers show quite some variation in basic gene expression, the changes in gene expression due to exposure to a toxic compound are rather consistent, thus showing the feasibility of this type of experiments with a limited number of human samples.

Gene expression microarrays only measure the response at the mRNA transcription level of a gene, which only gives a rather rough estimate of its corresponding changes in protein expression level and the subsequent metabolic changes. Proteomics studies, aiming to characterize the expression of all proteins in a cell, tissue or organism are needed to understand the functional relevance of proteins regulated due to a toxic insult. In addition, metabolomics studies, aiming to characterize the global metabolite profiles in a system (cell, tissue or organism) under a given set of conditions, may elucidate the effects of the induced changes in protein expression. The liver is responsible for the production and secretion of
a large variety of plasma proteins and endogenous molecules and is also a major target for drug-induced toxicity. Hence, secreted protein or metabolite profiles can also reveal relevant toxicity signatures. To obtain a broader insight about the drug induced liver injury and to discover clinically significant biomarkers, comparison of data from transcriptomics, proteomics and metabolomics experiments would be necessary.

Further gene expression studies using RNA-Seq (RNA sequencing) can be considered for the better prediction of biomarkers for DILI. RNA-Seq can look at different populations of RNA, which include total RNA, small RNA, such as microRNA, transferRNA, and ribosomal profiling, in addition to mRNA transcripts. MicroRNAs (miRNA) are non-coding RNAs that play key roles in the post-transcriptional regulation of gene expression and participate in physiological and pathological regulatory processes, including liver diseases. Changes in the expression levels of specific miRNAs have been reported in different liver diseases, indicating their potential use as biomarkers for DILI [219, 220, 221]. Other advantages of RNA-Seq compared to microarrays include high sensitivity; discovery of novel genes; ability to quantify a large dynamic range of expression levels allowing the identification of more differentially expressed genes with higher fold changes [222].

So far, toxicogenomics studies using PCLS model were scarcely reported in the literature. The presence of all the different liver cell types in the PCLS model allows for studying the interaction between different cells in response to a toxic insult but at the same can also contributes to an extra level of complexity in the study. Gene expression is invariably heterogeneous between different cell types, and it is sometimes difficult to attribute the observed changes in gene expression to any of the different cell types. Single cell transcriptome studies using for instance Laser Scanning Dissection Microscopy, would provide information about the response of each of the different cell types and identify the most responsive cell types in the PCLS [223]. The limited lifetime of the PCLS model was also considered as one of the main disadvantage and limited their extensive use in the toxicology research in comparison to other in vitro models. However, recently, significant improvements in the incubation medium were reported which facilitate the extension of the viability of the PCLS up to 5 days [224]. So with the increased viability up to five days, it seems that sub-chronic toxicity studies can be performed in the near future.

The results of the studies described in this thesis show the ability of human PCLS to properly reflect drug-induced liver injury as observed in the clinic and to identify human specific toxicity markers using toxicogenomics analysis. The use of human tissue will not only greatly contribute to the replacement, reduction and refinement (3R’s) of animals for scientific purposes, but also enables a better risk assessment by avoiding interspecies extrapolation.