From the Department of Physiology and Pharmacology,
Karolinska Institutet, Stockholm, Sweden

HEPATIC 3D SPHEROIDS AS A MODEL SYSTEM FOR DRUG-INDUCED LIVER INJURY AND CYTOCHROME P450 INDUCTION

Delilah F. G. Hendriks

Stockholm 2018

All previously published papers were reproduced with permission from the publisher.
Cover image adapted from Sci. Rep. 2016 6:25187.
Published by Karolinska Institutet.
Printed by E-print AB 2018

ISBN 978-91-7831-081-4

Hepatic 3D spheroids as a model system for drug-induced liver injury and cytochrome P450 induction

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Public defence takes place on Friday June 15th 2018, 09.00 a.m.
Samuelssonsalen, Karolinska Institutet, Tomtebodavägen 6, Solna

by

Delilah F. G. Hendriks

Principal Supervisor:
Prof. Magnus Ingelman-Sundberg
Karolinska Institutet
Department of Physiology and Pharmacology

Co-supervisors:
Associate Prof. Inger Johansson
Karolinska Institutet
Department of Physiology and Pharmacology

Associate Prof. Souren Mkrtchian
Karolinska Institutet
Department of Physiology and Pharmacology

Opponent:
Prof. Mathieu Vinken
Vrije Universiteit Brussel
Department of Toxicology

Examination Board:
Prof. Bengt Fadeel
Karolinska Institutet
Institute of Environmental Medicine

Associate Prof. Kent Jardemark
Karolinska Institutet
Department of Physiology and Pharmacology

Prof. Hans Lennernäs
Uppsala Universitet
Department of Pharmacy

ABSTRACT

Drug-induced liver injury (DILI) is a major cause of post-marketing drug withdrawals and restricted-use warnings. In addition, unexpected adverse hepatic drug reactions occurring in the clinical phases of development are a major reason for drug attrition. Currently, there is an unmet need for reliable *in vitro* models to faithfully study the impact of drugs on the human liver.

In this thesis, we developed and extensively characterized a novel three-dimensional (3D) spheroid culture system comprised of primary human hepatocytes (PHH). We found that the proteomes of PHH in 3D spheroid culture closely resemble those observed in the liver *in vivo*, whereas in conventional two-dimensional (2D) monolayer cultures PHH rapidly lose their mature phenotype due to dedifferentiation.

PHH spheroids retain stable molecular phenotypes and liver-specific functionalities for multiple weeks in culture. These features allow prediction of DILI events, including those that may be delayed in onset, in a more phenotypically adequate system. Moreover, the PHH spheroid system was found suitable to detect the liability of drugs to induce cholestasis and to identify concomitant toxicity mechanisms.

Our results also indicate that PHH spheroids can be used to screen drugs for cytochrome P450 3A4 (CYP3A4) induction. Importantly, PHH in 3D spheroid culture could identify a clinically relevant atypical mechanism of CYP3A4 induction that was not possible to detect in the corresponding 2D monolayer cultures.

In conclusion, the PHH spheroid system presented here constitutes a versatile *in vitro* model to study liver biology and to assess the metabolic and toxicological profiles of drugs and drug candidates.

LIST OF SCIENTIFIC PAPERS

I. Lauschke VM, Vorrink SU, Moro SML, Rezayee F, Nordling Å, **Hendriks DFG**, Bell CC, Sison-Young R, Park BK, Goldring CE, Ellis E, Johansson I, Mkrtchian S, Andersson TB, Ingelman-Sundberg M. Massive rearrangements of cellular microRNA signatures are key drivers of hepatocyte dedifferentiation. *Hepatology. 2016; 64(5):1743-1756.*

II. Bell CC*****, **Hendriks DFG***, Moro SML*****, Ellis E, Walsh J, Renblom A, Fredriksson Puigvert L, Dankers ACA, Jacobs F, Snoeys J, Sison-Young RL, Jenkins RE, Nordling Å, Mkrtchian S, Park BK, Kitteringham NR, Goldring CEP, Lauschke VM, Ingelman-Sundberg M. Characterization of primary human hepatocyte spheroids as a model system for drug-induced liver injury, liver function and disease. *Sci. Rep. 2016; 6:25187.*

III. **Hendriks DFG***, Fredriksson Puigvert L*****, Messner S, Moritz W, Ingelman-Sundberg M. Hepatic 3D spheroid models for the detection and study of compounds with cholestatic liability. *Sci. Rep. 2016; 6:35434*.

IV. Parmentier C*****, **Hendriks DFG***, Heyd B, Bachellier P, Ingelman-Sundberg M, Richert L. Assessment of inter-individual variability towards drug-induced cholestasis in 2D-sandwich and 3D-spheroid cultures of primary human hepatocytes. *Toxicol. Lett. – in revision*.

V. **Hendriks DFG**, Vorrink SU, Sim SC, Ullah S, Templin M, Sachse C, Andersson TB, Johansson I, Jones BC, Lauschke VM, Ingelman-Sundberg M. Primary human hepatocyte spheroids constitute a sensitive platform to screen drugs and drug candidates for CYP3A4 induction. *Manuscript*.

*****Equal contribution.

CONTENTS

ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
ADME	Absorption, distribution, metabolism, and excretion
ADR	Adverse drug reaction
ALF	Acute liver failure
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APAP	Acetaminophen
BA	Bile acid
BSEP	Bile salt export pump
CAR	Constitutive androstane receptor
CPZ	Chlorpromazine
CsA	Cyclosporine A
CYP	Cytochrome P450
DDI	Drug-drug interaction
DILI	Drug-induced liver injury
DME	Drug metabolizing enzyme
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMA	European Medicines Agency
FDA	Food and Drug Administration
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
HLC	Hepatocyte-like cell
iPSC	Induced pluripotent stem cell
LPS	Lipopolysaccharide
MRP	Multi-drug resistance protein
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NPC	Non-parenchymal cell
NR	Nuclear receptor
PHH	Primary human hepatocytes
PK/PD	Pharmacokinetics/pharmacodynamics
PXR	Pregnane X receptor
RXR	Retinoid X receptor

1 INTRODUCTION

1.1 ADVERSE DRUG REACTIONS

Novel drugs for prevention, treatment, or cure of diseases are constantly being developed, optimized, and tailored to patient-specific needs. Undoubtedly in the vast majority of cases the benefits of drug therapy outweigh the associated side effects and risks. Nevertheless, the occurrence of adverse drug reactions (ADRs) remains a significant problem in clinical practice, in part due to the increased complexity of therapeutic regimes associated with the rise in global multimorbidity (1). ADRs are suspected to directly cause or contribute to 5-18% of all in-hospital deaths (2-4) and are ranked as the seventh most common cause of death in the general population of Sweden (5). Approximately 5% of all hospital admissions are associated with ADRs and it is estimated that 5-10% of all hospitalized patients experience an ADR (6-11). As such, a significant economic burden is placed on healthcare systems, with reported annual costs of 30.1 billion USD in the United States due to increased hospitalization rates and prolonged hospital stays (12). The incidence and burden of ADRs may however significantly exceed the reported numbers since ADRs are notoriously under-reported, which is a major challenge in pharmacovigilance worldwide (13-16).

Bringing a new drug from concept to market is a highly protracted and costly process, taking on average 13-15 years with associated costs estimated at 1.3 billion USD (17, 18). Since the 1990s, the performance of the pharmaceutical industry has been alarming, with low numbers of new drug approvals by the Food and Drug Administration (FDA) (19-23). However, AstraZeneca recently reported a marked improvement in their project success rates from candidate drug nomination to phase III completion from 4% in 2005-2010 to 19% in 2012-2016 (24), suggesting productivity may be on the rise again. In addition, the FDA approved 46 new drugs in 2017, the highest number since 1996 (25).

Still, a major challenge the pharmaceutical industry currently faces is to address and reduce the high attrition rates in drug development. While in the early 1990s adverse pharmacokinetic and bioavailability profiles were the most common reason for attrition, the contribution of these factors has significantly decreased in recent years. Instead, lack of efficacy and safety issues are now two of the most frequent causes of attrition (22, 26, 27). Analysis of 605 compounds terminated from development between 2000 and 2010 revealed that non-clinical toxicology and clinical safety issues accounted for over 50% of all failures (27). In the same period, 32% of newly FDA-approved therapeutics were affected by post-market safety events, including withdrawals, restricted-use warnings, and issuance of safety communications (28). Thus, unexpected ADRs are of major concern for public health and remain a key challenge for the pharmaceutical industry during both pre- and post-marketing stages of drug development.

1.2 DRUG-INDUCED LIVER INJURY

1.2.1 Epidemiology

Between 1953 and 2013, 462 medicinal products were withdrawn from the pharmaceutical market in one or more countries because of severe ADRs, including death which was documented as the reason for a quarter of these cases. Hepatotoxicity was reported as the most frequent reason for withdrawal, accounting for 18% of all cases (29). Indeed, drug-induced liver injury (DILI) continues to be a major concern for public health, surpassing viral hepatitis as the most common cause of acute liver failure (ALF) in the United States (30). Almost 50% of ALF cases are due to intentional or accidental acetaminophen (APAP) overdose while approximately 11% are due to idiosyncratic drug reactions (31). The latter cause of ALF is often more severe and associated with poor outcome, having a mere 27% transplant-free survival rate (31, 32). Fortunately, the incidence of idiosyncratic DILI is rare, crudely estimated at ~19 cases per 100,000 individuals annually (33). However, this is likely an underestimation due to serious under-reporting because of the lack of surveillance systems and the concomitant challenges in its recognition and diagnosis (34). Despite its infrequency, the clinical outcomes can be severe. One in five patients will develop chronic liver injury and in nearly 10% of all cases it is, directly or indirectly, the cause of fatality or need for liver transplantation (35-37).

1.2.2 Classification

Conventionally, DILI has been classified into either having an underlying intrinsic or idiosyncratic mechanism of toxicity. DILI is referred to as intrinsic when the toxicity is clearly dose-dependent and occurs in a predictable and reproducible manner. On the other hand, idiosyncratic DILI is often delayed in onset and is the result of a complex interplay between drug properties, individual sensitivity factors, and environmental factors (38). Other than APAP, there are few drugs currently used in the clinic known to pose the risk of intrinsic liver injury, because this type of drug liability can normally be detected in drug development, resulting in either drug termination, changing the route of drug administration, or continuation of the drug but at doses well below the toxicity threshold (39). In recent years however, intrinsic and idiosyncratic DILI have thought to be coinciding rather than existing as two separate entities, where it is hypothesized that other factors such as inflammatory stress can sensitize certain individuals towards intrinsic hepatotoxins, ultimately resulting in idiosyncratic liver injury (40, 41). Evidence substantiating this hypothesis is provided by the finding that pharmacological doses of APAP in patients hospitalized for acute viral hepatitis can provoke serious liver injury (42). Further supporting this notion is the fact that in around 10% of ALF cases linked to APAP toxicity occurs at the recommended dose (43, 44).

1.2.3 Clinical presentation

Clinical symptoms of idiosyncratic DILI are often vague and nonspecific (*e.g.* lethargy, nausea, vomiting, and abdominal pain). In more serious cases liver-specific symptoms such as jaundice and encephalopathy may be present (45). Because of the diverse disease representation, idiosyncratic DILI can phenotypically mimic virtually all primary liver diseases, in particular acute viral hepatitis. As such, this inevitably leads to frequent misdiagnoses that can negatively impact on patient care (46). Currently, reliable and objective biomarkers of DILI are scarce, making it a diagnosis of exclusion based on reviewing the patient's history to exclude competing etiologies and relying on blood tests, hepatobiliary imaging, and occasionally liver biopsies (47).

Biochemically, the pattern of DILI can be subdivided into hepatocellular, cholestatic or a mixed pattern based on the ratio of elevation of baseline alanine aminotransferase (ALT) to baseline alkaline phosphatase (ALP) (48-50). In case of hepatocellular injury, ALT is markedly elevated while ALP elevations are modest. However, generally, the degree of ALT elevation poorly correlates with the severity of disease and often underestimates the degree of injury (51). Compared to other patterns of DILI, the hepatocellular type is more likely to progress to ALF (52). Cholestasis and mixed patterns are frequently observed in the clinic, accounting in some reports for up to half of all DILI cases (33, 53). While mortality rates are lower for cholestatic and mixed patterns of injury, normalization of liver function tests is protracted and the risk for chronic injury is higher (54). Other less commonly observed patterns of DILI include steatohepatitis, granulomatous hepatitis, neoplasms, and vascular abnormalities (55).

An important feature that aids in making the diagnosis of DILI is recognition of specific patterns of liver injury and the concomitant latencies that are characteristic for certain drugs. Drug classes frequently associated with DILI include antimicrobials (in particular amoxicillin-clavulanate), cardiovascular agents, central nervous system agents, and antineoplastics (56). Moreover, reports of idiosyncratic liver injury from herbal products and dietary supplements have been on the rise in recent years (57). Ongoing efforts to more confidently and rapidly recognize DILI in the clinic are aimed at identification of novel DILI-specific biomarkers by analyzing blood and tissue samples from idiosyncratic DILI cases. Emerging examples include microRNA-122, high mortality group box 1, and keratin 18. Though before such markers can be implemented into the clinic, further characterization is needed regarding the sensitivity and specificity of these markers as well as their stability in body fluids for reliable detection (58, 59).

1.2.4 Risk factors

A plethora of factors have been proposed to affect the susceptibility to idiosyncratic DILI and the subsequent outcomes. These include characteristics of the drug, environmental factors, and genetic and non-genetic factors of the host. Drug-specific risk factors include high

lipophilicity (60), high daily oral doses (60, 61), and the propensity to cause interactions with other drugs (62, 63). Extensive hepatic metabolism has also been identified as a risk factor in the pathogenesis of DILI (64, 65). Reactive drug metabolites can covalently bind to proteins, forming drug-protein adducts that could induce direct toxicity or elicit an immune response (66). In addition, the liability of drugs to inhibit certain bile acid transporters, including the bile salt export pump (BSEP) (67) and multi-drug resistance proteins (MRP) 2-4 (68), is considered a risk factor for liver injury. Specifically, the potency to inhibit BSEP was higher among drugs causing cholestatic or mixed patterns of liver injury than among drugs causing hepatocellular injury (67).

A variety of non-genetic host risk factors have been proposed including age, gender, comorbidities, and concomitant drug use. Although it is well recognized that aging induces pharmacokinetic and pharmacodynamic changes, agedness has not univocally been identified as a risk factor for idiosyncratic DILI (69). However, DILI with persistent or chronic hepatic abnormalities are more often seen in older patients, possibly due to the age-related reduction in tissue repair capacity (70, 71). Conversely, young patients under the age of two appear to be at increased risk for hepatotoxicity induced by valproic acid (72). It is considered controversial as to whether females in general are at higher risk to develop DILI, though strong evidence is provided that female patients presenting with DILI are more likely to progress to ALF (73). Age and gender differences are also found in the clinical phenotype of DILI: where cholestasis was more frequent in male patients over the age of 60, hepatocellular injury was more frequent in younger female patients (74), though due to the limited study size further validation of this observation is needed. Underlying chronic viral infections, *e.g.* with hepatitis C or human immunodeficiency viruses, have been associated with an increased risk of liver injury caused by antituberculosis drugs (75, 76). Lifestyle factors that are thought to play a role in the pathogenesis of idiosyncratic DILI include diet, alcohol consumption, and the gut microbiome, but these are not currently recognized as *bona fide* risk factors (77).

Genetic variation in drug-metabolizing enzymes (DMEs) and drug transporters may result in an imbalance between formation and detoxification of reactive drug metabolites or impaired hepatic transport, ultimately predisposing the patient to specific drug hepatotoxicities. Genetic studies on idiosyncratic DILI are challenging, due to its rare incidence, the number of different drugs implicated in DILI, and the wide range of liver injury phenotypes (78). However, significant progress has been made in recent years, employing both genome-wide association studies (GWAS) and candidate gene association studies using *a priori* information. A well-established example is the association between certain *N*-acetyltransferase 2 (*NAT2*) genotypes and isoniazid-induced liver injury, which has been observed in multiple independent studies (79-81). Possession of the **2* allele of uridine diphosphate-glucuronosyltransferase 2B7 (*UGT2B7*) appears to constitute a risk factor for diclofenac-induced liver injury (82, 83). Several polymorphisms in *ABCB11*, which encodes BSEP, have been proposed to increase the risk of liver injury from different drugs (84-86).

However, conflicting reports exist (87) and additional studies in larger and varied populations are needed to validate these findings.

Certain human leukocyte antigen (*HLA*) alleles may also constitute important risk factors for idiosyncratic immune-mediated DILI reactions since the major histocompatibility complex proteins mediate antigen presentation essential for T cell immune responses. To date, only few drug-specific *HLA* risk alleles have been uncovered by GWAS. Examples include *HLA-B*57:01* with flucloxacillin (odds ratio (OR) = 80) (88), *HLA-DQA1*02:01* with lapatinib (OR = 9) (89), and *HLA-B*35:02* with minocycline (OR = 29.6) (90). Further characterization of the heterogeneity of the genetic risk factors predisposing a patient to idiosyncratic DILI is awaited. In particular, whole genome sequencing could provide novel insights into the role of rare variants (91). However, given that especially *HLA* genes are hyperpolymorphic and the fact that additional risk factors may be required, prospectively predicting idiosyncratic DILI events prior to drug prescription remains extraordinarily challenging.

1.3 DRUG-INDUCED PHARMACOKINETIC ALTERATIONS

1.3.1 Drug-drug interactions

Drug-drug interactions (DDIs) are highly concerning in the clinic with regard to unfavorable therapeutic responses and occurrence of ADRs. While the list of potential DDIs is extensive, many are not necessarily clinically relevant (92). Still, clinically important DDIs frequently occur and are a major source of ADRs (93), including cases of fatality (94, 95), and have been the reason for multiple restricted-use warnings and post-marketing drug withdrawals (96). DDIs are expected to only become a bigger clinical issue as drug prescription and multi-drug therapies are steadily on the rise, which is in part driven by the high rates of comorbidities and the growing aging population (97-99). Analysis of drug use in the United States between 2007 and 2010 revealed that 47.5% of the population take at least one prescription drug, while 10.1% take five or more simultaneously (100). Elderly patients are especially at an increased risk to develop DDI-related ADRs due to the increased prevalence of polypharmacy and age-related changes impacting on drug pharmacokinetics (101-104). This growing problem is also recognized by regulatory agencies and accordingly the European Medicines Agency (EMA) recently issued a strategy to report ADRs and to identify common DDIs or drug-disease interactions in the elderly (105). In theory, DDIs could be considered predictable and preventable (106-108), yet medication prescription errors still frequently occur, in part due to inadequate knowledge about DDIs of healthcare providers (109, 110). In addition, various factors are known to influence the likelihood of DDI occurrence including age, genetic background, and disease and nutritional statuses, which make accurately predicting patient-specific clinical outcomes highly complex (111).

1.3.2 Cytochrome P450-mediated drug interactions

DDIs influencing pharmacokinetic parameters result in changes in the absorption, distribution, metabolism, and excretion (ADME) profile of the affected drug, characterized by altered plasma drug concentration-time profiles (112). The vast majority of DDIs are metabolism-mediated and involve induction or inhibition of DMEs or transporters. The clinical consequences may include 1) reduced or loss of intensity or duration of drug effects due to decreased systemic drug exposure, 2) enhanced efficacy due to increased systemic drug exposure, or 3) development of ADRs due to either increased systemic drug exposure or increased formation of reactive drug metabolites (113, 114). The majority of clinically significant DDIs documented in the literature are mediated via changes in the activity of cytochrome P450 (CYP) enzymes (115-117). This is not surprising given that they are responsible for the metabolism of ~75% of all drugs (118). In one report, the prevalence of a potential CYP-mediated DDI in elderly patients taking 5 or more drugs was estimated at 80%, and the probability of at least one CYP-mediated DDI was 50% when taking 5-9 drugs, which increased to 100% when taking 20 or more drugs (119). The impact of CYP-mediated DDIs has long been recognized and both the FDA and EMA provide extensive guidelines on approaches to evaluate potential clinical DDIs through CYP induction or inhibition studies in drug development (120).

The CYP superfamily is involved in many important biological processes including cholesterol homeostasis as well as the metabolism of steroids, fatty acids, and prostaglandins (121, 122). CYPs are however predominantly known for catalyzing the oxidative metabolism of drugs and other xenobiotics (123). Although 57 putatively functional human CYP enzymes exist (124), only 8 isoenzymes are considered to be clinically important (*i.e.* CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5) (125). Significant inter-individual differences in basal CYP expression and activities exist, owing to a wide variety of factors including age, gender, genetic polymorphisms, epigenetic changes, disease state, and diet (126, 127). Likewise, tremendous inter-individual variabilities in response to CYP induction and inhibition are observed due to both environmental and physiological factors (128).

CYP-mediated DDIs arise more frequently from CYP inhibition than from CYP induction and the clinical outcomes are often more serious (129, 130). Inhibition of CYP metabolism is an instantaneous and direct reaction that can be either reversible or irreversible in nature and may result in increased plasma concentrations of the affected drug, leading to toxicity (131). A classic example is the ketoconazole-mediated inhibition of CYP3A4-mediated metabolism of terfenadine leading to prolonged electrocardiographic QT intervals (132), increasing the risk for torsades de pointes. CYP induction is considered to be less of a safety issue but can result in therapeutic failures due to increased metabolic clearance of the victim drug and therefore is an undesirable property that should be considered during drug development (133). Though not as common, CYP induction can also result in toxicity due to increased

formation of reactive drug metabolites. In addition, toxicity may arise if clinicians do not recognize the need to reduce the dose of the induced medication upon discontinuation of the inducing agent, as can be the case when patients self-medicate with the CYP3A4 inducing agent St John's wort (134, 135). CYP induction is a much slower and indirect process, most commonly occurring through increased gene transcription or rarely through stabilization of the mRNA or protein (as is the case for CYP2E1 (136)). These processes involve a multitude of molecular players, and CYP induction responses are consequently subject to large inter-individual differences (137). Besides impacting on other medications, drugs can also unfavorably induce their own metabolism (*i.e.* auto-induction), which is also pertinent to consider during drug development. This is exemplified by the case of AZD1208, a PIM kinase inhibitor developed for the treatment of acute myeloid leukemia (138). This compound was recently terminated from development because of its unfavorable pharmacokinetic profile observed in the clinic, which was attributed to the acceleration of its own metabolism through CYP3A4 induction leading to unstable plasma concentration profiles (139, 140).

1.3.3 Molecular mechanisms of CYP3A4 induction

DDIs involving CYP3A4 are especially important to consider, as it is the predominant CYP isoform expressed in the human liver and intestine and is implicated in the metabolism of many marketed drugs (141). Various drugs currently used in the clinic are known to induce CYP3A4 (*e.g.* rifampicin (142), phenobarbital (143), and phenytoin (144)). On a molecular level, CYP3A4 induction is thought to predominantly occur via activation of the nuclear receptor (NR) pregnane X receptor (PXR; *NR1I2*) (145). Upon activation, PXR forms a heterodimer with the retinoid X receptor (RXR) in the nucleus. The PXR/RXR complex then binds to distinct responsive elements within the promoter region of the *CYP3A4* gene to regulate its transcription (146, 147). Expression of CYP3A4 is also under the control of the constitutive androstane receptor (CAR; *NR1I3*) (148). Upon activation, CAR translocates from the cytoplasm to the nucleus to form a heterodimer with RXR that binds to specific responsive elements in the promoters of its target genes to control their transcription. It is noteworthy to mention that significant species differences exist in the response to CYP inducers, owing to cross-species variations in the ligand-binding domain sequences of PXR and CAR (149). For example, rifampicin is a potent human PXR activator but has little effect on mouse PXR (150). Reciprocally, TCPOBOP is a potent mouse CAR ligand but does not activate human CAR (151). These prominent differences represent a challenge in drug development since evidently animal models cannot be employed to screen for human CYP induction (152).

In humans, PXR and CAR can be activated by a variety of structurally diverse compounds, some of which act both on PXR and CAR (*e.g.* phenobarbital) (153, 154). Moreover, in addition to *CYP3A4*, PXR and CAR regulate the expression of a largely overlapping set of target genes, including *CYP2B6*, *CYP2C9*, *CYP2C19*, and *CYP3A5* (155). However, differences in their preference of gene regulation exist. PXR displays strong binding to all

functional responsive elements in the promoters of both *CYP2B6* and *CYP3A4*, whereas CAR only weakly binds to the ER6 motif in *CYP3A4*, but strongly binds to the DR4 motif in the phenobarbital responsive element module (PRBEM) in the promoter region of *CYP2B6*, explaining its preferential induction of CYP2B6 over CYP3A4 (156, 157). Furthermore, an important difference between these NRs is that CAR can constitutively transactivate target genes in the absence of activators, while PXR cannot (158). CAR activity is normally restrained through cytosolic sequestration (159) but upon stimuli, either through direct ligand binding or indirect activation, CAR translocates to the nucleus to regulate the expression of its target genes (160-163). On the other hand, PXR activation is conventionally thought to depend on direct ligand binding. However, reports of indirect regulation of PXR-mediated induction of CYP3A4 by protein kinase A (PKA) (164) and protein kinase C (PKC) (165) exist, possibly through altering the phosphorylation status of PXR. Therefore, it can be anticipated that additional ways of indirect PXR activation resulting in CYP3A4 induction exist.

Besides PXR and CAR, other NRs reported to be directly or indirectly involved in the regulation of CYP3A4 expression include the vitamin D receptor (VDR) (166) and the glucocorticoid receptor (GR) (167). In addition, the liver-enriched transcription factor hepatocyte nuclear factor 4 alpha (HNF4α) has been shown to regulate PXR- and CAR-mediated transcriptional activation of *CYP3A4* (168). Various NRs are known to share the same responsive elements in the promotors of their target genes and to compete for common coactivators and corepressors, which are necessary for the tuning of the transcriptional response (169, 170). For example, PXR and CAR share several coactivators, including the peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) and the steroid receptor coactivators 1 and 2 (SRC1-2) (171). As such, a complex cross-talk between these NR signaling pathways is created.

1.4 PRECLINICAL LIVER MODELS

Clinical safety issues are currently one of the primary reasons for the attrition of drug candidates in phases I and II (24, 27, 172, 173). A recent review of AstraZeneca's productivity remarkably indicated that for compounds terminated in the clinical phase due to safety issues the level of confidence in their preclinical safety profile was low, whereas when confidence levels were high no compounds were terminated in the clinical phase because of safety issues but rather due to efficacy issues (173). Adding to this observation, Morgan *et al.* (2018) note that *'…there remain continued instances of idiosyncratic or unexplained toxicity. We and others continue to explore new in vitro and in vivo models to improve translation to the clinic'* (24). The introduction of drug metabolism and pharmacokinetic studies in drug development has led to increased understanding of the pharmacokinetic/pharmacodynamic (PK/PD) properties of drug candidates. Still, analysis of AstraZeneca's attrition data from 2005 to 2010 revealed that unfavorable PK/PD profiles were the cause of 15% of all project closures in phase I (173).

The liver is persistently being reported as one of the major organ systems associated with (late-stage) closures of drug development programs and post-marketing withdrawals (173, 174). Recent examples include the attrition of fasiglifam, which was terminated only in phase III due to concerns about liver safety (175, 176), the termination of AZD1979 due to unexplained ALT elevations in the clinic (24), and the restricted-use warnings of flupirtine, pazopanib, and temozolomide due to signs of hepatotoxicity that remained unnoticed during drug development (177). Not surprisingly, the EMA and FDA recognize the problem of drug hepatotoxicity and accordingly have issued guidelines to aid in the assessment and interpretation of DILI signals during the preclinical stage of development (178). The recent termination of AZD1208 because of its unfavorable PK profile in humans (*i.e.* CYP3A4 auto-induction) that was not identified in the preclinical phase of development (139, 140) also underlines the shortcomings of the current preclinical models to accurately predict drug PK profiles. Undeniably, refinements of the preclinical strategies to predict the metabolic and toxicological profiles of drug candidates need to be made. In the following paragraphs conventional preclinical models and emerging novel models to assess the impact of drugs on the human liver are discussed.

1.4.1 Animal models

Safety tests in two or more different animal species are required before a drug candidate can proceed to being tested in the clinic (179). Preclinical assessments in animals could provide essential information regarding the PK/PD and toxicological profiles of drug candidates. Retrospective analyses, however, indicate that the concordance between animal and human toxicity is poor, with true positive human toxicity concordance rates of 63% and 43% for non-rodent and rodent models, respectively (180). Furthermore, animals may show increased sensitivity towards toxicity of drugs at doses that are considered safe for humans (*e.g.* APAP (mice) and ibuprofen (dogs)) (39). With specific regard to DILI, the presence of toxicity in animal models does not typically halt the progression of drug candidates into clinical development unless clear dose-dependent indicators are seen (181), due to the fact that preclinical prediction of clinical hepatotoxicity is notoriously poor (182, 183). This may stem from the apparent and significant inter-species variation in xenobiotic metabolism (184), explained by differences in the structure, substrate affinities, catalytic activities, and induction of DMEs (185, 186). Consequently, the use of animal models to predict drug metabolism and hepatotoxicity events in humans is limited.

The case of fialuridine is an excellent example of how inter-species differences can cause detrimental effects in the clinic. This drug, a nucleoside analogue developed for the treatment of chronic hepatitis B virus infection, showed great promise concerning its toxicity profile in preclinical *in vivo* studies in mice, rats, and cynomolgus monkeys (187). Clinical phase I studies in patients were promising, resulting in significant suppression of serum hepatitis B virus DNA levels (188) and hence a subsequent phase II trial with 15 patients was started in 1993. Unexpectedly, however, in the 13th week of treatment, one patient developed liver

failure and lactic acidosis, leading to the termination of the study. Severe hepatotoxicity with progressive lactic acidosis was observed in seven patients, resulting in the death of five and two others requiring emergency liver transplants. An additional three patients displayed mild hepatotoxicity. Analyses of the hepatic tissues revealed fat accumulation in the hepatocytes and abnormal mitochondria (189). Additional follow-up experiments confirmed the absence of toxicity in rodents after 10 weeks of treatment at 1,000-fold concentrated doses compared to those used in humans (187). Later mechanistic evaluations *in vitro* indicated that species differences in the subcellular expression pattern of the equilibrative nucleoside transporter 1 (ENT1), for which fialuridine is a substrate, may account for the observed human-specific toxicity of fialuridine. Besides expression on the plasma membrane, this transporter was found to be exclusively expressed on the human, but not mouse, mitochondrial membrane. The ENT1-mediated uptake of fialuridine in human mitochondria may explain its selective mitochondrial toxicity exerted in humans (190, 191). Indeed, fialuridine-induced hepatotoxicity could exclusively be detected in chimeric mice with humanized livers (192).

1.4.2 Conventional *in vitro* models

Recommendations for toxicity testing made by the U.S. National Academy of Sciences in 2007 urged a paradigm shift from extensive usage of *in vivo* models to *in vitro* systems employing human primary cells or cell lines to not only decrease animal usage but also to obtain increased efficiency and better mechanistic understanding of human ADRs (193). To date, a multitude of different human liver-derived *in vitro* models have been developed. Immortalized human liver cell lines, such as the HepG2 or HepaRG cell lines, are often employed to obtain an initial indication of the crude DILI risk of drug candidates using supraphysiological drug concentrations (194-197). Though these cell lines are well-established, cheap, easy to handle, and allow generation of reproducible data in a high-throughput setting, they are limited by their immature phenotypes. CYP activities and expression of DMEs and hepatic transporters are drastically lower in these cell lines compared to primary human hepatocytes (PHH) (198-200), which is also reflected in their reduced sensitivity towards drug hepatotoxicity compared to PHH and the lack of CYP inducibility in the case of HepG2 cells (200, 201). To overcome the limited liver-specific functionalities, cell lines overexpressing specific DMEs or NRs have been established for improved toxicity prediction and assessments of CYP enzyme induction (202-204), yet the overall molecular phenotypes still remain drastically different from the human liver *in vivo*.

PHH have long been considered the gold standard cell source to create *in vitro* liver models (205). When kept in suspension, gene expression patterns are well maintained and are comparable to those observed in the liver of origin (206). PHH suspension cultures are mainly useful for the prediction of metabolic clearance and CYP inhibition-mediated DDIs (207-210), while assessments of drug hepatotoxicity and CYP induction are not feasible due to their limited life span (*i.e.* a couple hours) (211). When seeded on a layer of rat-tail collagen as two-dimensional (2D) monolayers, PHH can be kept viable for several days in

culture. However, the plating on a rigid substratum induces major morphological and functional alterations (212). Cells flatten, cell-cell contacts are reduced, and the loss of polarity hampers formation of bile canalicular networks and leads to a rapid reduction in DME expression and decline in metabolic activities (206, 212-214). Nevertheless, PHH 2D monolayers are still widely used for drug hepatotoxicity studies (215). Reports on their sensitivity towards DILI vary greatly but in general are not considered satisfactory (200). In addition, they are often also the model of choice to evaluate drugs for their CYP induction liability (216, 217). Yet, the recent case of the termination of AZD1208 from development due to unexpected CYP3A4 auto-induction observed *in vivo*, which was not identified in preclinical studies employing 2D monolayer cultures of three different PHH donors (140), questions the robustness of this model to make accurate predictions of clinical CYP induction.

1.4.3 Emerging novel *in vitro* models

A relevant *in vitro* liver model should possess a variety of characteristics to study liver biology and to assess the metabolic and toxicological profile of drugs. We (218) recently formulated that a system should:

- Closely reflect *in vivo* liver physiology and morphology
- Remain viable and functionally stable for multiple weeks in culture
- Allow co-culturing of all liver cell types, *i.e.* parenchymal and non-parenchymal cells
- Allow taking genetic predispositions into consideration
- Be compatible with higher throughput applications
- Use low cell numbers to reduce costs and minimize material usage

In recent years, much emphasis has been placed on moving from the conventional simple 2D hepatocyte culture systems to more organotypic culture systems that include both hepatic parenchymal and non-parenchymal cell (NPC) types. This enables cells to adopt a more tissue-like structure, where homo- and heterotypic cell contacts and interactions with the environment can be made. Accordingly, these improvements positively impact on the cellular phenotypes *in vitro* (219, 220). Hepatocytes *in vivo* are highly polarized epithelial cells that form thin branching hepatic plates, usually of only one cell thickness, which are separated from the hepatic sinusoidal vascular network by the space of Disse. In the liver sinusoids, different NPC types reside including Kupffer cells, liver sinusoidal endothelial cells (LSECs), as well as various lymphocytes (221). Hepatocytes contain distinct canalicular-apical domains that are separated from the sinusoidal-basolateral domains by tight junctions. The basal membranes of hepatocytes are in contact with extracellular matrix (ECM) components and hepatic stromal stellate cells in the blood plasma-filled space of Disse. The lateral membranes of hepatocytes are used to establish contact with neighboring hepatocytes. During liver development, hepatocytes form apical domains enclosed by tight junctions at the lateral membrane between two adjacent cells which merge together to form a complex bile

canalicular network later in development (222-225). Hepatocyte polarity is critical for proper liver function and loss of polarity is associated with major pathophysiological changes including liver diseases such as cholestasis and hepatocellular carcinoma (226).

One of the first attempts made to reestablish hepatocyte polarity *in vitro* is based on culturing hepatocytes within two layers of ECM components (most commonly collagen or Matrigel®), termed sandwich cultures (227). In this configuration, cell spreading and formation of stress fibers are reduced, cell-cell contacts are improved, hepatocytes secrete a variety of ECM proteins, and importantly hepatocyte polarity is reestablished (228, 229). These changes positively impact on the viability and functionality of hepatocytes (230). Importantly, over the course of several days functional bile canalicular networks are created (231), which is a critical aspect for assessments of drug PK profiles (232). PHH sandwich cultures are therefore often the model of choice for studies of hepatobiliary drug transport and drug-induced cholestasis (233, 234). A large toxicity study in PHH sandwich cultures using 200 DILI-positive and 144 DILI-negative compounds screened at $100xC_{max}$ levels impressively detected no false positives, yet only 51% sensitivity was achieved (235). This rather low sensitivity may be due to the acute 24 hour drug exposure setting as well as the fact that sandwich culturing cannot prevent hepatocyte dedifferentiation, as typical patterns of epithelial-to-mesenchymal transition (EMT) are observed after two weeks of culture (236). Moreover, expression of DMEs has been reported to be unstable over time (237) and activities of important CYPs are gradually lost during the first days of culture (238).

Substantial advances have also been made with regard to the fabrication of functional microscale liver subunits where the microenvironment can be precisely controlled, which has been shown to positively impact on the stability and functionality of cells (239-241). A notable example is the development of micropatterned co-cultures of PHH and supportive cells. In this model, PHH are cultured on 2D collagen-coated islands that are surrounded by supportive stroma (*i.e.* mouse embryonic 3T3-J2 fibroblasts), thereby allowing the establishment of important heterotypic interactions between the hepatocytes and stromal cells, which are of importance for hepatocyte functionality (242). Accordingly, expression of DMEs, CYP activities, secretion of albumin and urea, and functional bile canalicular networks could be retained for several weeks in culture, and hepatotoxicity was predicted with 66% sensitivity and 90% specificity in a screen of 35 DILI-positive and 10 DILI-negative compounds tested at $100xC_{max}$ levels (242, 243). This model has since been used for various applications, including studies of drug metabolism (244-246), host-pathogen interactions (247), and nutritional state changes (248). Further improvements were recently made by establishing co-cultures of PHH with Kupffer cells to study the impact of pro-inflammatory cytokines on hepatic functionality (249). However, the use of mouse embryonic non-liver-derived cells as stroma to support hepatocyte longevity and functionality, as well as the use of rat-tail collagen rather than liver-specific ECM reduce the human relevance. Furthermore, the 2D monolayer format of the hepatocyte islands limits the

physiological relevance of this model to mimicking hepatic cords rather than advanced hepatic sinusoidal structures (250).

Numerous efforts have focused on further improving the physiological relevance *in vitro* by creating three-dimensional (3D) organotypic cultures, including scaffold-based or scaffold-free multi-well plates and perfusion bioreactors (251-253). In 3D spheroid culture, hepatocytes spontaneously aggregate into tissue-like structures over the course of several days. Currently, the value of such systems has predominantly been illustrated with human liver cell lines. In relation to the respective 2D monolayer cultures, enhanced molecular phenotypes and liver-specific functionalities have been observed when HepG2 or HepaRG cells are cultured as 3D spheroids, which may lead to improved drug toxicity responses (254-259). Though PHH are considered the most relevant cell source to mimic the human liver, few studies have evaluated their behavior in 3D spheroid culture. In a perfusion bioreactor, PHH spheroids formed functional bile canalicular networks, displayed stable *CYP* expression during 2 weeks of culture, and were in a long-term setting responsive towards 2 CYP inducers (260). In another study, responsiveness to drug toxicity of PHH spheroids cultured in a microscale bioreactor was shown using APAP as a model hepatotoxin (261). In these perfusion bioreactor set-ups, *in vivo* hemodynamics can be mimicked, physiological parameters (*e.g.* pH and oxygen levels) can be tightly controlled, and a continuous nutrient supply and removal of metabolic by-products is established (262). However, large cell numbers are required and the bioreactor set-up approach impedes higher throughput applications including drug toxicity screening.

A static scaffold-free 96-well hanging drop system was developed a few years ago that represents a higher throughput approach requiring substantially fewer cells (263). In this system, randomly organized spheroid co-cultures of PHH and non-parenchymal Kupffer and endothelial cells showed stable viability and albumin production for 5 weeks in culture. Using 3 hepatotoxins it was shown that repeated-dose toxicity studies could be performed for up to 14 days (263). Using a set of 69 DILI-positive and 41 DILI-negative compounds, DILI was predicted with 59% sensitivity and 80% specificity in this system (264), indicating no substantial improvements compared to reports in PHH sandwich cultures and the micropatterned co-culture system mentioned earlier. In another study, PHH spheroids generated in a 384-well magnetic 3D culture system were evaluated as a novel high-throughput model to assess CYP induction and inhibition (265). While basal CYP activities of PHH were higher in 3D spheroid culture compared to the corresponding 2D monolayer cultures, the response to a set of 6 prototypical CYP inducers and inhibitors was similar in both culture formats (265). Taken together, these studies provide proof-of-concept that PHH in 3D spheroid culture may have enhanced phenotypes and functionalities, whereas thorough evaluation of their responses to drugs has not been performed.

Microfluidic liver-on-a-chip approaches open up the possibility to create highly organized hepatic microenvironments with tightly controllable dynamic flow conditions (266, 267). A

3D-configured microfluidic chip was recently constructed that enables configuring four relevant murine liver cell types (*i.e.* hepatocytes, stellate, Kupffer, and endothelial cells) into liver sinusoidal structures. This model was found suitable to study primary immune responses seen by neutrophil recruitment in the chip upon lipopolysaccharide (LPS) stimulation (268). Also promising is the construction of multiple organs-on-a-chips that allow studying delicate interactions between the liver and other organs. Interesting examples include, amongst others, a gut-liver chip to reproduce the first pass metabolism of drugs (269, 270), a liver-pancreatic islet chip as a type 2 diabetes mellitus model (271), a liver-kidney chip for assessment of drug nephrotoxicity dependent on hepatic metabolism (272), a skin-intestine-liver-kidney chip for long-term systemic drug toxicity testing (273), an intestine-liver-cancer-connective tissue chip for evaluating anti-cancer drug therapies (274), and an impressive proof-of-concept of 13-organs-on-a-chip (275). The integration of 3D printing into these microfluidic chips is expected to further facilitate the establishment of micro-livers *in vitro*, since cells and ECM can be assembled in a layer-by-layer process and the spatial distributions of materials and cells can be tightly controlled in an automated manner (276-279). The downscaling enabled by these innovative technologies substantially reduces the number of cells needed, though also makes it more complex to control the culture environment. Furthermore, many of the microfluidic chips to date have relied on hydrophobic poly(dimethylsiloxane) (PDMS) scaffolds, although these can bind to lipophilic drugs and their metabolites which confounds drug exposures (280). In addition, the associated costs are high and the complexity of these systems affect the throughput and the ability to perform diverse biochemical analyses, limiting their current use in drug development (281, 282).

An alternative source of liver cells that could overcome certain limitations of PHH (*e.g.* shortage of donors, originating from diseased livers, and the inability to be expanded *in vitro* (283)) are hepatocyte-like cells (HLCs) derived from induced pluripotent stem cells (iPSCs) (284, 285). Any somatic tissue can in principle be used to generate iPSCs and hence this enables retrospectively identifying underlying genetic risk factors that confer a patient susceptible towards idiosyncratic DILI. In addition, HLCs may be valuable to obtain a better understanding of disease processes and allow the potential identification of new therapeutic interventions (286, 287). However, with the current differentiation protocols HLCs do not reach mature hepatocyte phenotypes (288, 289). Instead, they display immature, fetal-like characteristics and expression of DMEs and activities of CYPs are drastically lower in comparison to PHH (290-293) and high expression of alpha-fetoprotein (AFP), a fetal hepatocyte biomarker, is frequently observed in these cultures (294). Like other liver cells, when maintained in 3D culture, the phenotypes of HLCs are improved, as seen by enhanced expression of DMEs, increased CYP activities, improved CYP induction responses, and formation of bile canalicular networks (295, 296). Recent studies showcased the utility of HLCs to assess inter-individual differences regarding CYP metabolism (297) and drug toxicity sensitivity (298, 299). Furthermore, their value in studying the mechanisms underlying idiosyncratic DILI was recently demonstrated. HLCs derived from iPSCs

generated from patients presenting with pazopanib-induced hepatotoxicity displayed greater sensitivity towards the toxicity of this drug *in vitro* compared to HLCs from patients insensitive to pazopanib hepatotoxicity (300).

Collectively, it is evident that complex organotypic liver models are a major research interest and consequently the progress in the field is rapid. Ample evidence has been provided that the physiology and functionality of liver cells cultured in these systems more closely resemble the liver *in vivo*. However, most studies have employed human liver cell lines or primary rat hepatocytes as cell sources. While HLCs derived from iPSCs are a promising avenue, improved differentiation protocols are needed and as such primary human liver cells remain the preferred cell source to model the human liver *in vitro*. Comprehensive characterization of primary human liver cells with regard to their molecular phenotypes and functionalities as well as their drug responses in these complex 3D culture systems is awaited.

2 AIMS

The overall aim of this thesis was to develop and characterize a novel *in vitro* 3D spheroid culture system comprised of PHH for assessments of drug metabolism and hepatotoxicity.

The specific aims were to:

I. Study the early changes of the dedifferentiation process of PHH in 2D monolayer culture

II. Characterize the molecular phenotypes and functionalities of PHH in 3D spheroid culture and assess the long-term stability

III. Assess the suitability of PHH 3D spheroid cultures as a screening model for DILI, with emphasis on reactions that may be delayed in onset

IV. Assess the suitability of PHH 3D spheroid cultures to detect and study drug-induced cholestasis

V. Study the differences between PHH in sandwich and 3D spheroid culture for prediction of drug-induced cholestasis

VI. Assess the suitability of PHH 3D spheroid cultures to detect the liability of drugs to induce CYP3A4

3 RESULTS AND DISCUSSION

3.1 ALTERATIONS OF THE LIVER ARCHITECTURE MAJORLY IMPACT ON HEPATOCYTE PHENOTYPES *IN VITRO* (STUDIES I AND II)

PHH rapidly lose their liver-specific functionalities when cultured as 2D monolayers *in vitro* due to the hepatocytes undergoing dedifferentiation (213, 214). This greatly hampers the ability to accurately study liver biology as well as to predict drug responses. In-depth characterization of this dedifferentiation process is needed to formulate strategies to improve hepatic phenotypes *in vitro* and to increase the overall understanding of hepatocyte plasticity.

In **study I**, we characterized the dedifferentiation process of PHH during the first 24 hours of cultivation as 2D monolayers using comprehensive transcriptomic and proteomic analyses. In this period, 4,042 differentially expressed transcripts were identified (Fig. 1A). The transcriptional changes could be grouped into an early (< 4 hours) and late (> 16 hours) response. Pathway analyses of the differentially expressed genes and proteins revealed early changes in immunity and energy balance, whereas changes in major metabolic pathways occurred later. Already 16 hours after cultivation, changes in the expression patterns of important ADME genes were observed, including downregulation of various *CYPs* and *NRs* (Fig. 1B).

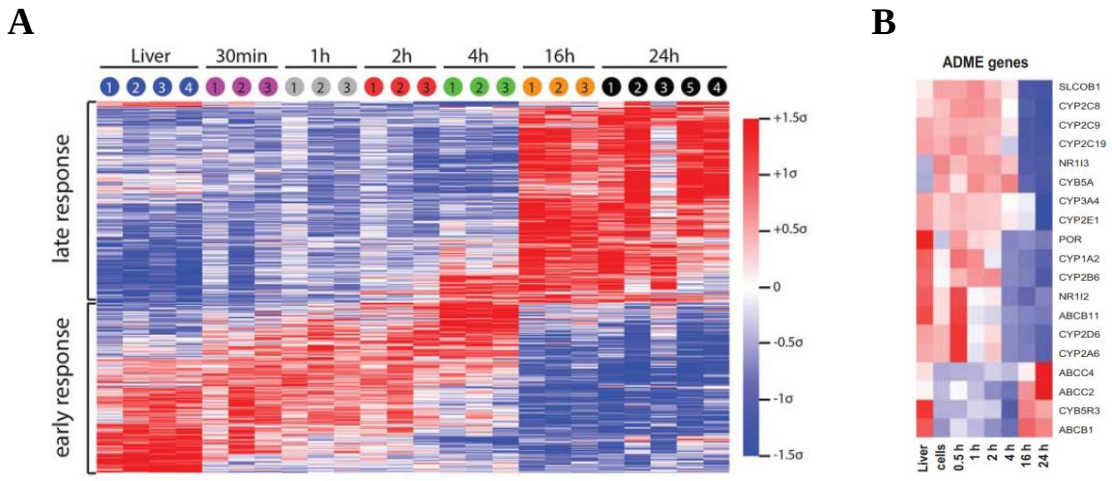

Figure 1. PHH rapidly dedifferentiate within the first 24 hours in 2D monolayer culture**. (A)** Heatmap showcasing differentially expressed genes in PHH from five different donors (indicated by number). **(B)** Heatmap showcasing average expression patterns of ADME genes in PHH from three different donors. Reproduced from paper I.

We also observed that changes in the expression of non-coding RNAs preceded the changes in the expression of coding RNAs. Additional mechanistic investigations revealed an important role for microRNAs in driving hepatocyte dedifferentiation. The results from **study I**, as well as other studies (214, 301), clearly demonstrate that in 2D monolayer culture PHH rapidly lose their mature phenotype. The reduced physiological relevance of the 2D monolayer culture format is thought to be a key reason for the rapid dedifferentiation of PHH.

Current efforts are therefore focused on better mimicking the structural organization of the liver *in vivo* using diverse 3D culture strategies. Preliminary indications suggest that PHH in 3D spheroid culture may have improved phenotypes, but extensive characterizations had not been performed to date.

In **study II** we aimed to comprehensively characterize 3D spheroid cultures of PHH with regard to molecular phenotypes and drug responses. Because the 96-well Gravity PLUS^{TM} Hanging Drop System had previously been successfully used to generate multi-liver cell type spheroids (263), we first employed this platform to generate 3D spheroid cultures of PHH. However, despite extensive optimization efforts, this system was not deemed feasible in our hands, amongst others due to the technique being extremely laborious and recurrent batch-to-batch variations of the plates affecting data reproducibility. Therefore, we employed Corning® Costar® 96-well Ultra-Low Attachment plates for spheroid formation. These plates are coated with a hydrophilic, neutrally charged hydrogel that prevents cells from attaching and forces them in suspension, thereby allowing spontaneous aggregation of the cells into spheroids. Using this platform, PHH robustly formed a single spheroid per well of consistent size (1,500 cells/well, ~200 µm diameter) after 4-5 days in culture and were deemed mature from day 7 (Fig. 2).

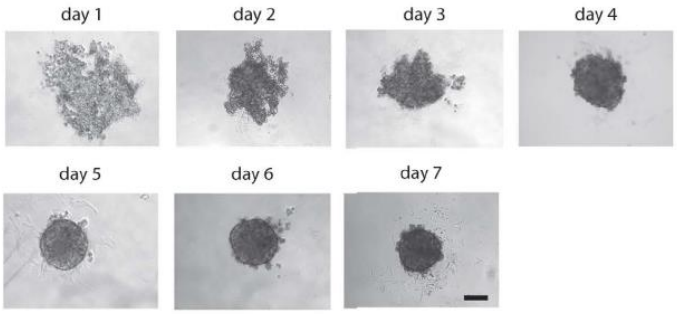

Figure 2. PHH spontaneously self-aggregate into compact 3D spheroid structures during the first week of culture. Scale bar = 100 µm. Reproduced from paper II.

We then compared the proteomes of PHH in 3D spheroid culture (day 7 after seeding when spheroids had formed) to 1) the proteomes found in the human liver *in vivo* and 2) the proteomes of PHH when cultured as 2D monolayer after short-term (24 hours) and long-term (7 days) culture. The proteomes of PHH in 3D spheroid culture closely clustered together with the proteomes of the liver *in vivo*, whereas the proteomes of PHH in 2D monolayer culture were already drastically different after 24 hours (Fig. 3A). In line with the findings from **study I**, pathway analyses revealed major changes in metabolic pathways in PHH 2D monolayer culture, whereas few changes were observed in PHH 3D spheroid culture. Furthermore, using whole proteome analyses we found that PHH in 3D spheroid cultures retained their inter-individual variability, since 3D spheroid cultures from PHH clustered more closely together with the respective liver of origin than with the livers or 3D spheroid cultures from PHH from other donors (Fig. 3B).

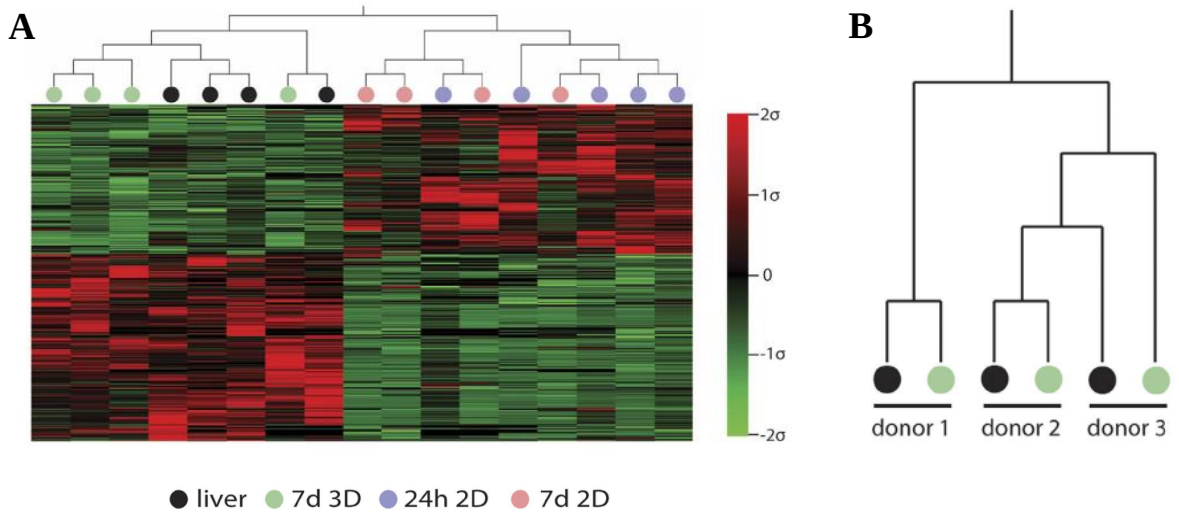

Figure 3. PHH in 3D spheroid culture, but not in 2D monolayer culture, closely resemble the human liver *in vivo* at the proteome level. **(A)** Heatmap showcasing differentially expressed proteins (n = 574). **(B)** Whole proteome analyses of PHH in 3D spheroid cultures and the respective liver of origin. Reproduced from paper II.

3.2 MOLECULAR PHENOTYPES OF PHH ARE STABLY MAINTAINED IN 3D SPHEROID CULTURE FOR MULTIPLE WEEKS (STUDY II)

Having confirmed that PHH in 3D spheroid culture closely reflect the human liver *in vivo*, we investigated in **study II** the stability of the system during long-term culture regarding morphology, viability, and liver-specific functionalities. H&E staining demonstrated long-term preservation of spheroid morphology, though spheroids were noticeably smaller at day 35 compared to day 8 (Fig. 4A). This was associated with both a gradual increase in spheroid compaction as well as a small measure of continuous cell death. Using three different donors, we found that PHH in 3D spheroid culture could be maintained viable for at least four weeks after aggregation. Albumin secretion, an important liver-specific function, was stable during this period too, with a noticeable decline only at day 35. Interestingly, we found evidence of hepatic zonation patterns in the PHH spheroids, seen by the marginal overlap of CYP3A4 and albumin protein stainings (Fig. 4B). CYP3A4 is predominantly expressed in the perivenous region of the liver, whereas albumin is predominantly present in the periportal region (302). We hypothesize that different hepatocytes within a spheroid retain the phenotype and functionality according to which area in the liver they initially originated from.

Reactive drug metabolite formation is considered an important mechanism of DILI (303). Hence, a reliable DILI screening model should amongst others display activity of important DMEs. Results from **study II** indicate that metabolic activities of CYP1A2, CYP2C9, CYP2D6, and CYP3A4 remain stable during four weeks of culture relative to the activities observed in PHH spheroids at day 8. Metabolic activity of CYP2C8 gradually decreased over time; the reason for this decline remains to be elucidated, though we consistently observed this trend with 3D spheroid cultures from different PHH donors, excluding inter-donor variability as the cause. Notably, remarkable variations in CYP activity between single spheroids formed from the same PHH donor were observed, indicating a certain degree of

heterogeneity of the system. This could be resulting from different ratios of perivenous *vs.* periportal hepatocytes within different spheroids, transdifferentiation of the cells, or variable oxygen levels across the culture plate.

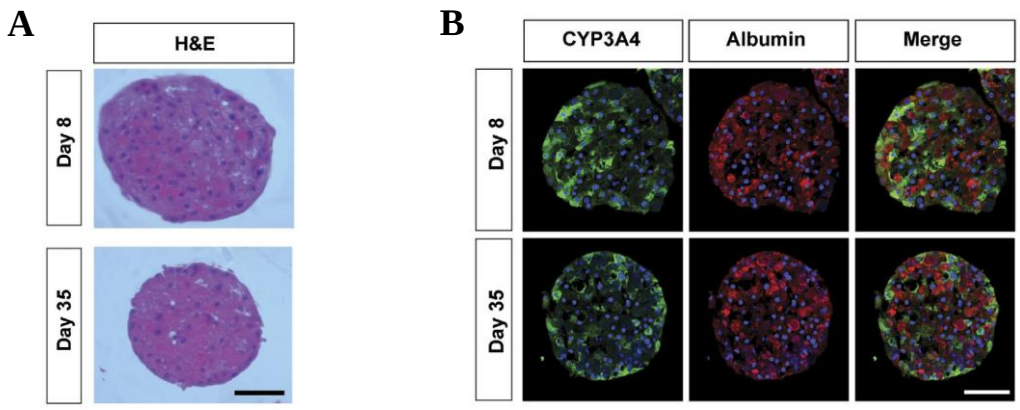

Figure 4. PHH spheroids have stable phenotypes during four weeks of culture after aggregation. **(A)** H&E staining of spheroid morphology. **(B)** Immunohistochemical analysis of CYP3A4 and albumin protein expression. Scale bars = 100 µm. Reproduced from paper II.

3.3 PHH SPHEROIDS ARE A SUITABLE SYSTEM TO SCREEN FOR DRUG-INDUCED LIVER INJURY (STUDIES II AND III)

There is currently an unmet but urgent need for novel preclinical DILI screening models. Since PHH in 3D spheroid culture possess *in vivo*-relevant phenotypes which can be stably maintained for several weeks, we assessed the potential of the system to retrospectively identify drugs with reports of causing liver injury *in vivo*. Since it is well known that DILI may be delayed in onset we specifically focused in **study II** on assessing the differences in sensitivity between acute (single 48 hour exposure) and chronic treatment (7 or 28 days of repeated exposure). Strikingly, the sensitivity of PHH in 3D spheroid culture towards the toxicity of all five hepatotoxins tested (amiodarone, bosentan, diclofenac, fialuridine, and tolcapone) markedly increased upon prolonging the drug exposures. While EC_{50} values for all drugs except tolcapone exceeded $30xC_{max}$ levels after a single 48 hour exposure, they decreased to $1\text{-}30xC_{max}$ levels (amiodarone, bosentan, and diclofenac) or below $1xC_{max}$ (fialuridine and tolcapone) after 7 days of repeated exposure. A further decrease in the EC_{50} values of all drugs was observed after 28 days of exposure (Fig. 5A). Similar results were obtained in **study III**, where a decrease in the EC_{50} values of all hepatotoxins tested (*i.e.* acetaminophen, bosentan, chlorpromazine, tetracycline, and troglitazone) was observed when the exposures were extended from 8 to 14 days.

An interesting finding of **study II** was the remarkable toxicity pattern of fialuridine upon prolonged exposure. A single 48 hour exposure at concentrations up to $100xC_{max}$ levels (C_{max} = 1 µM) did not induce any toxicity. However, when PHH spheroids were repeatedly exposed for 7 or 28 days, fialuridine toxicity could be clearly detected at physiologically relevant concentrations (EC_{50} = 0.1 µM at 28 days) (Fig. 5B). Hepatotoxicity exerted by

fialuridine appeared only after multiple weeks of treatment *in vivo* (189), presumably due to the gradual depletion of mitochondrial DNA leading to mitochondrial structural defects and lipid accumulation in hepatocytes (304, 305). This is an exemplar case of DILI that is delayed in onset. Such toxicity manifestations would likely be missed during the preclinical phase of development, as most currently used *in vitro* DILI screens are focused on acute drug toxicity assessments using a single exposure. Our findings therefore strongly argue to also assess the risk of delayed onset DILI events using a physiologically relevant and stable system, such as the PHH spheroid system presented here.

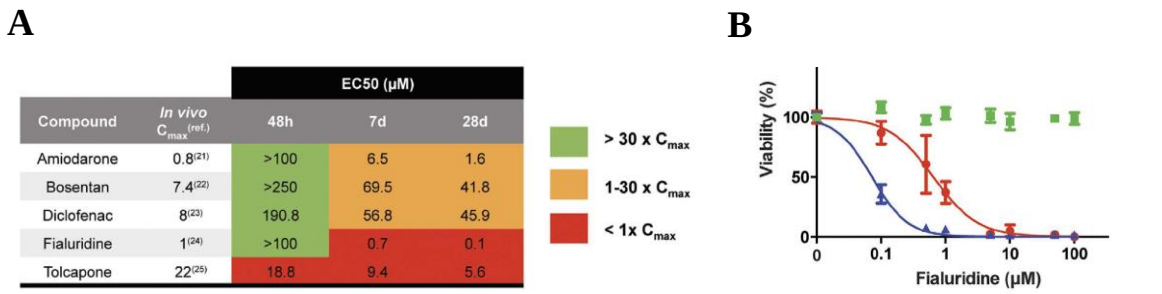

Figure 5. PHH spheroids are a suitable platform to assess DILI events that may be delayed in onset. **(A)** EC_{50} values of five hepatotoxins observed in PHH spheroids after a single 48 hour exposure or repeated exposures for 7 or 28 days. **(B)** Toxicity profile of fialuridine in PHH spheroids after 48 hours (green), 7 days (red), and 28 days (blue). Reproduced from paper II.

3.4 CLINICAL PATTERNS AND MECHANISMS OF DRUG-INDUCED LIVER INJURY ARE REFLECTED IN PHH SPHEROIDS (STUDIES II AND III)

The clinical phenotypes of DILI are extremely heterogeneous and may mimic virtually all primary liver diseases. In **studies II** and **III** we assessed whether specific patterns of DILI, including drug-induced steatosis and cholestasis, could be identified in the PHH spheroid system. In **study II**, we observed that upon a single 48 hour exposure to cyclosporine A (CsA), known to induce steatosis *in vivo* (306), neutral lipids were rapidly enriched in PHH spheroids. Interestingly, co-exposure to the antioxidant α-tocopherol (α-TOH) prevented the CsA-induced lipid accumulation (Fig. 6). This is in agreement with previous reports that antioxidants may have a beneficial role in mitigating CsA-induced liver injury (307, 308). Non-alcoholic fatty liver disease (NAFLD) is a major cause of liver disease worldwide and its prevalence is rapidly increasing (309). Yet, currently no FDA-approved drug therapies exist for the treatment of non-alcoholic steatohepatitis (NASH), the more aggressive form of NAFLD (310). These preliminary findings suggest that the PHH spheroid system could be used to study hepatic steatosis, which if further characterized may be used as a model to screen for compounds that prevent, inhibit the progression, or reverse steatosis.

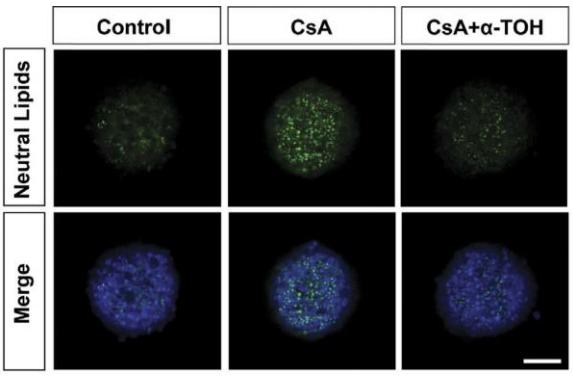

Figure 6. PHH spheroids reflect patterns of drug-induced steatosis. Cyclosporine A (CsA, 30 µM) rapidly induces neutral lipid accumulation after a single 48 hour exposure, a process that is prevented in the presence of the antioxidant α-tocopherol (α-TOH, 10 µM). Reproduced from paper II.

In order to constitute a relevant system for cholestatic liver disease, the presence of bile canalicular networks with functional bile acid (BA) transporters is essential. Accordingly, in **study III** we evaluated the protein expression of multi-drug resistance protein 2 (MRP2) and the bile salt export pump (BSEP), two major canalicular BA transporters whose functional inhibition is often associated with drug-induced cholestasis (311), and evaluated their responsiveness towards a mixture of five human BAs (Fig. 7A). MRP2 was abundantly expressed in PHH spheroids, confirming our findings from **study II** which indicated stable MRP2 expression in PHH spheroids during four weeks of culture after aggregation. The basal expression of BSEP was low but could accordingly be induced by the BA mixture (30x concentrated compared to average human plasma levels), presumably through activation of the farnesoid X receptor (312).

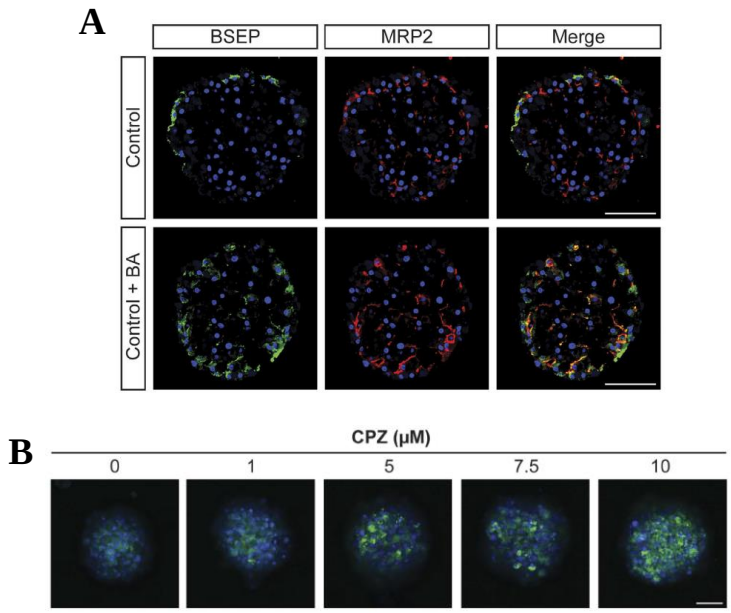

Figure 7. PHH spheroids can be used as a model for drug-induced cholestasis. **(A)** Immunohistochemical analysis of MRP2 and BSEP protein expression in the presence and absence of a 30x concentrated BA mixture for 8 days. **(B)** Assessment of BA accumulation using the fluorescently-labelled BA derivative tauro-nor-THCA-25-DBD upon exposure to increasing chlorpromazine (CPZ) concentrations after 8 days of repeated treatment. Scale bars = 100 µm. Reproduced from paper III.

We then focused on recapitulating patterns of drug-induced cholestasis in the PHH spheroid system using the model cholestatic compound chlorpromazine (CPZ) (313). Repeated exposure to CPZ for 8 days induced a dose-dependent accumulation of the fluorescently-labelled BA derivative tauro-nor-THCA-25-DBD (Fig. 7B). Further mechanistic investigations revealed that CPZ inhibits *ABCB11* mRNA expression (encoding BSEP) and disrupts the F-actin cytoskeleton which is of importance for proper insertion of BA transporters. These data are in line with previous observations in HepaRG cells (314). Interestingly, when PHH spheroids were exposed to CPZ in the presence of the BA mixture, patterns indicative of a synergistic increase in oxidative stress and BA toxicity were observed, seen by induction of mRNA expression of the Nrf2 target sulfiredoxin 1 (*SRXN1*) (315) and death receptor 5 (*DR5*) (316), respectively. The proposed mechanisms of CPZ-mediated cholestatic hepatotoxicity are illustrated in Fig. 8.

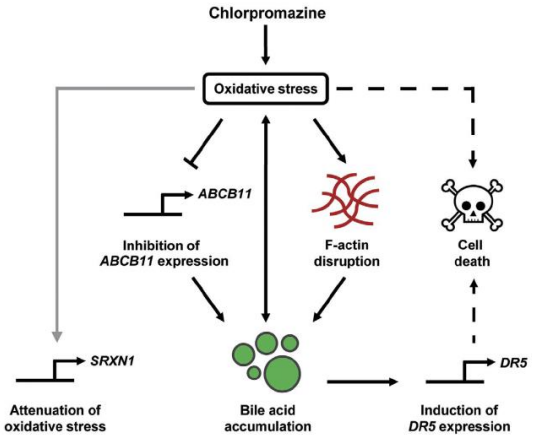

Figure 8. Currently identified mechanisms associated with chlorpromazine (CPZ)-induced cholestatic hepatotoxicity. Oxidative stress is rapidly observed upon CPZ exposure which has been associated with the disruption of the F-actin cytoskeleton (314). In combination with the inhibition of *ABCB11* mRNA expression, BAs accumulate at toxic levels which pose additional oxidative stress and likely activate death receptor signaling, evident from the induction of death receptor 5 *(DR5)* mRNA expression. Reproduced from paper III.

3.5 PHH SPHEROIDS CAN IDENTIFY THE LIABILITY OF DRUGS TO INDUCE CHOLESTATIC LIVER INJURY (STUDIES III AND IV)

Cholestatic and mixed hepatocellular/cholestatic liver injuries are two serious clinical manifestations of DILI with high incidence rates (317). Currently, preclinical prediction of the liability of drug candidates to induce cholestasis is mainly aimed at assessing the potency to inhibit BSEP, which is achieved using either membrane vesicles or sandwich-cultured hepatocytes (318). However, the underlying mechanisms may be more complex and likely involve a multitude of players mediating BA homeostasis including NRs, BA conjugating enzymes, and other BA transporters (311). Furthermore, drug-induced cholestasis is often delayed in onset and subject to adaptive responses, making it challenging to predict (319). Therefore, there is a need for models that assess the risk for drug-induced cholestasis in a holistic manner with a focus on capturing toxicity events that may be delayed in onset.

In **study III** we assessed whether PHH spheroids could represent a suitable model to screen for the liability of drugs to induce cholestasis. To this end, we used a drug and concentrated BA mixture co-exposure strategy that was previously successfully employed in hepatocyte sandwich cultures (320). The risk of cholestasis is determined based on the drug's ability to interfere with the disposal of an added BA mixture that is otherwise non-toxic to the cells. We observed a synergistic increase in toxicity upon co-exposure to a 30x concentrated BA mixture for hepatotoxins reported to be associated with cholestasis *in vivo* (bosentan, chlorpromazine, and troglitazone) (317), while this was not the case for the non-cholestatic hepatotoxins tested (acetaminophen and tetracycline). This indicates that the cholestatic drugs exclusively interfere with the disposal of the added BAs resulting in an increase in toxicity, a phenomenon that was more pronounced when the exposures were prolonged from 8 to 14 days (Fig. 9A-B). Most notably, the cholestatic liability of chlorpromazine was only evident after 14 days of repeated exposure. Further validation of the model with 7 cholestasis-positive and 4 cholestasis-negative hepatotoxins in 3D spheroid cultures formed from a pool of 10 PHH donors resulted in the correct classification of the cholestatic risk of all drugs after 14 days of exposure with the exception of ticlopidine. The risk for developing ticlopidine-induced cholestasis has been associated with an idiosyncratic immune-mediated component (321), which evidently cannot be captured in the PHH spheroid system.

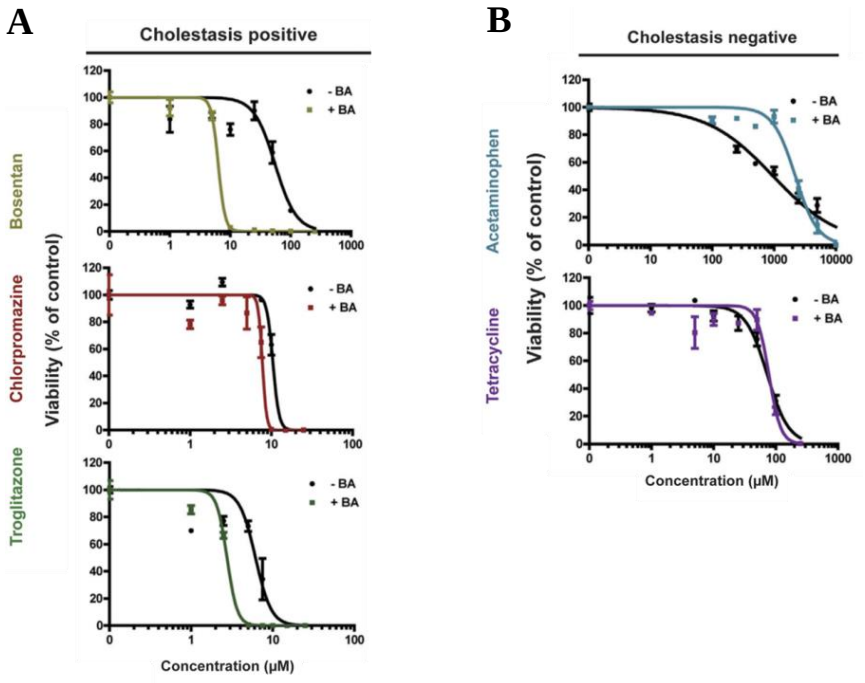

Figure 9. PHH spheroids can identify the liability of drugs to induce cholestasis after 14 days of repeated exposure. **(A)** Increased toxicity in the presence of an otherwise non-toxic 30x concentrated BA mixture is selectively observed for hepatotoxins with reports of causing cholestasis *in vivo* (bosentan, chlorpromazine, and troglitazone), **(B)** but not for hepatotoxins with no such reports (acetaminophen and tetracycline). Reproduced from paper III.

Since PHH sandwich cultures are considered the preferred model for studies of hepatobiliary transport and cholestasis (233), we sought to compare the PHH spheroid system to the current benchmark for assessing drug-induced cholestasis. The findings in **study IV** indicate that PHH sandwich cultures are indeed a robust model to detect the cholestatic risk of compounds, though remarkable differences between different PHH donors were apparent with regard to their sensitivity towards the cholestatic toxicity of certain drugs (*i.e.* bosentan, chlorpromazine, and troglitazone). Interestingly, a direct comparison of sandwich cultures and 3D spheroid cultures from the same PHH donor revealed that prolonged exposures in 3D spheroid culture could increase the sensitivity to detect the cholestatic risk of certain compounds. Chlorpromazine showed no cholestatic liability after a single 48 hour exposure in PHH sandwich cultures, nor after 72 hours exposure in 3D spheroid cultures of the same PHH donor. However, the synergistic toxicity of chlorpromazine and BAs could be unraveled when the exposures were extended in 3D spheroid culture, with the most pronounced effect visible after 14 days (Fig. 10A-C). The implications of this observation remain to be further investigated as the sandwich-3D spheroid comparison was only performed with one PHH donor, since other PHH donors either did not aggregate into spheroids or could not be stably maintained for extended culture periods. Additional comparative studies are needed to comment on the suitability and sensitivity of both systems for the prediction and study of drug-induced cholestasis.

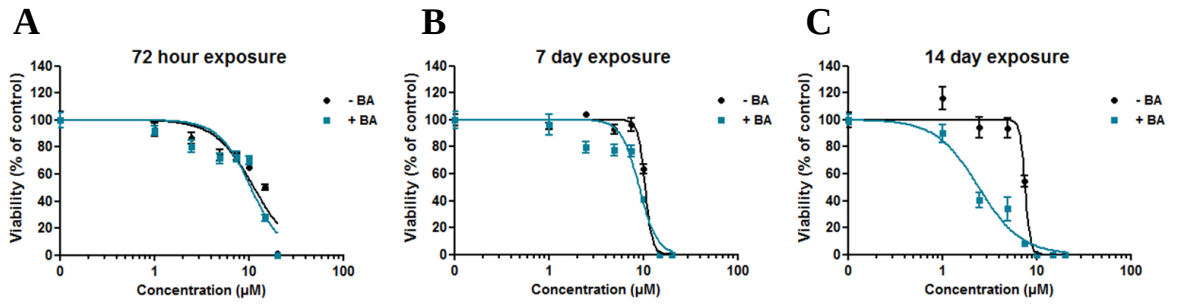

Figure 10. The cholestatic liability of chlorpromazine becomes increasingly apparent when the BA co-exposures are prolonged in 3D spheroid cultures of PHH. After 72 hours **(A)** and 7 days **(B)** no clear indications are evident, whereas after 14 days **(C)** chlorpromazine and BAs clearly pose synergistic toxicity. Adapted from manuscript IV.

3.6 CLINICALLY RELEVANT CYP3A4 INDUCTION EVENTS ARE DETECTED IN PHH SPHEROIDS (STUDY V)

CYP3A4 is involved in the metabolism of many clinically used drugs (141). Changes in its enzymatic activity, *e.g.* via induction, can therefore have detrimental outcomes including DDI-mediated therapeutic failures or development of ADRs. As such, early assessment of the liability of drug candidates to induce CYP3A4 during the preclinical phase of development is crucial. PHH 2D monolayer cultures are often the preferred model for CYP induction studies. However, the applicability of this model is questionable, as we previously observed in **study I** that PHH in 2D monolayer culture rapidly dedifferentiate and that expression of *NRs*,

important for mediating CYP expression, is drastically decreased within 24 hours. Therefore, in **study V** we examined whether the PHH spheroid system could constitute a more relevant and sensitive model to predict drug-mediated CYP3A4 induction. Induction of *CYP3A4* mRNA expression of a panel of 11 drugs with reports of inducing CYP3A4 activity *in vivo* could be clearly detected in PHH spheroids at physiologically relevant concentrations (at or below $1xC_{max}$) and with magnitudes comparable to those found *in vivo*. In contrast, no induction of *CYP3A4* mRNA expression was observed for a set of 13 drugs with no reports of inducing CYP3A4 *in vivo* (Fig. 11A-B). Applying the recommendations issued by the EMA to classify the liability of drugs to induce CYPs (322), the PHH spheroid system impressively achieved 100% sensitivity and 100% specificity.

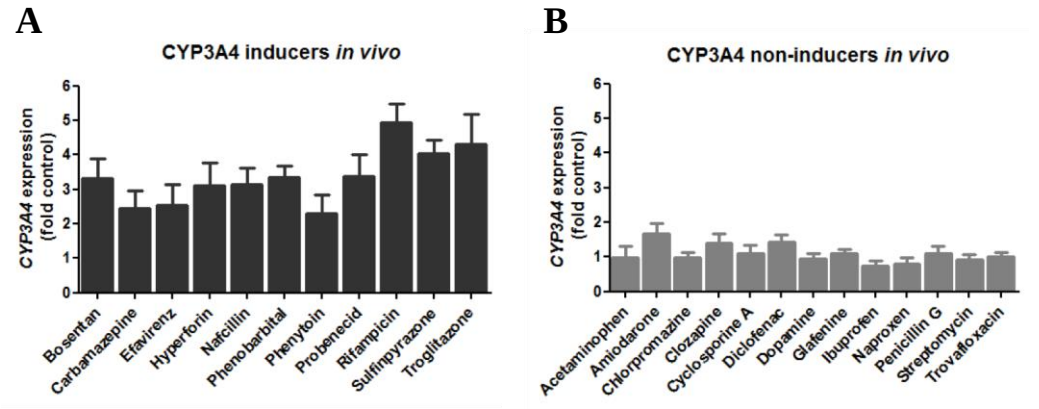

Figure 11. PHH spheroids correctly detect clinical CYP3A4 induction. **(A)** *CYP3A4* mRNA expression is induced upon exposure to drugs reported to induce CYP3A4 activity *in vivo*, **(B)** whereas this is not the case upon exposure to drugs not known to induce CYP3A4 *in vivo*. All drugs were screened at $1xC_{max}$ levels, except probenecid ($0.2xC_{max}$). Reproduced from manuscript V.

A crucial finding of this study was that PHH only when maintained in 3D spheroid culture, but not in 2D monolayer culture, could detect the CYP3A4 induction capacity of AZD1208 (at $0.2xC_{max}$) on mRNA and protein level at levels which were comparable to the magnitude of change observed for the prototypical inducer rifampicin (Fig. 12A-B). AZD1208 had eluded preclinical CYP3A4 induction screens employing HepaRG cells and 2D monolayer cultures of PHH from three different donors, but showed unexpected CYP3A4 auto-induction in the clinic leading to its termination (140). We hypothesized that AZD1208 induces CYP3A4 via an indirect mechanism rather than direct NR ligand binding since the latter mechanism can normally be identified in PHH 2D monolayer culture. Using gene knockdown experiments, we found that AZD1208 requires PXR and is partially dependent on CAR to exert its CYP3A4 induction. Preliminary indications suggest that ERK signaling is involved in this indirect pathway leading to CYP3A4 induction, which appears to be sensitive towards the presence of epidermal growth factor (EGF). Not surprisingly, ERK signaling is drastically altered in PHH 2D monolayers during hepatocyte dedifferentiation (212) which could explain the absence of AZD1208-mediated CYP3A4 induction in 2D monolayer culture. Collectively, our data suggest that the PHH spheroid system can robustly detect drug-

mediated CYP3A4 induction at clinically relevant concentrations, and as such may constitute a valuable novel preclinical model to screen drug candidates for CYP3A4 induction.

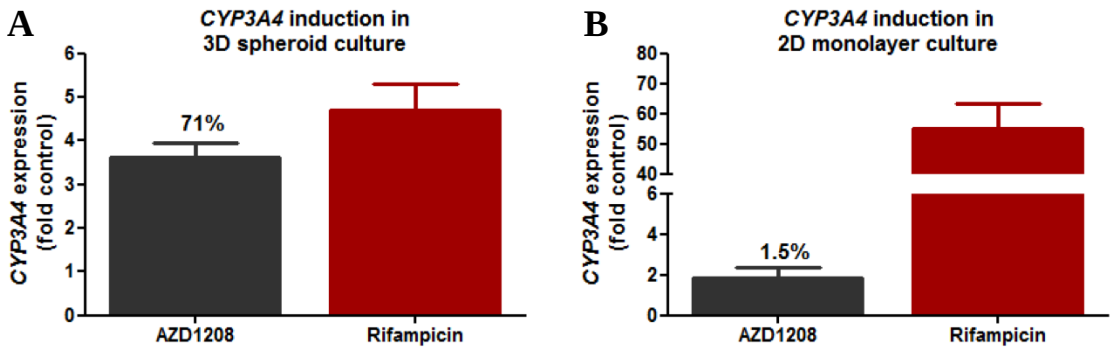

Figure 12. Detection of AZD1208-mediated CYP3A4 induction in PHH is dependent on the culture format. **(A)** PHH in 3D spheroid culture, **(B)** but not in 2D monolayer culture, can detect AZD1208-mediated induction of *CYP3A4* mRNA expression at levels comparable to rifampicin. Percentages indicate the extent of induction relative to rifampicin. Reproduced from manuscript V.

4 SUMMARY

The findings in this thesis can be summarized as follows:

- PHH dedifferentiation in 2D monolayer culture is a rapid process involving major changes on transcript and protein level during the first 24 hours, limiting the *in vivo* relevance of this model (**study I**).

- In the 3D spheroid system developed here PHH are cultured under serum-free and defined chemical conditions. Unlike in 2D monolayer culture, PHH in 3D spheroid culture possess relevant hepatic phenotypes comparable to those found in the human liver *in vivo* and inter-individual variability can be retained (**study II**).

- PHH in 3D spheroid culture remain viable, form bile canalicular networks, and have stable liver-specific functionalities for several weeks in culture. These features allow screening for drug responses and toxicity events that may be delayed in onset (**study II**).

- PHH spheroids can be used to identify the liability of compounds to induce cholestasis and to study mechanistic aspects thereof. The PHH spheroid system may have additional sensitivity over PHH sandwich cultures due to the ability to assess chronic drug-induced cholestasis events (**studies III** and **IV**).

- The propensity of drugs to induce CYP3A4 can be accurately assessed in PHH spheroids at clinically relevant concentrations. PHH in 3D spheroid culture, but not in 2D monolayer culture, are able to detect an atypical mechanism of CYP3A4 induction of clinical importance (**study V**).

5 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Unexpected adverse hepatic drug events are a significant threat to patient safety and negatively impact on the productivity of the pharmaceutical industry. If liver safety concerns are not recognized in the preclinical stage of drug development, human safety is put at risk. DILI is a major cause of post-marketing drug withdrawals and restricted-use warnings and is one of the major reasons for drug attrition during the clinical phases of development. Hence, it is clear that a translational gap between preclinical predictions of drug behavior and clinical outcomes currently exists.

Post-marketing DILI events predominantly occur due to idiosyncratic reactions, often only occurring in isolated cases and several years post-marketing. Inherent to its definition, the nature of idiosyncratic type of drug reactions is multifaceted, requiring a complex interplay between various risk factors including drug properties, the environment, and genetic and non-genetic factors of the patient. As such, it is highly unlikely that the risk for idiosyncratic liver injury events is recognized during drug development which in its current state merely aims to estimate the risk of ADR occurrence in the general population.

A clear paradigm shift from a one-size-fits-all strategy to a new era of personalized medicine is ongoing where pharmacogenetic testing is slowly gaining appreciation in the clinic (323). In recent years, GWAS analyses have associated some common genetic variants with idiosyncratic DILI (*e.g.* certain *HLA* alleles). However, when used to prospectively predict patient-specific liver injury risks prior to drug prescription, these tests would have a high negative predictive value, yet a low positive predictive value due to the low incidence of idiosyncratic DILI (324). Instead, such tests serve more value as a diagnostic tool where liver injury patterns can be linked to particular drugs, thereby allowing effective continuation of other co-medicated drugs (325). Unless it is made possible to comprehensively assess patient-specific drug responses *in vitro* using suitable patient's material, it is unlikely that the majority of idiosyncratic drug reactions will be predictable prior to prescription.

Adverse liver safety profiles during the clinical phase of drug development often appear in several subjects. This implies that the mechanism of hepatotoxicity is intrinsic in nature and thus in theory should have been predictable, highlighting the ongoing existence of a translational gap between preclinical liver safety prediction and clinical outcome. Animal models lack predictive power due to important inter-species differences, exemplified by various cases of unexpected toxicities in the clinic with serious injuries or fatalities as a result (*e.g.* fialuridine (189), TGN1412 (326), and BIA 10-2474 (327)). Therefore, contemporary research places much emphasis on developing novel human-based *in vitro* models to improve the preclinical drug toxicity testing strategies (24).

PHH in 2D monolayer cultures have long been the preferred *in vitro* system to model the human liver. However, there is an increased recognition that in this culture format PHH rapidly lose their mature phenotype due to dedifferentiation (214). The results from this thesis

substantiate this, where we observed that PHH in 2D monolayer culture very rapidly lose the expression of crucial hepatic genes. Consequently, accurately predicting clinical drug metabolism and toxicity profiles is impeded. It is now recognized that a major reason for the rapid decline of hepatocyte functionality is the lack of physiological relevance of such simple 2D culture systems. The liver *in vivo* has a complex 3D organization where hepatocytes are highly polarized which is of vital importance for proper hepatocyte functionality. Accordingly, various novel 3D culture platforms have been proposed to create more physiologically relevant *in vitro* liver models, ranging in complexity from sandwich cultures to complex microfluidic chips, each with unique advantages and shortcomings.

To constitute a suitable model to be implemented in drug development, several characteristics should be considered. These include the robustness, versatility, throughput, and cost-effectiveness of the system. In this regard, 3D spheroid cultures generated in multi-well plates constitute an attractive approach. In this thesis, we developed and characterized a scaffold-free 96-well PHH spheroid system, using only 1,500 cells per well to generate a single spheroid of a defined size. In contrast to a previously constructed multi-liver cell type spheroid system where media conditions were not disclosed (263, 328), PHH spheroids in our system are maintained in chemically defined and serum-free conditions, thus allowing wide-spread use of this model among researchers and industry.

Our results indicate that the PHH spheroid system is a highly phenotypically relevant *in vitro* model, closely resembling the human liver *in vivo*. Importantly, we found indications that inter-individual variability is retained in PHH 3D spheroid culture, opening up the possibility to study inter-individual differences in drug response and toxicity (329). Because molecular phenotypes and liver-specific functions of PHH are largely stable for multiple weeks in 3D spheroid culture, this system is a valuable model to assess DILI events that may be delayed in onset. This was exemplified by the striking toxicity pattern of fialuridine that required prolonged exposures to exert its toxicity. Furthermore, since PHH form bile canalicular networks in 3D spheroid culture, the system can be used to predict and study chronic drug-induced cholestasis. Recent reports from our lab indicate that the PHH spheroid system could predict DILI with 69% sensitivity and 100% specificity using a panel of 70 DILI-positive and 53 DILI-negative compounds after two weeks of repeated exposure (330). Remarkably, the system outperformed other proposed DILI screening models employing PHH (235, 243), including also the multi-liver cell type spheroid system mentioned earlier (264), despite relying on lower drug concentrations ($20xC_{max}$ compared to $100xC_{max}$). The discrepancy between these two spheroid models might be related to differences in the choice of compounds, inter-donor variabilities, inclusion of NPCs, or differences in media composition. Furthermore, the PHH spheroid system was also recently shown to detect and reflect drug toxicity patterns in a more appropriate manner than other conventionally used models, including 2D cultures of HepaRG cells or HLCs (331), as well as PHH sandwich cultures (332).

In addition to showing promise for assessments of drug hepatotoxicity, the PHH spheroid system may represent a suitable platform to screen drugs for CYP3A4 induction. PHH in 3D spheroid culture stably express important *NRs* and *CYPs* at levels comparable to those found in the liver *in vivo* (333). Furthermore, we found that clinical CYP3A4 induction patterns were accurately reflected at physiologically relevant concentrations. We convincingly showed that 3D spheroid cultivation of PHH is necessary to identify an atypical mechanism leading to CYP3A4 induction. This was exemplified by the detection of AZD1208-mediated induction of CYP3A4 exclusively in PHH 3D spheroid culture, but not in 2D monolayer cultures from the same donor, indicating that activity of signaling pathways of importance for CYP induction are drastically altered upon cultivation of PHH as 2D monolayers. As such, the PHH spheroid system may constitute a promising novel preclinical model to predict CYP3A4 induction liabilities of drug candidates.

While our results are encouraging and provide a step forward in establishing a more relevant *in vitro* human liver system, certain observations and limitations should be noted. We observed significant donor-to-donor variation of PHH fractions with regard to the ability to aggregate into compact spheroids, as well as differences in the long-term viability and stability of hepatic phenotypes. Though not specific to the chosen 3D spheroid culture system, this necessitates extensive initial screenings to find suitable PHH donors that perform well in 3D spheroid culture. Furthermore, in this thesis, we mainly focused on 3D spheroid cultures consisting of solely PHH. While it is true that parenchymal hepatocytes are the main cell type of the liver, hepatic NPCs are known to support hepatocyte function and play crucial roles in certain liver diseases. Our preliminary investigations suggest that spheroid co-cultures of PHH with crude primary hepatic NPC fractions can be generated and that the Kupffer cells are responsive to LPS stimulation. Further characterization of the long-term stability of each cell type in these spheroid co-cultures is needed. It will be tremendously interesting to observe whether complex diseases with crucial roles for hepatic NPCs (*e.g.* NAFLD (334) and fibrosis (335)) can be reflected in such co-cultures. Indeed, 3D spheroid co-cultures of HepaRG cells and hepatic stellate cells were recently presented as a potential drug-induced fibrosis model (336).

While the 3D spheroid culture system employed here has several features attractive for drug development (*e.g.* few cells needed for drug toxicity screenings and a higher throughput compared to other complex systems), perfusion is lacking and as such various physiological parameters cannot be controlled that could be of importance for hepatic functionality. Integration of PHH spheroids into microfluidic chips is therefore considered a promising avenue in which the microenvironment can be more tightly regulated and potential interactions with other organs can be generated. It is anticipated that such organotypic *in vitro* models will increase our understanding of human bodily functions, are of value for disease modelling and screening of novel therapeutic interventions, and may aid in enhancing safety assessments in drug development (337-340).

ACKNOWLEDGEMENTS

There have been many people that have supported me during my PhD studies. I want to express my gratitude to all my colleagues, friends, and relatives. In particular, I would like to say a few words to the following people:

First and foremost, my principal supervisor **Magnus Ingelman-Sundberg**, thank you for accepting me as an internship student and for subsequently enabling me to continue practicing exciting science in your group as your one-to-last PhD student. Your enthusiasm and passion for science are really inspiring.

My co-supervisors **Inger Johansson** and **Souren Mkrtchian**, thank you both for your guidance throughout these years and the fruitful scientific discussions. It was a pleasure to learn from the fresh and different looks on science you both have.

A few words to some important past spheroid girls: **Sabrina Moro**, thank you for getting me started in the lab and being the supervisor of my Master's thesis, I really learned so much from you; **Sarah Sim**, **Catherine Bell**, and **Lisa Fredriksson Puigvert**, thank you all for the pleasant joint efforts in numerous spheroid projects.

And to notable current MIS group members: **Volker Lauschke**, I am really appreciative of your critical input and out-of-the-box thinking; **Åsa Nordling**, thank you for the ever challenging job to keep the lab up and running while simultaneously also being such a caring person; **Mikael Kozyra**, **Yitian Zhou**, and **Nuria Vilarnau**, good luck with your own PhD journeys, I am sure you will all do great; **Sabine Vorrink** and **Tracey Hurrell**, thank you both for making life inside and outside the lab so much more entertaining - I certainly enjoyed all the endless fika times, badminton sessions and travels.

Big thanks also to all my students that have helped with hard work and great input in various research projects: **Sarianna Tuovinen**, **Muriëlle van der Horst**, **Matthew Driban**, **Nahid Baghdadi**, and **Achilleas Fardellas**, my best wishes to your future endeavours.

Finally, thanks and love to my **family** for your unconditional support and believe in me throughout these dynamic years.

REFERENCES

1.Coleman JJ, Pontefract SK. Adverse drug reactions. Clin Med (Lond). 2016;16(5):481-5. Epub 2016/10/05.
2.Juntti-Patinen L, Neuvonen PJ. Drug-related deaths in a university central hospital. Eur J Clin Pharmacol. 2002;58(7):479-82. Epub 2002/10/22.
3.Ebbesen J, Buajordet I, Erikssen J, Brors O, Hilberg T, Svaar H, et al. Drug-related deaths in a department of internal medicine. Arch Intern Med. 2001;161(19):2317-23. Epub 2001/11/09.
4.Cabello AJP, Gavilan ED, Jimenez FJG, Rodriguez CM, Del Castillo JDL, Canas EP. Drug-related mortality among inpatients: a retrospective observational study. European Journal of Clinical Pharmacology. 2016;72(6):731-6.
5.Wester K, Jonsson AK, Spigset O, Druid H, Hagg S. Incidence of fatal adverse drug reactions: a population based study. Br J Clin Pharmacol. 2008;65(4):573-9. Epub 2007/12/12.
6.Pirmohamed M, James S, Meakin S, Green C, Scott AK, Walley TJ, et al. Adverse drug reactions as cause of admission to hospital: prospective analysis of 18 820 patients. BMJ. 2004;329(7456):15-9. Epub 2004/07/03.
7.Kongkaew C, Noyce PR, Ashcroft DM. Hospital admissions associated with adverse drug reactions: A systematic review of prospective observational studies. Annals of Pharmacotherapy. 2008;42(7-8):1017-25.
8.Lazarou J, Pomeranz BH, Corey PN. Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. JAMA. 1998;279(15):1200-5. Epub 1998/04/29.
9.Krahenbuhl-Melcher A, Schlienger R, Lampert M, Haschke M, Drewe J, Krahenbuhl S. Drug-related problems in hospitals: a review of the recent literature. Drug Saf. 2007;30(5):379-407. Epub 2007/05/03.
10.Bouvy JC, De Bruin ML, Koopmanschap MA. Epidemiology of adverse drug reactions in Europe: a review of recent observational studies. Drug Saf. 2015;38(5):437-53. Epub 2015/03/31.
11.Davies EC, Green CF, Taylor S, Williamson PR, Mottram DR, Pirmohamed M. Adverse Drug Reactions in Hospital In-Patients: A Prospective Analysis of 3695 Patient-Episodes. PLoS ONE. 2009;4(2).
12.Sultana J, Cutroneo P, Trifiro G. Clinical and economic burden of adverse drug reactions. J Pharmacol Pharmacother. 2013;4(Suppl 1):S73-7. Epub 2013/12/19.
13.Hazell L, Shakir SA. Under-reporting of adverse drug reactions : a systematic review. Drug Saf. 2006;29(5):385-96. Epub 2006/05/13.
14.Irujo M, Beitia G, Bes-Rastrollo M, Figueiras A, Hernandez-Diaz S, Lasheras B. Factors that influence under-reporting of suspected adverse drug reactions among community pharmacists in a Spanish region. Drug Safety. 2007;30(11):1073-82.
15.Tandon VR, Mahajan V, Khajuria V, Gillani Z. Under-reporting of adverse drug reactions: A challenge for pharmacovigilance in India. Indian Journal of Pharmacology. 2015;47(1):65-71.
16.Lopez-Gonzalez E, Herdeiro MT, Figueiras A. Determinants of Under-Reporting of Adverse Drug Reactions A Systematic Review. Drug Safety. 2009;32(1):19-31.
17.Hughes JP, Rees S, Kalindjian SB, Philpott KL. Principles of early drug discovery. Br J Pharmacol. 2011;162(6):1239-49. Epub 2010/11/26.
18.DiMasi JA, Grabowski HG, Hansen RW. Innovation in the pharmaceutical industry: New estimates of R&D costs. Journal of Health Economics. 2016;47:20-33.
19.Paul SM, Mytelka DS, Dunwiddie CT, Persinger CC, Munos BH, Lindborg SR, et al. How to improve R&D productivity: the pharmaceutical industry's grand challenge. Nature Reviews Drug Discovery. 2010;9(3):203-14.
20.Bunnage ME. Getting pharmaceutical R&D back on target. Nature Chemical Biology. 2011;7(6):335-9.
21.Pammolli F, Magazzini L, Riccaboni M. The productivity crisis in pharmaceutical R&D. Nat Rev Drug Discov. 2011;10(6):428-38. Epub 2011/06/02.
22.Hay M, Thomas DW, Craighead JL, Economides C, Rosenthal J. Clinical development success rates for investigational drugs. Nat Biotechnol. 2014;32(1):40-51. Epub 2014/01/11.
23.Khanna I. Drug discovery in pharmaceutical industry: productivity challenges and trends. Drug Discov Today. 2012;17(19-20):1088-102. Epub 2012/05/26.
24.Morgan P, Brown DG, Lennard S, Anderton MJ, Barrett JC, Eriksson U, et al. Impact of a five-dimensional framework on R&D productivity at AstraZeneca. Nat Rev Drug Discov. 2018;17(3):167-81. Epub 2018/01/20.
25.Mullard A. 2017 FDA drug approvals. Nat Rev Drug Discov. 2018;17(2):81-5. Epub 2018/01/20.
26.Kola I, Landis J. Can the pharmaceutical industry reduce attrition rates? Nature Reviews Drug Discovery. 2004;3(8):711-5.
27.Waring MJ, Arrowsmith J, Leach AR, Leeson PD, Mandrell S, Owen RM, et al. An analysis of the attrition of drug candidates from four major pharmaceutical companies. Nature Reviews Drug Discovery. 2015;14(7):475-86.
28.Downing NS, Shah ND, Aminawung JA, Pease AM, Zeitoun JD, Krumholz HM, et al. Postmarket Safety Events Among Novel Therapeutics Approved by the US Food and Drug Administration Between 2001 and 2010. Jama-Journal of the American Medical Association. 2017;317(18):1854-63.
29.Onakpoya IJ, Heneghan CJ, Aronson JK. Post-marketing withdrawal of 462 medicinal products because of adverse drug reactions: a systematic review of the world literature. BMC Med. 2016;14:10. Epub 2016/02/05.

30.Ostapowicz G, Fontana RJ, Schiodt FV, Larson A, Davern TJ, Han SH, et al. Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. Ann Intern Med. 2002;137(12):947-54. Epub 2002/12/18.
31.Reuben A, Koch DG, Lee WM. Drug-induced acute liver failure: results of a U.S. multicenter, prospective study. Hepatology. 2010;52(6):2065-76. Epub 2010/10/16.
32.Tujios SR, Lee WM. Acute liver failure induced by idiosyncratic reaction to drugs: Challenges in diagnosis and therapy. Liver International. 2018;38(1):6-14.
33.Bjornsson ES, Bergmann OM, Bjornsson HK, Kvaran RB, Olafsson S. Incidence, presentation, and outcomes in patients with drug-induced liver injury in the general population of Iceland. Gastroenterology. 2013;144(7):1419-25, 25 e1-3; quiz e19-20. Epub 2013/02/20.
34.Bell LN, Chalasani N. Epidemiology of idiosyncratic drug-induced liver injury. Semin Liver Dis. 2009;29(4):337-47. Epub 2009/10/15.
35.Fontana RJ, Hayashi PH, Gu JZ, Reddy KR, Barnhart H, Watkins PB, et al. Idiosyncratic Drug-Induced Liver Injury Is Associated With Substantial Morbidity and Mortality Within 6 Months From Onset. Gastroenterology. 2014;147(1):96-U189.
36.Chalasani N, Bonkovsky HL, Fontana R, Lee W, Stolz A, Talwalkar J, et al. Features and Outcomes of 899 Patients With Drug-Induced Liver Injury: The DILIN Prospective Study. Gastroenterology. 2015;148(7):1340-52 e7. Epub 2015/03/11.
37.Hayashi PH, Rockey DC, Fontana RJ, Tillmann HL, Kaplowitz N, Barnhart HX, et al. Death and liver transplantation within 2 years of onset of drug-induced liver injury. Hepatology. 2017;66(4):1275-85. Epub 2017/05/26.
38.Leise MD, Poterucha JJ, Talwalkar JA. Drug-induced liver injury. Mayo Clin Proc. 2014;89(1):95-106. Epub 2014/01/07.
39.Mosedale M, Watkins PB. Drug-Induced Liver Injury: Advances in Mechanistic Understanding That Will Inform Risk Management. Clinical Pharmacology & Therapeutics. 2017;101(4):469-80.
40.Ganey PE, Luyendyk JP, Maddox JF, Roth RA. Adverse hepatic drug reactions: inflammatory episodes as consequence and contributor. Chem Biol Interact. 2004;150(1):35-51. Epub 2004/11/04.
41.Roth RA, Ganey PE. Intrinsic versus Idiosyncratic Drug-Induced Hepatotoxicity-Two Villains or One? Journal of Pharmacology and Experimental Therapeutics. 2010;332(3):692-7.
42.Yaghi C, Honein K, Boujaoude J, Slim R, Moucari R, Sayegh R. Influence of acetaminophen at therapeutic doses on surrogate markers of severity of acute viral hepatitis. Gastroenterol Clin Biol. 2006;30(5):763-8. Epub 2006/06/28.
43.Larson AM, Polson J, Fontana RJ, Davern TJ, Lalani E, Hynan LS, et al. Acetaminophen-induced acute liver failure: Results of a United States multicenter, prospective study. Hepatology. 2005;42(6):1364-72.
44.Gulmez SE, Larrey D, Pageaux GP, Lignot S, Lassalle R, Jove J, et al. Transplantation for Acute Liver Failure in Patients Exposed to NSAIDs or Paracetamol (Acetaminophen) The Multinational Case-Population SALT Study. Drug Safety. 2013;36(2):135-44.
45.Yamashita YI, Imai K, Mima K, Nakagawa S, Hashimoto D, Chikamoto A, et al. Idiosyncratic drug-induced liver injury: A short review. Hepatol Commun. 2017;1(6):494-500. Epub 2018/02/07.
46.Lu RJ, Zhang Y, Tang FL, Zheng ZW, Fan ZD, Zhu SM, et al. Clinical characteristics of drug-induced liver injury and related risk factors. Exp Ther Med. 2016;12(4):2606-16. Epub 2016/10/06.
47.Chalasani NP, Hayashi PH, Bonkovsky HL, Navarro VJ, Lee WM, Fontana RJ, et al. ACG Clinical Guideline: The Diagnosis and Management of Idiosyncratic Drug-Induced Liver Injury. American Journal of Gastroenterology. 2014;109(7):950-66.
48.Watkins PB, Seeff LB. Drug-induced liver injury: summary of a single topic clinical research conference. Hepatology. 2006;43(3):618-31. Epub 2006/02/24.
49.Aithal GP, Watkins PB, Andrade RJ, Larrey D, Molokhia M, Takikawa H, et al. Case definition and phenotype standardization in drug-induced liver injury. Clin Pharmacol Ther. 2011;89(6):806-15. Epub 2011/05/06.
50.Benichou C. Criteria of drug-induced liver disorders. Report of an international consensus meeting. J Hepatol. 1990;11(2):272-6. Epub 1990/09/01.
51.Abboud G, Kaplowitz N. Drug-induced liver injury. Drug Saf. 2007;30(4):277-94. Epub 2007/04/06.
52.Katarey D, Verma S. Drug-induced liver injury. Clin Med (Lond). 2016;16(Suppl 6):s104-s9. Epub 2016/12/14.
53.Sgro C, Clinard F, Ouazir K, Chanay H, Allard C, Guilleminet C, et al. Incidence of drug-induced hepatic injuries: a French population-based study. Hepatology. 2002;36(2):451-5. Epub 2002/07/27.
54.Verma S, Kaplowitz N. Diagnosis, management and prevention of drug-induced liver injury. Gut. 2009;58(11):1555-64. Epub 2009/10/17.
55.Ramachandran R, Kakar S. Histological patterns in drug-induced liver disease. J Clin Pathol. 2009;62(6):481-92. Epub 2009/05/29.
56.Bjornsson ES. Hepatotoxicity by Drugs: The Most Common Implicated Agents. Int J Mol Sci. 2016;17(2):224. Epub 2016/02/11.

57.Navarro VJ, Khan I, Bjornsson E, Seeff LB, Serrano J, Hoofnagle JH. Liver injury from herbal and dietary supplements. Hepatology. 2017;65(1):363-73. Epub 2016/09/30.
58.Robles-Diaz M, Medina-Caliz I, Stephens C, Andrade RJ, Lucena MI. Biomarkers in DILI: One More Step Forward. Front Pharmacol. 2016;7:267. Epub 2016/09/07.
59.Church RJ, Watkins PB. The transformation in biomarker detection and management of drug-induced liver injury. Liver Int. 2017;37(11):1582-90. Epub 2017/04/08.
60.Chen M, Borlak J, Tong W. High lipophilicity and high daily dose of oral medications are associated with significant risk for drug-induced liver injury. Hepatology. 2013;58(1):388-96. Epub 2012/12/22.
61.Lammert C, Einarsson S, Saha C, Niklasson A, Bjornsson E, Chalasani N. Relationship between daily dose of oral medications and idiosyncratic drug-induced liver injury: search for signals. Hepatology. 2008;47(6):2003-9. Epub 2008/05/06.
62.Chalasani N, Bjornsson E. Risk factors for idiosyncratic drug-induced liver injury. Gastroenterology. 2010;138(7):2246-59. Epub 2010/04/17.
63.Yu K, Geng XC, Chen MJ, Zhang J, Wang BS, Ilic K, et al. High Daily Dose and Being a Substrate of Cytochrome P450 Enzymes Are Two Important Predictors of Drug-Induced Liver Injury. Drug Metabolism and Disposition. 2014;42(4):744-50.
64.Knowles SR, Uetrecht J, Shear NH. Idiosyncratic drug reactions: the reactive metabolite syndromes. Lancet. 2000;356(9241):1587-91.
65.Lammert C, Bjornsson E, Niklasson A, Chalasani N. Oral medications with significant hepatic metabolism at higher risk for hepatic adverse events. Hepatology. 2010;51(2):615-20. Epub 2009/10/20.
66.Faulkner L, Meng XL, Park BK, Naisbitt DJ. The importance of hapten - protein complex formation in the development of drug allergy. Current Opinion in Allergy and Clinical Immunology. 2014;14(4):293-300.
67.Dawson S, Stahl S, Paul N, Barber J, Kenna JG. In vitro inhibition of the bile salt export pump correlates with risk of cholestatic drug-induced liver injury in humans. Drug Metab Dispos. 2012;40(1):130-8. Epub 2011/10/04.
68.Morgan RE, van Staden CJ, Chen Y, Kalyanaraman N, Kalanzi J, Dunn RT, 2nd, et al. A multifactorial approach to hepatobiliary transporter assessment enables improved therapeutic compound development. Toxicol Sci. 2013;136(1):216-41. Epub 2013/08/21.
69.Ortega-Alonso A, Stephens C, Lucena MI, Andrade RJ. Case Characterization, Clinical Features and Risk Factors in Drug-Induced Liver Injury. Int J Mol Sci. 2016;17(5). Epub 2016/05/18.
70.Fontana RJ, Hayashi PH, Barnhart H, Kleiner DE, Reddy KR, Chalasani N, et al. Persistent liver biochemistry abnormalities are more common in older patients and those with cholestatic drug induced liver injury. Am J Gastroenterol. 2015;110(10):1450-9. Epub 2015/09/09.
71.Medina-Caliz I, Robles-Diaz M, Garcia-Munoz B, Stephens C, Ortega-Alonso A, Garcia-Cortes M, et al. Definition and risk factors for chronicity following acute idiosyncratic drug-induced liver injury. J Hepatol. 2016;65(3):532-42. Epub 2016/05/18.
72.Felker D, Lynn A, Wang S, Johnson DE. Evidence for a potential protective effect of carnitine-pantothenic acid co-treatment on valproic acid-induced hepatotoxicity. Expert Rev Clin Pharmacol. 2014;7(2):211-8. Epub 2014/01/24.
73.Robles-Diaz M, Lucena MI, Kaplowitz N, Stephens C, Medina-Caliz I, Gonzalez-Jimenez A, et al. Use of Hy's law and a new composite algorithm to predict acute liver failure in patients with drug-induced liver injury. Gastroenterology. 2014;147(1):109-18 e5. Epub 2014/04/08.
74.Lucena MI, Andrade RJ, Kaplowitz N, Garcia-Cortes M, Fernandez MC, Romero-Gomez M, et al. Phenotypic characterization of idiosyncratic drug-induced liver injury: the influence of age and sex. Hepatology. 2009;49(6):2001-9. Epub 2009/05/29.
75.Lomtadze N, Kupreishvili L, Salakaia A, Vashakidze S, Sharvadze L, Kempker RR, et al. Hepatitis C Virus Co-Infection Increases the Risk of Anti-Tuberculosis Drug-Induced Hepatotoxicity among Patients with Pulmonary Tuberculosis. PLoS ONE. 2013;8(12).
76.Shu CC, Lee CH, Lee MC, Wang JY, Yu CJ, Lee LN. Hepatotoxicity due to first-line anti-tuberculosis drugs: a five-year experience in a Taiwan medical centre. International Journal of Tuberculosis and Lung Disease. 2013;17(7):934-9.
77.Fontana RJ. Pathogenesis of Idiosyncratic Drug-Induced Liver Injury and Clinical Perspectives. Gastroenterology. 2014;146(4):914-U437.
78.Daly AK, Day CP. Genetic association studies in drug-induced liver injury. Semin Liver Dis. 2009;29(4):400-11. Epub 2009/10/15.
79.Huang YS, Chern HD, Su WJ, Wu JC, Lai SL, Yang SY, et al. Polymorphism of the N-acetyltransferase 2 gene as a susceptibility risk factor for antituberculosis drug-induced hepatitis. Hepatology. 2002;35(4):883-9. Epub 2002/03/27.
80.Cho HJ, Koh WJ, Ryu YJ, Ki CS, Nam MH, Kim JW, et al. Genetic polymorphisms of NAT2 and CYP2E1 associated with antituberculosis drug-induced hepatotoxicity in Korean patients with pulmonary tuberculosis. Tuberculosis (Edinb). 2007;87(6):551-6. Epub 2007/10/24.

81.Chan SL, Chua APG, Aminkeng F, Chee CBE, Jin S, Loh M, et al. Association and clinical utility of NAT2 in the prediction of isoniazid-induced liver injury in Singaporean patients. PLoS ONE. 2017;12(10):e0186200. Epub 2017/10/17.
82.Daly AK, Aithal GP, Leathart JB, Swainsbury RA, Dang TS, Day CP. Genetic susceptibility to diclofenac-induced hepatotoxicity: contribution of UGT2B7, CYP2C8, and ABCC2 genotypes. Gastroenterology. 2007;132(1):272-81. Epub 2007/01/24.
83.Urban TJ, Shen Y, Stolz A, Chalasani N, Fontana RJ, Rochon J, et al. Limited contribution of common genetic variants to risk for liver injury due to a variety of drugs. Pharmacogenet Genomics. 2012;22(11):784-95. Epub 2012/09/13.
84.Lang C, Meier Y, Stieger B, Beuers U, Lang T, Kerb R, et al. Mutations and polymorphisms in the bile salt export pump and the multidrug resistance protein 3 associated with drug-induced liver injury. Pharmacogenet Genomics. 2007;17(1):47-60. Epub 2007/02/01.
85.Ulzurrun E, Stephens C, Crespo E, Ruiz-Cabello F, Ruiz-Nunez J, Saenz-Lopez P, et al. Role of chemical structures and the 1331T>C bile salt export pump polymorphism in idiosyncratic drug-induced liver injury. Liver Int. 2013;33(9):1378-85. Epub 2013/05/25.
86.Chen R, Wang J, Tang SW, Zhang Y, Lv XZ, Wu SS, et al. Role of polymorphic bile salt export pump (BSEP, ABCB11) transporters in anti-tuberculosis drug-induced liver injury in a Chinese cohort. Scientific Reports. 2016;6.
87.Kagawa T, Hirose S, Arase Y, Oka A, Anzai K, Tsuruya K, et al. No Contribution of the ABCB11 p.444A Polymorphism in Japanese Patients with Drug-Induced Cholestasis. Drug Metabolism and Disposition. 2015;43(5):691-7.
88.Daly AK, Donaldson PT, Bhatnagar P, Shen YF, Pe'er I, Floratos A, et al. HLA-B(star)5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. Nature Genetics. 2009;41(7):816-U71.
89.Spraggs CF, Budde LR, Briley LP, Bing N, Cox CJ, King KS, et al. HLA-DQA1*02:01 Is a Major Risk Factor for Lapatinib-Induced Hepatotoxicity in Women With Advanced Breast Cancer. Journal of Clinical Oncology. 2011;29(6):667-73.
90.Urban TJ, Nicoletti P, Chalasani N, Serrano J, Stolz A, Daly AK, et al. Minocycline hepatotoxicity: Clinical characterization and identification of HLA-B *35:02 as a risk factor. J Hepatol. 2017;67(1):137-44. Epub 2017/03/23.
91.Urban TJ, Daly AK, Aithal GP. Genetic basis of drug-induced liver injury: present and future. Semin Liver Dis. 2014;34(2):123-33. Epub 2014/06/01.
92.Magro L, Moretti U, Leone R. Epidemiology and characteristics of adverse drug reactions caused by drug-drug interactions. Expert Opin Drug Saf. 2012;11(1):83-94. Epub 2011/10/26.
93.Obreli Neto PR, Nobili A, de Lyra DP, Jr., Pilger D, Guidoni CM, de Oliveira Baldoni A, et al. Incidence and predictors of adverse drug reactions caused by drug-drug interactions in elderly outpatients: a prospective cohort study. J Pharm Pharm Sci. 2012;15(2):332-43. Epub 2012/05/15.
94.Ferslew KE, Hagardorn AN, Harlan GC, McCormick WF. A fatal drug interaction between clozapine and fluoxetine. J Forensic Sci. 1998;43(5):1082-5. Epub 1998/09/08.
95.Hung IF, Wu AK, Cheng VC, Tang BS, To KW, Yeung CK, et al. Fatal interaction between clarithromycin and colchicine in patients with renal insufficiency: a retrospective study. Clin Infect Dis. 2005;41(3):291-300. Epub 2005/07/12.
96.Huang SM, Lesko LJ. Drug-drug, drug-dietary supplement, and drug-citrus fruit and other food interactions: what have we learned? J Clin Pharmacol. 2004;44(6):559-69. Epub 2004/05/18.
97.Ward BW. Multiple chronic conditions and labor force outcomes: A population study of U.S. adults. Am J Ind Med. 2015;58(9):943-54. Epub 2015/06/24.
98.Gu Q, Dillon CF, Burt VL. Prescription drug use continues to increase: U.S. prescription drug data for 2007-2008. NCHS Data Brief. 2010(42):1-8. Epub 2010/09/22.
99.Payne RA. The epidemiology of polypharmacy. Clin Med (Lond). 2016;16(5):465-9. Epub 2016/10/05.
100.Percha B, Altman RB. Informatics confronts drug-drug interactions. Trends in Pharmacological Sciences. 2013;34(3):178-84.
101.Mallet L, Spinewine A, Huang A. The challenge of managing drug interactions in elderly people. Lancet. 2007;370(9582):185-91. Epub 2007/07/17.
102.Koper D, Kamenski G, Flamm M, Bohmdorfer B, Sonnichsen A. Frequency of medication errors in primary care patients with polypharmacy. Fam Pract. 2013;30(3):313-9. Epub 2012/11/08.
103.Marengoni A, Pasina L, Concoreggi C, Martini G, Brognoli F, Nobili A, et al. Understanding adverse drug reactions in older adults through drug-drug interactions. Eur J Intern Med. 2014;25(9):843-6. Epub 2014/10/15.
104.Cusack BJ. Pharmacokinetics in older persons. Am J Geriatr Pharmacother. 2004;2(4):274-302. Epub 2005/05/21.
105.Cerreta F, Eichler HG, Rasi G. Drug Policy for an Aging Population - The European Medicines Agency's Geriatric Medicines Strategy. New England Journal of Medicine. 2012;367(21):1972-4.
106.Gurwitz JH, Field TS, Harrold LR, Rothschild J, Debellis K, Seger AC, et al. Incidence and preventability of adverse drug events among older persons in the ambulatory setting. JAMA. 2003;289(9):1107-16. Epub 2003/03/08.

107.Strandell J, Bate A, Lindquist M, Edwards IR. Drug-drug interactions - a preventable patient safety issue? Br J Clin Pharmacol. 2008;65(1):144-6. Epub 2007/07/20.
108.Juurlink DN, Mamdani M, Kopp A, Laupacis A, Redelmeier DA. Drug-drug interactions among elderly patients hospitalized for drug toxicity. JAMA. 2003;289(13):1652-8. Epub 2003/04/04.
109.Ko Y, Malone DC, Skrepnek GH, Armstrong EP, Murphy JE, Abarca J, et al. Prescribers' knowledge of and sources of information for potential drug-drug interactions: a postal survey of US prescribers. Drug Saf. 2008;31(6):525-36. Epub 2008/05/20.
110.Payne TH, Hines LE, Chan RC, Hartman S, Kapusnik-Uner J, Russ AL, et al. Recommendations to improve the usability of drug-drug interaction clinical decision support alerts. J Am Med Inform Assoc. 2015;22(6):1243-50. Epub 2015/04/02.
111.Ansari J. Drug interaction and pharmacist. J Young Pharm. 2010;2(3):326-31. Epub 2010/11/03.
112.Palleria C, Di Paolo A, Giofre C, Caglioti C, Leuzzi G, Siniscalchi A, et al. Pharmacokinetic drug-drug interaction and their implication in clinical management. J Res Med Sci. 2013;18(7):601-10. Epub 2014/02/12.
113.Leucuta SE, Vlase L. Pharmacokinetics and metabolic drug interactions. Curr Clin Pharmacol. 2006;1(1):5-20. Epub 2008/08/01.
114.Li AP, Jurima-Romet M. Overview: pharmacokinetic drug-drug interactions. Adv Pharmacol. 1997;43:1-6. Epub 1997/01/01.
115.Guengerich FP. Role of cytochrome P450 enzymes in drug-drug interactions. Adv Pharmacol. 1997;43:7-35. Epub 1997/01/01.
116.Ogu CC, Maxa JL. Drug interactions due to cytochrome P450. Proc (Bayl Univ Med Cent). 2000;13(4):421-3. Epub 2006/01/04.
117.Lin JH, Lu AY. Inhibition and induction of cytochrome P450 and the clinical implications. Clin Pharmacokinet. 1998;35(5):361-90. Epub 1998/12/05.
118.Guengerich FP. Cytochrome p450 and chemical toxicology. Chem Res Toxicol. 2008;21(1):70-83. Epub 2007/12/07.
119.Doan J, Zakrzewski-Jakubiak H, Roy J, Turgeon J, Tannenbaum C. Prevalence and risk of potential cytochrome P450-mediated drug-drug interactions in older hospitalized patients with polypharmacy. Ann Pharmacother. 2013;47(3):324-32. Epub 2013/03/14.
120.Prueksaritanont T, Chu X, Gibson C, Cui D, Yee KL, Ballard J, et al. Drug-drug interaction studies: regulatory guidance and an industry perspective. AAPS J. 2013;15(3):629-45. Epub 2013/04/02.
121.Nebert DW, Russell DW. Clinical importance of the cytochromes P450. Lancet. 2002;360(9340):1155-62. Epub 2002/10/22.
122.Nebert DW, Dalton TP. The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. Nat Rev Cancer. 2006;6(12):947-60. Epub 2006/11/28.
123.Anzenbacher P, Anzenbacherova E. Cytochromes P450 and metabolism of xenobiotics. Cell Mol Life Sci. 2001;58(5-6):737-47. Epub 2001/07/05.
124.Lewis DF. 57 varieties: the human cytochromes P450. Pharmacogenomics. 2004;5(3):305-18. Epub 2004/04/23.
125.Huang SM, Strong JM, Zhang L, Reynolds KS, Nallani S, Temple R, et al. New era in drug interaction evaluation: US Food and Drug Administration update on CYP enzymes, transporters, and the guidance process. J Clin Pharmacol. 2008;48(6):662-70. Epub 2008/04/02.
126.Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacol Ther. 2013;138(1):103-41. Epub 2013/01/22.
127.Tracy TS, Chaudhry AS, Prasad B, Thummel KE, Schuetz EG, Zhong XB, et al. Interindividual Variability in Cytochrome P450-Mediated Drug Metabolism. Drug Metab Dispos. 2016;44(3):343-51. Epub 2015/12/19.
128.Lin JH, Lu AY. Interindividual variability in inhibition and induction of cytochrome P450 enzymes. Annu Rev Pharmacol Toxicol. 2001;41:535-67. Epub 2001/03/27.
129.Bjornsson TD, Callaghan JT, Einolf HJ, Fischer V, Gan L, Grimm S, et al. The conduct of in vitro and in vivo drug-drug interaction studies: a Pharmaceutical Research and Manufacturers of America (PhRMA) perspective. Drug Metab Dispos. 2003;31(7):815-32. Epub 2003/06/20.
130.Wienkers LC, Heath TG. Predicting in vivo drug interactions from in vitro drug discovery data. Nat Rev Drug Discov. 2005;4(10):825-33.
131.Dresser GK, Spence JD, Bailey DG. Pharmacokinetic-pharmacodynamic consequences and clinical relevance of cytochrome P450 3A4 inhibition. Clinical Pharmacokinetics. 2000;38(1):41-57.
132.Honig PK, Wortham DC, Zamani K, Conner DP, Mullin JC, Cantilena LR. Terfenadine-Ketoconazole Interaction - Pharmacokinetic and Electrocardiographic Consequences. Jama-Journal of the American Medical Association. 1993;269(12):1513-8.
133.Clinical Implications of CYP Induction-Mediated Drug–Drug Interactions. Encyclopedia of Drug Metabolism and Interactions.
134.Henderson L, Yue QY, Bergquist C, Gerden B, Arlett P. St John's wort (Hypericum perforatum): drug interactions and clinical outcomes. Br J Clin Pharmacol. 2002;54(4):349-56. Epub 2002/10/24.
135.Doligalski CT, Tong Logan A, Silverman A. Drug interactions: a primer for the gastroenterologist. Gastroenterol Hepatol (N Y). 2012;8(6):376-83. Epub 2012/08/31.

136.Gonzalez FJ. The 2006 Bernard B. Brodie Award Lecture. Cyp2e1. Drug Metab Dispos. 2007;35(1):1-8. Epub 2006/10/06.
137.Tang C, Lin JH, Lu AY. Metabolism-based drug-drug interactions: what determines individual variability in cytochrome P450 induction? Drug Metab Dispos. 2005;33(5):603-13. Epub 2005/01/28.
138.Keeton EK, McEachern K, Dillman KS, Palakurthi S, Cao Y, Grondine MR, et al. AZD1208, a potent and selective pan-Pim kinase inhibitor, demonstrates efficacy in preclinical models of acute myeloid leukemia. Blood. 2014;123(6):905-13. Epub 2013/12/24.
139.Cortes J, Tamura K, DeAngelo DJ, de Bono J, Lorente D, Minden M, et al. Phase I studies of AZD1208, a PIM kinase inhibitor, in patients with recurrent or refractory acute myelogenous leukemia or advanced solid tumors. Cancer Research. 2016;76.
140.Jones BC, Rollison H, Johansson S, Kanebratt KP, Lambert C, Vishwanathan K, et al. Managing the Risk of CYP3A Induction in Drug Development: A Strategic Approach. Drug Metab Dispos. 2017;45(1):35-41. Epub 2016/10/26.
141.Guengerich FP. Cytochrome P-450 3A4: regulation and role in drug metabolism. Annu Rev Pharmacol Toxicol. 1999;39:1-17. Epub 1999/05/20.
142.Backman JT, Olkkola KT, Neuvonen PJ. Rifampin drastically reduces plasma concentrations and effects of oral midazolam. Clin Pharmacol Ther. 1996;59(1):7-13. Epub 1996/01/01.
143.Rutledge DR, Pieper JA, Mirvis DM. Effects of chronic phenobarbital on verapamil disposition in humans. J Pharmacol Exp Ther. 1988;246(1):7-13. Epub 1988/07/01.
144.Backman JT, Olkkola KT, Ojala M, Laaksovirta H, Neuvonen PJ. Concentrations and effects of oral midazolam are greatly reduced in patients treated with carbamazepine or phenytoin. Epilepsia. 1996;37(3):253-7. Epub 1996/03/01.
145.Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, et al. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell. 1998;92(1):73-82. Epub 1998/03/07.
146.Goodwin B, Hodgson E, Liddle C. The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. Mol Pharmacol. 1999;56(6):1329-39. Epub 1999/11/26.
147.Matsumura K, Saito T, Takahashi Y, Ozeki T, Kiyotani K, Fujieda M, et al. Identification of a novel polymorphic enhancer of the human CYP3A4 gene. Mol Pharmacol. 2004;65(2):326-34. Epub 2004/01/27.
148.Goodwin B, Hodgson E, D'Costa DJ, Robertson GR, Liddle C. Transcriptional regulation of the human CYP3A4 gene by the constitutive androstane receptor. Mol Pharmacol. 2002;62(2):359-65. Epub 2002/07/20.
149.Reschly EJ, Krasowski MD. Evolution and function of the NR1I nuclear hormone receptor subfamily (VDR, PXR, and CAR) with respect to metabolism of xenobiotics and endogenous compounds. Curr Drug Metab. 2006;7(4):349-65. Epub 2006/05/27.
150.Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA. The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. J Clin Invest. 1998;102(5):1016-23. Epub 1998/09/03.
151.Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, et al. Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. J Biol Chem. 2000;275(20):15122-7. Epub 2000/04/05.
152.Ihunnah CA, Jiang M, Xie W. Nuclear receptor PXR, transcriptional circuits and metabolic relevance. Biochim Biophys Acta. 2011;1812(8):956-63. Epub 2011/02/08.
153.Banerjee M, Robbins D, Chen T. Targeting xenobiotic receptors PXR and CAR in human diseases. Drug Discov Today. 2015;20(5):618-28. Epub 2014/12/03.
154.Willson TM, Kliewer SA. PXR, CAR and drug metabolism. Nat Rev Drug Discov. 2002;1(4):259-66. Epub 2002/07/18.
155.Wang YM, Ong SS, Chai SC, Chen T. Role of CAR and PXR in xenobiotic sensing and metabolism. Expert Opin Drug Metab Toxicol. 2012;8(7):803-17. Epub 2012/05/05.
156.Faucette SR, Sueyoshi T, Smith CM, Negishi M, Lecluyse EL, Wang H. Differential regulation of hepatic CYP2B6 and CYP3A4 genes by constitutive androstane receptor but not pregnane X receptor. J Pharmacol Exp Ther. 2006;317(3):1200-9. Epub 2006/03/04.
157.Xie W, Barwick JL, Simon CM, Pierce AM, Safe S, Blumberg B, et al. Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. Genes Dev. 2000;14(23):3014-23. Epub 2000/12/15.
158.Baes M, Gulick T, Choi HS, Martinoli MG, Simha D, Moore DD. A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. Mol Cell Biol. 1994;14(3):1544-52. Epub 1994/03/01.
159.Yang H, Wang H. Signaling control of the constitutive androstane receptor (CAR). Protein Cell. 2014;5(2):113-23. Epub 2014/01/30.
160.Li L, Sinz MW, Zimmermann K, Wang H. An insulin-like growth factor 1 receptor inhibitor induces CYP3A4 expression through a pregnane X receptor-independent, noncanonical constitutive androstane receptor-related mechanism. J Pharmacol Exp Ther. 2012;340(3):688-97. Epub 2011/12/16.

161.Kanno Y, Inoue Y, Inouye Y. 5-aminoimidazole-4-carboxamide-1-beta-ribofuranoside (AICAR) prevents nuclear translocation of constitutive androstane receptor by AMP-activated protein kinase (AMPK) independent manner. J Toxicol Sci. 2010;35(4):571-6. Epub 2010/08/06.
162.Saito K, Moore R, Negishi M. p38 Mitogen-activated protein kinase regulates nuclear receptor CAR that activates the CYP2B6 gene. Drug Metab Dispos. 2013;41(6):1170-3. Epub 2013/03/30.
163.Mutoh S, Sobhany M, Moore R, Perera L, Pedersen L, Sueyoshi T, et al. Phenobarbital indirectly activates the constitutive active androstane receptor (CAR) by inhibition of epidermal growth factor receptor signaling. Sci Signal. 2013;6(274):ra31. Epub 2013/05/09.
164.Ding X, Staudinger JL. Induction of drug metabolism by forskolin: the role of the pregnane X receptor and the protein kinase a signal transduction pathway. J Pharmacol Exp Ther. 2005;312(2):849-56. Epub 2004/10/02.
165.Ding X, Staudinger JL. Repression of PXR-mediated induction of hepatic CYP3A gene expression by protein kinase C. Biochem Pharmacol. 2005;69(5):867-73. Epub 2005/02/16.
166.Drocourt L, Ourlin JC, Pascussi JM, Maurel P, Vilarem MJ. Expression of CYP3A4, CYP2B6, and CYP2C9 is regulated by the vitamin D receptor pathway in primary human hepatocytes. J Biol Chem. 2002;277(28):25125-32. Epub 2002/05/07.
167.Pascussi JM, Drocourt L, Gerbal-Chaloin S, Fabre JM, Maurel P, Vilarem MJ. Dual effect of dexamethasone on CYP3A4 gene expression in human hepatocytes. Sequential role of glucocorticoid receptor and pregnane X receptor. Eur J Biochem. 2001;268(24):6346-58. Epub 2001/12/12.
168.Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, et al. The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. Nat Med. 2003;9(2):220-4. Epub 2003/01/07.
169.Pascussi JM, Gerbal-Chaloin S, Duret C, Daujat-Chavanieu M, Vilarem MJ, Maurel P. The tangle of nuclear receptors that controls xenobiotic metabolism and transport: crosstalk and consequences. Annu Rev Pharmacol Toxicol. 2008;48:1-32. Epub 2007/07/05.
170.Pavek P. Pregnane X Receptor (PXR)-Mediated Gene Repression and Cross-Talk of PXR with Other Nuclear Receptors via Coactivator Interactions. Front Pharmacol. 2016;7:456. Epub 2016/12/10.
171.di Masi A, De Marinis E, Ascenzi P, Marino M. Nuclear receptors CAR and PXR: Molecular, functional, and biomedical aspects. Mol Aspects Med. 2009;30(5):297-343. Epub 2009/05/12.
172.Harrison RK. Phase II and phase III failures: 2013-2015. Nature Reviews Drug Discovery. 2016;15(12):817-8.
173.Cook D, Brown D, Alexander R, March R, Morgan P, Satterthwaite G, et al. Lessons learned from the fate of AstraZeneca's drug pipeline: a five-dimensional framework. Nat Rev Drug Discov. 2014;13(6):419-31. Epub 2014/05/17.
174.Watkins PB. Drug safety sciences and the bottleneck in drug development. Clin Pharmacol Ther. 2011;89(6):788-90. Epub 2011/05/20.
175.Takeda announces termination of fasiglifam (TAK-875) development. 2013. Last accessed May 2018. https://www.takeda.com/en-us/newsroom/news-releases/2013/takeda-announces-termination-of-fasiglifam-tak-875-development/.
176.Otieno MA, Snoeys J, Lam W, Ghosh A, Player MR, Pocai A, et al. Fasiglifam (TAK-875): Mechanistic Investigation and Retrospective Identification of Hazards for Drug Induced Liver Injury. Toxicol Sci. 2017. Epub 2017/02/17.
177.Kullak-Ublick GA, Andrade RJ, Merz M, End P, Benesic A, Gerbes AL, et al. Drug-induced liver injury: recent advances in diagnosis and risk assessment. Gut. 2017;66(6):1154-64. Epub 2017/03/28.
178.Hughes B. Industry concern over EU hepatotoxicity guidance. Nat Rev Drug Discov. 2008;7(9):719. Epub 2009/01/28.
179.National Research Council (U.S.). Science, medicine, and animals : a circle of discovery. Washington, DC: National Research Council, National Academies Press; 2004. 52 p. p.
180.Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, et al. Concordance of the toxicity of pharmaceuticals in humans and in animals. Regulatory Toxicology and Pharmacology. 2000;32(1):56-67.
181.Baldrick P. Safety evaluation to support first-in-man investigations II: Toxicology studies. Regulatory Toxicology and Pharmacology. 2008;51(2):237-43.
182.Boone L, Meyer D, Cusick P, Ennulat D, Bolliger AP, Everds N, et al. Selection and interpretation of clinical pathology indicators of hepatic injury in preclinical studies. Veterinary Clinical Pathology. 2005;34(3):182-8.
183.Ballet F. Preventing Drug-Induced Liver Injury: How Useful Are Animal Models? Digestive Diseases. 2015;33(4):477-85.
184.Caldwell J. Problems and opportunities in toxicity testing arising from species differences in xenobiotic metabolism. Toxicol Lett. 1992;64-65 Spec No:651-9. Epub 1992/12/01.
185.Martignoni M, Groothuis GM, de Kanter R. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert Opin Drug Metab Toxicol. 2006;2(6):875-94. Epub 2006/11/28.
186.Graham MJ, Lake BG. Induction of drug metabolism: species differences and toxicological relevance. Toxicology. 2008;254(3):184-91. Epub 2008/10/01.

187.Manning FJ, Swartz MN. Review of the Fialuridine (FIAU) clinical trials. Washington, DC: National Academy Press; 1995. ix, 269 p. p.
188.Fried MW, Dibisceglie AM, Straus SE, Savarese B, Beames MP, Hoofnagle JH. Fiau, a New Oral Antiviral Agent, Profoundly Inhibits Hbv DNA in Patients with Chronic Hepatitis-B. Hepatology. 1992;16(4):A127-A.
189.McKenzie R, Fried MW, Sallie R, Conjeevaram H, Di Bisceglie AM, Park Y, et al. Hepatic failure and lactic acidosis due to fialuridine (FIAU), an investigational nucleoside analogue for chronic hepatitis B. N Engl J Med. 1995;333(17):1099-105. Epub 1995/10/26.
190.Lee EW, Lai Y, Zhang H, Unadkat JD. Identification of the mitochondrial targeting signal of the human equilibrative nucleoside transporter 1 (hENT1): implications for interspecies differences in mitochondrial toxicity of fialuridine. J Biol Chem. 2006;281(24):16700-6. Epub 2006/04/06.
191.Lai Y, Tse CM, Unadkat JD. Mitochondrial expression of the human equilibrative nucleoside transporter 1 (hENT1) results in enhanced mitochondrial toxicity of antiviral drugs. J Biol Chem. 2004;279(6):4490-7. Epub 2003/11/11.
192.Xu D, Nishimura T, Nishimura S, Zhang H, Zheng M, Guo YY, et al. Fialuridine induces acute liver failure in chimeric TK-NOG mice: a model for detecting hepatic drug toxicity prior to human testing. PLoS Med. 2014;11(4):e1001628. Epub 2014/04/17.
193.Krewski D, Acosta D, Jr., Andersen M, Anderson H, Bailar JC, 3rd, Boekelheide K, et al. Toxicity testing in the 21st century: a vision and a strategy. J Toxicol Environ Health B Crit Rev. 2010;13(2-4):51-138. Epub 2010/06/25.
194.Saito J, Okamura A, Takeuchi K, Hanioka K, Okada A, Ohata T. High content analysis assay for prediction of human hepatotoxicity in HepaRG and HepG2 cells. Toxicol In Vitro. 2016;33:63-70. Epub 2016/02/28.
195.O'Brien PJ, Irwin W, Diaz D, Howard-Cofield E, Krejsa CM, Slaughter MR, et al. High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. Arch Toxicol. 2006;80(9):580-604. Epub 2006/04/07.
196.Garside H, Marcoe KF, Chesnut-Speelman J, Foster AJ, Muthas D, Kenna JG, et al. Evaluation of the use of imaging parameters for the detection of compound-induced hepatotoxicity in 384-well cultures of HepG2 cells and cryopreserved primary human hepatocytes. Toxicol In Vitro. 2014;28(2):171-81. Epub 2013/11/06.
197.Tolosa L, Pinto S, Donato MT, Lahoz A, Castell JV, O'Connor JE, et al. Development of a Multiparametric Cell-based Protocol to Screen and Classify the Hepatotoxicity Potential of Drugs. Toxicological Sciences. 2012;127(1):187-98.
198.Wisniewski JR, Vildhede A, Noren A, Artursson P. In-depth quantitative analysis and comparison of the human hepatocyte and hepatoma cell line HepG2 proteomes. J Proteomics. 2016;136:234-47. Epub 2016/01/31.
199.Sison-Young RL, Mitsa D, Jenkins RE, Mottram D, Alexandre E, Richert L, et al. Comparative Proteomic Characterization of 4 Human Liver-Derived Single Cell Culture Models Reveals Significant Variation in the Capacity for Drug Disposition, Bioactivation, and Detoxication. Toxicol Sci. 2015;147(2):412-24. Epub 2015/07/15.
200.Gerets HH, Tilmant K, Gerin B, Chanteux H, Depelchin BO, Dhalluin S, et al. Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. Cell Biol Toxicol. 2012;28(2):69-87. Epub 2012/01/20.
201.Sison-Young RL, Lauschke VM, Johann E, Alexandre E, Antherieu S, Aerts H, et al. A multicenter assessment of single-cell models aligned to standard measures of cell health for prediction of acute hepatotoxicity. Arch Toxicol. 2017;91(3):1385-400. Epub 2016/06/28.
202.Sinz MW. Evaluation of pregnane X receptor (PXR)-mediated CYP3A4 drug-drug interactions in drug development. Drug Metab Rev. 2013;45(1):3-14. Epub 2013/01/22.
203.Sinz M, Kim S, Zhu Z, Chen T, Anthony M, Dickinson K, et al. Evaluation of 170 xenobiotics as transactivators of human pregnane X receptor (hPXR) and correlation to known CYP3A4 drug interactions. Curr Drug Metab. 2006;7(4):375-88. Epub 2006/05/27.
204.Gustafsson F, Foster AJ, Sarda S, Bridgland-Taylor MH, Kenna JG. A Correlation Between the In Vitro Drug Toxicity of Drugs to Cell Lines That Express Human P450s and Their Propensity to Cause Liver Injury in Humans. Toxicological Sciences. 2014;137(1):189-211.
205.Zeilinger K, Freyer N, Damm G, Seehofer D, Knospel F. Cell sources for in vitro human liver cell culture models. Exp Biol Med (Maywood). 2016;241(15):1684-98. Epub 2016/07/08.
206.Richert L, Liguori MJ, Abadie C, Heyd B, Mantion G, Halkic N, et al. Gene expression in human hepatocytes in suspension after isolation is similar to the liver of origin, is not affected by hepatocyte cold storage and cryopreservation, but is strongly changed after hepatocyte plating. Drug Metab Dispos. 2006;34(5):870-9. Epub 2006/02/14.
207.Jouin D, Blanchard N, Alexandre E, Delobel F, David-Pierson P, Lave T, et al. Cryopreserved human hepatocytes in suspension are a convenient high throughput tool for the prediction of metabolic clearance. Eur J Pharm Biopharm. 2006;63(3):347-55. Epub 2006/04/20.
208.Mao J, Mohutsky MA, Harrelson JP, Wrighton SA, Hall SD. Predictions of cytochrome P450-mediated drug-drug interactions using cryopreserved human hepatocytes: comparison of plasma and protein-free media incubation conditions. Drug Metab Dispos. 2012;40(4):706-16. Epub 2012/01/10.

209.Desbans C, Hilgendorf C, Lutz M, Bachellier P, Zacharias T, Weber JC, et al. Prediction of fraction metabolized via CYP3A in humans utilizing cryopreserved human hepatocytes from a set of 12 single donors. Xenobiotica. 2014;44(1):17-27. Epub 2013/07/26.
210.Griffin SJ, Houston JB. Prediction of in vitro intrinsic clearance from hepatocytes: comparison of suspensions and monolayer cultures. Drug Metab Dispos. 2005;33(1):115-20. Epub 2004/12/21.
211.Richert L, Baze A, Parmentier C, Gerets HHJ, Sison-Young R, Dorau M, et al. Cytotoxicity evaluation using cryopreserved primary human hepatocytes in various culture formats. Toxicol Lett. 2016;258:207-15. Epub 2016/07/02.
212.Godoy P, Hengstler JG, Ilkavets I, Meyer C, Bachmann A, Muller A, et al. Extracellular Matrix Modulates Sensitivity of Hepatocytes to Fibroblastoid Dedifferentiation and Transforming Growth Factor beta-induced Apoptosis. Hepatology. 2009;49(6):2031-43.
213.Rodriguez-Antona C, Donato MT, Boobis A, Edwards RJ, Watts PS, Castell JV, et al. Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells. Xenobiotica. 2002;32(6):505-20. Epub 2002/08/06.
214.Elaut G, Henkens T, Papeleu P, Snykers S, Vinken M, Vanhaecke T, et al. Molecular mechanisms underlying the dedifferentiation process of isolated hepatocytes and their cultures. Current Drug Metabolism. 2006;7(6):629-60.
215.Gomez-Lechon MJ, Tolosa L, Conde I, Donato MT. Competency of different cell models to predict human hepatotoxic drugs. Expert Opin Drug Metab Toxicol. 2014;10(11):1553-68. Epub 2014/10/10.
216.Alexandre E, Baze A, Parmentier C, Desbans C, Pekthong D, Gerin B, et al. Plateable cryopreserved human hepatocytes for the assessment of cytochrome P450 inducibility: experimental condition-related variables affecting their response to inducers. Xenobiotica. 2012;42(10):968-79. Epub 2012/04/21.
217.Madan A, Graham RA, Carroll KM, Mudra DR, Burton LA, Krueger LA, et al. Effects of prototypical microsomal enzyme inducers on cytochrome P450 expression in cultured human hepatocytes. Drug Metab Dispos. 2003;31(4):421-31. Epub 2003/03/19.
218.Lauschke VM, Hendriks DF, Bell CC, Andersson TB, Ingelman-Sundberg M. Novel 3D Culture Systems for Studies of Human Liver Function and Assessments of the Hepatotoxicity of Drugs and Drug Candidates. Chem Res Toxicol. 2016;29(12):1936-55. Epub 2016/09/24.
219.Pampaloni F, Reynaud EG, Stelzer EH. The third dimension bridges the gap between cell culture and live tissue. Nat Rev Mol Cell Biol. 2007;8(10):839-45. Epub 2007/08/09.
220.Haycock JW. 3D cell culture: a review of current approaches and techniques. Methods Mol Biol. 2011;695:1-15. Epub 2010/11/03.
221.Bogdanos DP, Gao B, Gershwin ME. Liver immunology. Compr Physiol. 2013;3(2):567-98. Epub 2013/05/31.
222.Treyer A, Musch A. Hepatocyte polarity. Compr Physiol. 2013;3(1):243-87. Epub 2013/05/31.
223.Slim CL, van ISC, Lazaro-Dieguez F, Musch A. The special case of hepatocytes: unique tissue architecture calls for a distinct mode of cell division. Bioarchitecture. 2014;4(2):47-52. Epub 2014/04/29.
224.Zegers MM, Hoekstra D. Mechanisms and functional features of polarized membrane traffic in epithelial and hepatic cells. Biochem J. 1998;336 (Pt 2):257-69. Epub 1998/11/20.
225.Giepmans BN, van Ijzendoorn SC. Epithelial cell-cell junctions and plasma membrane domains. Biochim Biophys Acta. 2009;1788(4):820-31. Epub 2008/08/19.
226.Gissen P, Arias IM. Structural and functional hepatocyte polarity and liver disease. J Hepatol. 2015;63(4):1023-37. Epub 2015/06/28.
227.Dunn JC, Yarmush ML, Koebe HG, Tompkins RG. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. FASEB J. 1989;3(2):174-7. Epub 1989/02/01.
228.Ezzell RM, Toner M, Hendricks K, Dunn JC, Tompkins RG, Yarmush ML. Effect of collagen gel configuration on the cytoskeleton in cultured rat hepatocytes. Exp Cell Res. 1993;208(2):442-52. Epub 1993/10/01.
229.Berthiaume F, Moghe PV, Toner M, Yarmush ML. Effect of extracellular matrix topology on cell structure, function, and physiological responsiveness: hepatocytes cultured in a sandwich configuration. FASEB J. 1996;10(13):1471-84. Epub 1996/11/01.
230.Richert L, Binda D, Hamilton G, Viollon-Abadie C, Alexandre E, Bigot-Lasserre D, et al. Evaluation of the effect of culture configuration on morphology, survival time, antioxidant status and metabolic capacities of cultured rat hepatocytes. Toxicol In Vitro. 2002;16(1):89-99. Epub 2002/01/29.
231.LeCluyse EL, Audus KL, Hochman JH. Formation of extensive canalicular networks by rat hepatocytes cultured in collagen-sandwich configuration. Am J Physiol. 1994;266(6 Pt 1):C1764-74. Epub 1994/06/01.
232.Jones HM, Barton HA, Lai Y, Bi YA, Kimoto E, Kempshall S, et al. Mechanistic pharmacokinetic modeling for the prediction of transporter-mediated disposition in humans from sandwich culture human hepatocyte data. Drug Metab Dispos. 2012;40(5):1007-17. Epub 2012/02/22.
233.Swift B, Pfeifer ND, Brouwer KL. Sandwich-cultured hepatocytes: an in vitro model to evaluate hepatobiliary transporter-based drug interactions and hepatotoxicity. Drug Metab Rev. 2010;42(3):446-71. Epub 2010/01/30.

234.De Bruyn T, Chatterjee S, Fattah S, Keemink J, Nicolai J, Augustijns P, et al. Sandwich-cultured hepatocytes: utility for in vitro exploration of hepatobiliary drug disposition and drug-induced hepatotoxicity. Expert Opin Drug Metab Toxicol. 2013;9(5):589-616. Epub 2013/03/05.
235.Xu JJ, Henstock PV, Dunn MC, Smith AR, Chabot JR, de Graaf D. Cellular imaging predictions of clinical drug-induced liver injury. Toxicol Sci. 2008;105(1):97-105. Epub 2008/06/06.
236.Rowe C, Gerrard DT, Jenkins R, Berry A, Durkin K, Sundstrom L, et al. Proteome-wide analyses of human hepatocytes during differentiation and dedifferentiation. Hepatology. 2013;58(2):799-809. Epub 2013/03/26.
237.Wilkening S, Bader A. Influence of culture time on the expression of drug-metabolizing enzymes in primary human hepatocytes and hepatoma cell line HepG2. Journal of Biochemical and Molecular Toxicology. 2003;17(4):207-13.
238.Kimoto E, Walsky R, Zhang H, Bi YA, Whalen KM, Yang YS, et al. Differential modulation of cytochrome P450 activity and the effect of 1-aminobenzotriazole on hepatic transport in sandwich-cultured human hepatocytes. Drug Metab Dispos. 2012;40(2):407-11. Epub 2011/10/28.
239.Khetani SR, Bhatia SN. Engineering tissues for in vitro applications. Curr Opin Biotechnol. 2006;17(5):524-31. Epub 2006/09/19.
240.Griffith LG, Wells A, Stolz DB. Engineering liver. Hepatology. 2014;60(4):1426-34. Epub 2014/03/29.
241.Khetani SR, Berger DR, Ballinger KR, Davidson MD, Lin C, Ware BR. Microengineered liver tissues for drug testing. J Lab Autom. 2015;20(3):216-50. Epub 2015/01/27.
242.Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. Nat Biotechnol. 2008;26(1):120-6. Epub 2007/11/21.
243.Khetani SR, Kanchagar C, Ukairo O, Krzyzewski S, Moore A, Shi J, et al. Use of micropatterned cocultures to detect compounds that cause drug-induced liver injury in humans. Toxicol Sci. 2013;132(1):107-17. Epub 2012/11/16.
244.Ballard TE, Wang S, Cox LM, Moen MA, Krzyzewski S, Ukairo O, et al. Application of a Micropatterned Cocultured Hepatocyte System To Predict Preclinical and Human-Specific Drug Metabolism. Drug Metab Dispos. 2016;44(2):172-9. Epub 2015/11/27.
245.Chan TS, Yu H, Moore A, Khetani SR, Tweedie D. Meeting the challenge of predicting hepatic clearance of compounds slowly metabolized by cytochrome P450 using a novel hepatocyte model, HepatoPac. Drug Metab Dispos. 2013;41(12):2024-32. Epub 2013/08/21.
246.Wang WW, Khetani SR, Krzyzewski S, Duignan DB, Obach RS. Assessment of a micropatterned hepatocyte coculture system to generate major human excretory and circulating drug metabolites. Drug Metab Dispos. 2010;38(10):1900-5. Epub 2010/07/03.
247.March S, Ramanan V, Trehan K, Ng S, Galstian A, Gural N, et al. Micropatterned coculture of primary human hepatocytes and supportive cells for the study of hepatotropic pathogens. Nat Protoc. 2015;10(12):2027-53. Epub 2015/11/20.
248.Davidson MD, Ballinger KR, Khetani SR. Long-term exposure to abnormal glucose levels alters drug metabolism pathways and insulin sensitivity in primary human hepatocytes. Sci Rep. 2016;6:28178. Epub 2016/06/18.
249.Nguyen TV, Ukairo O, Khetani SR, McVay M, Kanchagar C, Seghezzi W, et al. Establishment of a hepatocyte-kupffer cell coculture model for assessment of proinflammatory cytokine effects on metabolizing enzymes and drug transporters. Drug Metab Dispos. 2015;43(5):774-85. Epub 2015/03/06.
250.Beckwitt CH, Clark AM, Wheeler S, Taylor DL, Stolz DB, Griffith L, et al. Liver 'organ on a chip'. Exp Cell Res. 2018;363(1):15-25. Epub 2018/01/02.
251.Godoy P, Hewitt NJ, Albrecht U, Andersen ME, Ansari N, Bhattacharya S, et al. Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. Arch Toxicol. 2013;87(8):1315-530. Epub 2013/08/27.
252.Lin C, Khetani SR. Advances in Engineered Liver Models for Investigating Drug-Induced Liver Injury. Biomed Res Int. 2016;2016:1829148. Epub 2016/10/12.
253.LeCluyse EL, Witek RP, Andersen ME, Powers MJ. Organotypic liver culture models: meeting current challenges in toxicity testing. Crit Rev Toxicol. 2012;42(6):501-48. Epub 2012/05/16.
254.Ramaiahgari SC, den Braver MW, Herpers B, Terpstra V, Commandeur JN, van de Water B, et al. A 3D in vitro model of differentiated HepG2 cell spheroids with improved liver-like properties for repeated dose high-throughput toxicity studies. Arch Toxicol. 2014;88(5):1083-95. Epub 2014/03/07.
255.Ramaiahgari SC, Waidyanatha S, Dixon D, DeVito MJ, Paules RS, Ferguson SS. Three-Dimensional (3D) HepaRG Spheroid Model With Physiologically Relevant Xenobiotic Metabolism Competence and Hepatocyte Functionality for Liver Toxicity Screening. Toxicol Sci. 2017;160(1):189-90. Epub 2017/10/28.
256.Gunness P, Mueller D, Shevchenko V, Heinzle E, Ingelman-Sundberg M, Noor F. 3D organotypic cultures of human HepaRG cells: a tool for in vitro toxicity studies. Toxicol Sci. 2013;133(1):67-78. Epub 2013/02/05.
257.Mueller D, Kramer L, Hoffmann E, Klein S, Noor F. 3D organotypic HepaRG cultures as in vitro model for acute and repeated dose toxicity studies. Toxicol In Vitro. 2014;28(1):104-12. Epub 2013/07/16.
258.Fey SJ, Wrzesinski K. Determination of drug toxicity using 3D spheroids constructed from an immortal human hepatocyte cell line. Toxicol Sci. 2012;127(2):403-11. Epub 2012/03/29.

259.Gaskell H, Sharma P, Colley HE, Murdoch C, Williams DP, Webb SD. Characterization of a functional C3A liver spheroid model. Toxicol Res (Camb). 2016;5(4):1053-65. Epub 2016/10/18.
260.Tostoes RM, Leite SB, Serra M, Jensen J, Bjorquist P, Carrondo MJ, et al. Human liver cell spheroids in extended perfusion bioreactor culture for repeated-dose drug testing. Hepatology. 2012;55(4):1227-36. Epub 2011/10/28.
261.Freyer N, Greuel S, Knöspel F, Gerstmann F, Storch L, Damm G, et al. Microscale 3D Liver Bioreactor for In Vitro Hepatotoxicity Testing under Perfusion Conditions. Bioengineering (Basel). 2018;5(1). Epub 2018/03/15.
262.Ebrahimkhani MR, Neiman JA, Raredon MS, Hughes DJ, Griffith LG. Bioreactor technologies to support liver function in vitro. Adv Drug Deliv Rev. 2014;69-70:132-57. Epub 2014/03/05.
263.Messner S, Agarkova I, Moritz W, Kelm JM. Multi-cell type human liver microtissues for hepatotoxicity testing. Arch Toxicol. 2013;87(1):209-13. Epub 2012/11/13.
264.Proctor WR, Foster AJ, Vogt J, Summers C, Middleton B, Pilling MA, et al. Utility of spherical human liver microtissues for prediction of clinical drug-induced liver injury. Arch Toxicol. 2017;91(8):2849-63. Epub 2017/06/13.
265.Desai PK, Tseng H, Souza GR. Assembly of Hepatocyte Spheroids Using Magnetic 3D Cell Culture for CYP450 Inhibition/Induction. Int J Mol Sci. 2017;18(5). Epub 2017/05/18.
266.Bhatia SN, Ingber DE. Microfluidic organs-on-chips. Nat Biotechnol. 2014;32(8):760-72. Epub 2014/08/06.
267.Usta OB, McCarty WJ, Bale S, Hegde M, Jindal R, Bhushan A, et al. Microengineered cell and tissue systems for drug screening and toxicology applications: Evolution of. Technology (Singap World Sci). 2015;3(1):1-26.
268.Du Y, Li N, Yang H, Luo C, Gong Y, Tong C, et al. Mimicking liver sinusoidal structures and functions using a 3D-configured microfluidic chip. Lab Chip. 2017;17(5):782-94. Epub 2017/01/24.
269.Choe A, Ha SK, Choi I, Choi N, Sung JH. Microfluidic Gut-liver chip for reproducing the first pass metabolism. Biomed Microdevices. 2017;19(1):4. Epub 2017/01/12.
270.Lee DW, Ha SK, Choi I, Sung JH. 3D gut-liver chip with a PK model for prediction of first-pass metabolism. Biomed Microdevices. 2017;19(4):100. Epub 2017/11/07.
271.Bauer S, Wennberg Huldt C, Kanebratt KP, Durieux I, Gunne D, Andersson S, et al. Functional coupling of human pancreatic islets and liver spheroids on-a-chip: Towards a novel human ex vivo type 2 diabetes model. Sci Rep. 2017;7(1):14620. Epub 2017/11/06.
272.Li Z, Jiang L, Zhu Y, Su W, Xu C, Tao T, et al. Assessment of hepatic metabolism-dependent nephrotoxicity on an organs-on-a-chip microdevice. Toxicol In Vitro. 2018;46:1-8. Epub 2017/10/03.
273.Maschmeyer I, Lorenz AK, Schimek K, Hasenberg T, Ramme AP, Hubner J, et al. A four-organ-chip for interconnected long-term co-culture of human intestine, liver, skin and kidney equivalents. Lab Chip. 2015;15(12):2688-99. Epub 2015/05/23.
274.Satoh T, Sugiura S, Shin K, Onuki-Nagasaki R, Ishida S, Kikuchi K, et al. A multi-throughput multi-organ-on-a-chip system on a plate formatted pneumatic pressure-driven medium circulation platform. Lab Chip. 2017;18(1):115-25. Epub 2017/12/01.
275.Miller PG, Shuler ML. Design and demonstration of a pumpless 14 compartment microphysiological system. Biotechnol Bioeng. 2016;113(10):2213-27. Epub 2016/04/14.
276.Yi HG, Lee H, Cho DW. 3D Printing of Organs-On-Chips. Bioengineering (Basel). 2017;4(1). Epub 2017/01/25.
277.Lee H, Cho DW. One-step fabrication of an organ-on-a-chip with spatial heterogeneity using a 3D bioprinting technology. Lab Chip. 2016;16(14):2618-25.
278.Bhise NS, Manoharan V, Massa S, Tamayol A, Ghaderi M, Miscuglio M, et al. A liver-on-a-chip platform with bioprinted hepatic spheroids. Biofabrication. 2016;8(1):014101. Epub 2016/01/12.
279.Nguyen DG, Funk J, Robbins JB, Crogan-Grundy C, Presnell SC, Singer T, et al. Bioprinted 3D Primary Liver Tissues Allow Assessment of Organ-Level Response to Clinical Drug Induced Toxicity In Vitro. PLoS One. 2016;11(7):e0158674. Epub 2016/07/07.
280.Starokozhko V, Groothuis GM. Judging the value of 'liver-on-a-chip' devices for prediction of toxicity. Expert Opin Drug Metab Toxicol. 2017;13(2):125-8. Epub 2016/10/12.
281.Materne EM, Tonevitsky AG, Marx U. Chip-based liver equivalents for toxicity testing--organotypicalness versus cost-efficient high throughput. Lab Chip. 2013;13(18):3481-95.
282.Kimura H, Sakai Y, Fujii T. Organ/body-on-a-chip based on microfluidic technology for drug discovery. Drug Metab Pharmacokinet. 2018;33(1):43-8. Epub 2017/11/13.
283.Palakkan AA, Hay DC, Anil Kumar PR, Kumary TV, Ross JA. Liver tissue engineering and cell sources: issues and challenges. Liver Int. 2013;33(5):666-76. Epub 2013/03/16.
284.Wobus AM, Loser P. Present state and future perspectives of using pluripotent stem cells in toxicology research. Arch Toxicol. 2011;85(2):79-117. Epub 2011/01/13.
285.Asgari S, Pournasr B, Salekdeh GH, Ghodsizadeh A, Ott M, Baharvand H. Induced pluripotent stem cells: a new era for hepatology. J Hepatol. 2010;53(4):738-51. Epub 2010/07/14.

286.Kia R, Sison RL, Heslop J, Kitteringham NR, Hanley N, Mills JS, et al. Stem cell-derived hepatocytes as a predictive model for drug-induced liver injury: are we there yet? Br J Clin Pharmacol. 2013;75(4):885-96. Epub 2012/06/19.
287.Anson BD, Kolaja KL, Kamp TJ. Opportunities for use of human iPS cells in predictive toxicology. Clin Pharmacol Ther. 2011;89(5):754-8. Epub 2011/03/25.
288.Gerbal-Chaloin S, Funakoshi N, Caillaud A, Gondeau C, Champon B, Si-Tayeb K. Human induced pluripotent stem cells in hepatology: beyond the proof of concept. Am J Pathol. 2014;184(2):332-47. Epub 2013/11/26.
289.Yu Y, Wang X, Nyberg SL. Potential and Challenges of Induced Pluripotent Stem Cells in Liver Diseases Treatment. J Clin Med. 2014;3(3):997-1017. Epub 2014/01/01.
290.Zhao D, Chen S, Duo S, Xiang C, Jia J, Guo M, et al. Promotion of the efficient metabolic maturation of human pluripotent stem cell-derived hepatocytes by correcting specification defects. Cell Res. 2013;23(1):157-61. Epub 2012/10/17.
291.Schwartz RE, Fleming HE, Khetani SR, Bhatia SN. Pluripotent stem cell-derived hepatocyte-like cells. Biotechnol Adv. 2014;32(2):504-13. Epub 2014/01/21.
292.Baxter M, Withey S, Harrison S, Segeritz CP, Zhang F, Atkinson-Dell R, et al. Phenotypic and functional analyses show stem cell-derived hepatocyte-like cells better mimic fetal rather than adult hepatocytes. J Hepatol. 2015;62(3):581-9. Epub 2014/12/03.
293.Ulvestad M, Nordell P, Asplund A, Rehnstrom M, Jacobsson S, Holmgren G, et al. Drug metabolizing enzyme and transporter protein profiles of hepatocytes derived from human embryonic and induced pluripotent stem cells. Biochem Pharmacol. 2013;86(5):691-702. Epub 2013/07/17.
294.Yi F, Liu GH, Izpisua Belmonte JC. Human induced pluripotent stem cells derived hepatocytes: rising promise for disease modeling, drug development and cell therapy. Protein Cell. 2012;3(4):246-50. Epub 2012/03/24.
295.Gieseck RL, 3rd, Hannan NR, Bort R, Hanley NA, Drake RA, Cameron GW, et al. Maturation of induced pluripotent stem cell derived hepatocytes by 3D-culture. PLoS ONE. 2014;9(1):e86372. Epub 2014/01/28.
296.Berger DR, Ware BR, Davidson MD, Allsup SR, Khetani SR. Enhancing the functional maturity of induced pluripotent stem cell-derived human hepatocytes by controlled presentation of cell-cell interactions in vitro. Hepatology. 2015;61(4):1370-81. Epub 2014/11/26.
297.Takayama K, Morisaki Y, Kuno S, Nagamoto Y, Harada K, Furukawa N, et al. Prediction of interindividual differences in hepatic functions and drug sensitivity by using human iPS-derived hepatocytes. Proc Natl Acad Sci U S A. 2014;111(47):16772-7. Epub 2014/11/12.
298.Ware BR, Berger DR, Khetani SR. Prediction of Drug-Induced Liver Injury in Micropatterned Co-cultures Containing iPSC-Derived Human Hepatocytes. Toxicol Sci. 2015;145(2):252-62. Epub 2015/02/27.
299.Li S, Guo J, Ying Z, Chen S, Yang L, Chen K, et al. Valproic acid-induced hepatotoxicity in Alpers syndrome is associated with mitochondrial permeability transition pore opening-dependent apoptotic sensitivity in an induced pluripotent stem cell model. Hepatology. 2015;61(5):1730-9. Epub 2015/01/22.
300.Choudhury Y, Toh YC, Xing J, Qu Y, Poh J, Huan L, et al. Patient-specific hepatocyte-like cells derived from induced pluripotent stem cells model pazopanib-mediated hepatotoxicity. Sci Rep. 2017;7:41238. Epub 2017/01/26.
301.Heslop JA, Rowe C, Walsh J, Sison-Young R, Jenkins R, Kamalian L, et al. Mechanistic evaluation of primary human hepatocyte culture using global proteomic analysis reveals a selective dedifferentiation profile. Archives of Toxicology. 2017;91(1):439-52.
302.Jungermann K, Kietzmann T. Zonation of parenchymal and nonparenchymal metabolism in liver. Annu Rev Nutr. 1996;16:179-203. Epub 1996/01/01.
303.Park BK, Boobis A, Clarke S, Goldring CE, Jones D, Kenna JG, et al. Managing the challenge of chemically reactive metabolites in drug development. Nat Rev Drug Discov. 2011;10(4):292-306. Epub 2011/04/02.
304.Lewis W, Levine ES, Griniuviene B, Tankersley KO, Colacino JM, Sommadossi JP, et al. Fialuridine and its metabolites inhibit DNA polymerase gamma at sites of multiple adjacent analog incorporation, decrease mtDNA abundance, and cause mitochondrial structural defects in cultured hepatoblasts. Proc Natl Acad Sci U S A. 1996;93(8):3592-7. Epub 1996/04/16.
305.Lewis W, Griniuviene B, Tankersley KO, Levine ES, Montione R, Engelman L, et al. Depletion of mitochondrial DNA, destruction of mitochondria, and accumulation of lipid droplets result from fialuridine treatment in woodchucks (Marmota monax). Lab Invest. 1997;76(1):77-87. Epub 1997/01/01.
306.Rezzani R. Cyclosporine A and adverse effects on organs: histochemical studies. Prog Histochem Cytochem. 2004;39(2):85-128. Epub 2004/09/10.
307.Erarslan E, Ekiz F, Uz B, Koca C, Turkcu UO, Bayrak R, et al. Effects of erdosteine on cyclosporine-A-induced hepatotoxicity in rats. Drug Chem Toxicol. 2011;34(1):32-7. Epub 2010/10/20.
308.Akbulut S, Elbe H, Eris C, Dogan Z, Toprak G, Yalcin E, et al. Effects of antioxidant agents against cyclosporine-induced hepatotoxicity. J Surg Res. 2015;193(2):658-66. Epub 2014/10/05.

309.Younossi ZM, Stepanova M, Afendy M, Fang Y, Younossi Y, Mir H, et al. Changes in the prevalence of the most common causes of chronic liver diseases in the United States from 1988 to 2008. Clin Gastroenterol Hepatol. 2011;9(6):524-30 e1; quiz e60. Epub 2011/03/29.
310.Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, et al. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. Nat Rev Gastroenterol Hepatol. 2018;15(1):11-20. Epub 2017/09/21.
311.Yang K, Kock K, Sedykh A, Tropsha A, Brouwer KLR. An Updated Review on Drug-Induced Cholestasis: Mechanisms and Investigation of Physicochemical Properties and Pharmacokinetic Parameters. Journal of Pharmaceutical Sciences. 2013;102(9):3037-57.
312.Ananthanarayanan M, Balasubramanian N, Makishima M, Mangelsdorf DJ, Suchy FJ. Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. J Biol Chem. 2001;276(31):28857-65. Epub 2001/06/02.
313.Regal RE, Billi JE, Glazer HM. Phenothiazine-induced cholestatic jaundice. Clin Pharm. 1987;6(10):787-94. Epub 1987/10/01.
314.Antherieu S, Bachour-El Azzi P, Dumont J, Abdel-Razzak Z, Guguen-Guillouzo C, Fromenty B, et al. Oxidative stress plays a major role in chlorpromazine-induced cholestasis in human HepaRG cells. Hepatology. 2013;57(4):1518-29. Epub 2012/11/24.
315.Soriano FX, Baxter P, Murray LM, Sporn MB, Gillingwater TH, Hardingham GE. Transcriptional regulation of the AP-1 and Nrf2 target gene sulfiredoxin. Mol Cells. 2009;27(3):279-82. Epub 2009/03/28.
316.Higuchi H, Bronk SF, Takikawa Y, Werneburg N, Takimoto R, El-Deiry W, et al. The bile acid glycochenodeoxycholate induces trail-receptor 2/DR5 expression and apoptosis. J Biol Chem. 2001;276(42):38610-8. Epub 2001/08/17.
317.Padda MS, Sanchez M, Akhtar AJ, Boyer JL. Drug-induced cholestasis. Hepatology. 2011;53(4):1377-87. Epub 2011/04/12.
318.Cheng YF, Woolf TF, Gan JP, He K. In vitro model systems to investigate bile salt export pump (BSEP) activity and drug interactions: A review. Chemico-Biological Interactions. 2016;255:23-30.
319.Vinken M. In vitro prediction of drug-induced cholestatic liver injury: a challenge for the toxicologist. Arch Toxicol. 2018. Epub 2018/03/27.
320.Chatterjee S, Richert L, Augustijns P, Annaert P. Hepatocyte-based in vitro model for assessment of drug-induced cholestasis. Toxicol Appl Pharmacol. 2014;274(1):124-36. Epub 2013/11/12.
321.Skurnik YD, Tcherniak A, Edlan K, Sthoeger Z. Ticlopidine-induced cholestatic hepatitis. Ann Pharmacother. 2003;37(3):371-5. Epub 2003/03/18.
322.European Medicines Agency. Guideline on the investigation of drug interactions. 2012. Last accessed May 2018.
http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf.
323.Scott SA. Personalizing medicine with clinical pharmacogenetics. Genet Med. 2011;13(12):987-95. Epub 2011/11/19.
324.Clare KE, Miller MH, Dillon JF. Genetic Factors Influencing Drug-Induced Liver Injury: Do They Have a Role in Prevention and Diagnosis? Curr Hepatol Rep. 2017;16(3):258-64. Epub 2017/09/01.
325.Aithal GP. Pharmacogenetic testing in idiosyncratic drug-induced liver injury: current role in clinical practice. Liver Int. 2015;35(7):1801-8. Epub 2015/03/27.
326.Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, Brunner MD, et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. N Engl J Med. 2006;355(10):1018-28. Epub 2006/08/16.
327.Moore N. Lessons from the fatal French study BIA-10-2474. BMJ. 2016;353:i2727. Epub 2016/05/20.
328.Messner S, Fredriksson L, Lauschke VM, Roessger K, Claudia E, Magdalena B, et al. Transcriptomic, Proteomic, and Functional Long-Term Characterization of Multicellular Three-Dimensional Human Liver Microtissues. Applied In Vitro Toxicology. 2018;4(1):1-12.
329.Lauschke VM, Ingelman-Sundberg M. The Importance of Patient-Specific Factors for Hepatic Drug Response and Toxicity. Int J Mol Sci. 2016;17(10). Epub 2016/10/19.
330.Vorrink S, Zhou Y, Ingelman-Sundberg M, Lauschke VM. Prediction of drug-induced hepatotoxicity using long-term stable primary hepatic 3D spheroid cultures in chemically defined conditions. Toxicol Sci. 2018. Epub 2018/03/29.
331.Bell CC, Lauschke VM, Vorrink SU, Palmgren H, Duffin R, Andersson TB, et al. Transcriptional, Functional, and Mechanistic Comparisons of Stem Cell-Derived Hepatocytes, HepaRG Cells, and Three-Dimensional Human Hepatocyte Spheroids as Predictive In Vitro Systems for Drug-Induced Liver Injury. Drug Metab Dispos. 2017;45(4):419-29. Epub 2017/02/01.
332.Bell CC, Dankers ACA, Lauschke VM, Sison-Young R, Jenkins R, Rowe C, et al. Comparison of Hepatic 2D Sandwich Cultures and 3D Spheroids for Long-term Toxicity Applications: A Multicenter Study. Toxicol Sci. 2018;162(2):655-66. Epub 2018/01/13.
333.Vorrink SU, Ullah S, Schmidt S, Nandania J, Velagapudi V, Beck O, et al. Endogenous and xenobiotic metabolic stability of primary human hepatocytes in long-term 3D spheroid cultures revealed by a combination of targeted and untargeted metabolomics. FASEB J. 2017;31(6):2696-708. Epub 2017/03/08.

334.Duarte N, Coelho IC, Patarrão RS, Almeida JI, Penha-Gonçalves C, Macedo MP. How Inflammation Impinges on NAFLD: A Role for Kupffer Cells. Biomed Res Int. 2015;2015:984578. Epub 2015/05/18.
335.Safadi R, Friedman SL. Hepatic fibrosis--role of hepatic stellate cell activation. MedGenMed. 2002;4(3):27. Epub 2002/07/15.
336.Leite SB, Roosens T, El Taghdouini A, Mannaerts I, Smout AJ, Najimi M, et al. Novel human hepatic organoid model enables testing of drug-induced liver fibrosis in vitro. Biomaterials. 2016;78:1-10. Epub 2015/11/17.
337.Ewart L, Dehne EM, Fabre K, Gibbs S, Hickman J, Hornberg E, et al. Application of Microphysiological Systems to Enhance Safety Assessment in Drug Discovery. Annu Rev Pharmacol Toxicol. 2018;58:65-82. Epub 2017/10/13.
338.Pridgeon CS, Schlott C, Wong MW, Heringa MB, Heckel T, Leedale J, et al. Innovative organotypic in vitro models for safety assessment: aligning with regulatory requirements and understanding models of the heart, skin, and liver as paradigms. Arch Toxicol. 2018;92(2):557-69. Epub 2018/01/25.
339.Underhill GH, Khetani SR. Bioengineered Liver Models for Drug Testing and Cell Differentiation Studies. Cell Mol Gastroenterol Hepatol. 2018;5(3):426-39 e1. Epub 2018/04/21.
340.Cole BK, Feaver RE, Wamhoff BR, Dash A. Non-alcoholic fatty liver disease (NAFLD) models in drug discovery. Expert Opin Drug Discov. 2018;13(2):193-205. Epub 2017/12/01.