REVIEW

Preclinical models of idiosyncratic drug-induced liver injury (iDILI): Moving towards prediction

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Received 22 September 2021; received in revised form 7 November 2021; accepted 10 November 2021

KEY WORDS
Drug-induced liver injury; Preclinical models; Mechanisms; Oxidative stress; Mitochondrial damage; Immune response; Personalized medicine

Abstract    Idiosyncratic drug-induced liver injury (iDILI) encompasses the unexpected harms that prescription and non-prescription drugs, herbal and dietary supplements can cause to the liver. iDILI remains a major public health problem and a major cause of drug attrition. Given the lack of biomarkers for iDILI prediction, diagnosis and prognosis, searching new models to predict and study mechanisms of iDILI is necessary. One of the major limitations of iDILI preclinical assessment has been the lack of correlation between the markers of hepatotoxicity in animal toxicological studies and clinically significant iDILI. Thus, major advances in the understanding of iDILI susceptibility and pathogenesis have come from the study of well-phenotyped iDILI patients. However, there are many gaps for explaining all the complexity of iDILI susceptibility and mechanisms. Therefore, there is a need to optimize preclinical human in vitro models to reduce the risk of iDILI during drug development. Here, the current experimental models and the future directions in iDILI modelling are thoroughly discussed, focusing on the human cellular models available to study the pathophysiological mechanisms of the disease and the most used in vivo animal iDILI models. We also comment about in silico approaches and the increasing relevance of patient-derived cellular models.

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Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences

https://doi.org/10.1016/j.apsb.2021.11.013
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1. Introduction

Drug-induced liver injury (DILI) includes the unexpected damage that prescription and non-prescription drugs, as well as herbal and dietary supplements (HDS) can cause to the liver. In contrast to intrinsic DILI, in which the damage is dose-related and occurs shortly after exposure to the drug, idiosyncratic DILI (hereinafter, iDILI) is multifactorial and unpredictable. iDILI onset is generally not dose-dependent (although the exposure to a threshold dose in each susceptible individual is needed) and has a longer latency period (from days to a few months)\(^1\). Recently, a third type of DILI—indirect DILI—has been described as a liver injury caused by the mechanism of action of the drug (what it does; i.e., rituximab by depletion of B cells reactivates silent hepatitis B virus which is responsible for the damage), in contrast to its idiosyncratic properties (what it is; i.e., the unexpected hepatotoxic reaction of amoxicillin-clavulanate)^2\).

iDILI is an uncommon event, with an incidence of less than 1:1000–1:10,000\(^3\)–^6\) in the general population, mostly detected in phase 3 trials or after-market release. It remains a major diagnostic and health challenge, being one of the most frequent reasons for drug withdrawal from the market\(^7\). iDILI also remains a major public health problem, since iDILI reactions represent a significant fraction of acute liver failure (ALF)^8\). Currently, there are more than 1000 drugs with iDILI potential, and the list is growing^7\).

iDILI may resemble acute and chronic liver diseases, and symptoms can include fever, nausea, vomiting, jaundice, dark urine and itching. In terms of severity, iDILI manifests a wide range of potential outcomes. Most patients make a full recovery after discontinuation of the culprit drug, but the progression of DILI is sometimes dramatic. In fact, up to 10% of patients with drug-induced jaundice fulfilling “Hy’s Law” criteria will go into ALF^9\). Some of these patients will require a liver transplantation or ultimately die, and others will develop chronic DILI. In addition, recently it has been observed that both the extent of drug metabolism and the levels of total bilirubin (TBIL) and alkaline phosphatase (ALP) at iDILI presentation, iDILI is roughly classified as hepatocellular when ALT or AST is hepatocellular, or cholestatic when ALP is °2 x ULN alone or if R-ratio is °5, mixed if the ratio is 2–5\(^12\). Age is a clear determinant of iDILI phenotype, with older age more frequently showing the cholestatic phenotype across different iDILI cohorts^14\).

However, these traditional biomarkers are not specific for iDILI. Therefore, iDILI is a diagnosis of exclusion, frequently assessed using the Roussel Uclaf Causality Assessment Method (RUCAM)/Council for International Organizations of Medical Sciences (CIOMS) scale. This scale includes a number of variables to determine the causality of a drug in an episode of hepatotoxicity, like temporal sequence, rechallenge, risk factors, exposure to other drugs and ruling out other causes of acute liver injury and re-exposure. RUCAM/CIOMS uses a continuous scoring system with seven major domains. A summation of both positive and negative points yields a total score which translates into categories of likelihood of iDILI (°1 excluded; 1–2 unlikely; 3–5 possible; 6–8 probable; and °9 highly probable)^12,15\).

REVThe pathogenesis behind this adverse drug reaction is believed to arise from the interaction between drug properties and host factors. The most widely agreed theory on the development of iDILI is the so-called “multiple determinant hypothesis”, according to which the interaction and intersection in time of different factors such as drug, gender, age, genetics, immune tolerance imbalance, microbiota and environmental and physiological determinants may increase the susceptibility to iDILI^16\). However, neither the host genetic factors nor the formation of chemically reactive metabolites (CRM) alone is capable of predicting the onset of iDILI. Moreover, both drug properties and host factors influence the probability of delayed onset of iDILI^17\). Therefore, numerous features must be influencing the susceptibility of individuals to the intrinsic toxic properties of drugs. iDILI mechanisms would include a multistep and multicellular disease process involving a plethora of molecular pathways^18\) (Fig. 1).

Given the lack of knowledge that remains in the field of iDILI prediction, diagnosis and outcome, the search for new models to predict and further understand pathophysiological mechanisms of iDILI is necessary, since a significant number of drugs causing iDILI escapes from detection in current preclinical testing during drug development. The assessment of risk for iDILI associated with a drug is still an iterative process based on accumulation of clinical data.

Therefore, the use of preclinical human-relevant in vitro models to study iDILI pathophysiology and reduce risks in drug development has become a necessity. Moreover, there is no widely accepted animal model and none of the existing in vitro and in silico models of hepatotoxicity are approved by the regulatory agencies for preclinical drug development.

Thus, in this review we focus on in vitro human cell-based culture models and in vivo animal models, highlighting the importance of patient-derived cellular models, also briefly addressing the existing in silico models. The spotlight is now on human liver cells as opposed to animal liver cells, since the latest are ultimately non-predictive of human iDILI for many drugs due to interspecies differences in drug-metabolizing enzymes and transporters (DMETs)^19\).
2. **In vitro models to assess toxicity pathways**

An *in vitro* model is defined as a test system, usually cell-based, displayed in a two-dimensional (2D) or three-dimensional (3D) configuration, in which specific endpoints or biomarkers are examined. These models are used during preclinical drug development to better understand the mechanisms of liver toxicity, to eliminate toxic compounds from the pharmaceutical pipeline and/or to design better molecules that ultimately would reduce attrition rates in clinical trials.

Human cellular models are currently the most used to study the pathophysiology of liver diseases and the iDILI potential of specific drugs, since they have shown more promising predictive power in identifying iDILI risks than animal cells. Table 1 summarizes the different human *in vitro* models used to evaluate liver pathophysiology and drug toxicity.

2.1. **Single-cell type in vitro models**

Single cell-type assays are still one of the most used to study cell injury mechanisms that lead to liver toxicity, despite hepatotoxicity being a complex and multilayer disorder.

2.1.1. **Primary human hepatocytes (PHH)**

The main event in the pathogenesis of DILI and most liver diseases is the death of hepatocytes. Therefore, and because they are able to retain some human-specific characteristics, PHHs cultures are considered the most suitable cell model for the establishment of liver cell culture models. PHHs can be obtained either from isolation of liver resections, liver tissues not suitable for transplantation obtained from surgical interventions or cryopreserved from commercial suppliers.

PHHs from liver biopsies extracted from living patients can be cultured in suspension for at least a few hours. Nevertheless, longer-term culture requires adhering hepatocytes to an extracellular matrix (ECM). Currently, the most popular culture configuration is a confluent monolayer of PHHs on adsorbed collagen-I (ECM sandwich cultures). Cell culture configurations will be discussed in the next sections.

PHHs do not survive in standard 2D culture for a long period of time, losing their morphology, metabolic activity and liver-specific functions over a few days. Therefore, there are difficulties in reproducing the pharmacokinetics of liver drug exposure in cultured PHHs, which restricts the types of assays that can be performed on these cells. In most cases, cells are exposed to potentially toxic compounds for a short time, which is not the best approach, since iDILI is a delayed response that usually occurs after long exposure to the drug. Indeed, in a study comparing different cell models for prediction of iDILI, PHHs were able to predict iDILI only when the exposition was made for 72 h, but not at 24 h, and correcting each drug dose with *in vivo*-observed $EC_{50}/C_{max}$. This study also highlights the poor performance of cell models in iDILI prediction. In the case of PHHs, one of the limitations that difficult the prediction of iDILI is that these cells greatly vary between donors and the protocol to obtain them from patients is difficult and invasive. The donor variability is a double-edged sword; on one hand, the relevance of interindividual variations in genetic polymorphisms can be explored; on the other hand, donor differences and cell alterations after isolation can lead to variance in experimental results and poor reproducibility.

Despite these disadvantages, the use of PHHs for liver diseases modelling and pharmaceutical drug screening has increased in the last years, thanks to advances in cryopreservation and extraction of cells that allow a more successful recovery and seeding of the hepatocytes after thawing.

Moreover, numerous efforts have been made to expand PHHs longevity and stability *in vitro* such as media supplements including nicotinamide and dimethyl sulfoxide (DMSO) together with different growth factors.

Recently, Upcyte, a new technology to expand the lifespan of PHHs, has been established. It is based on the transduction of hepatocytes with proliferation-inducing genes. Hepatocytes generated using Upcyte technology have better proliferation properties than PHHs and maintain functional phase I and II activities. Upcyte hepatocytes from different donors have already been used to screen the hepatotoxic potential of several compounds. However, as with any other cell model, results must be taken cautiously due to differences in protein expression compared to *in vivo* tissues. For instance, Upcyte hepatocytes have a significantly lower NADPH-cytochrome P450 reductase activity, and low expression of proteins of sinusoidal solute carrier transporters (except for NTCP and OATP2B1). In contrast, Upcyte cells have a well-preserved expression of canicular efflux pumps proteins, and two-dimensional sandwich configuration preserves the expression of organic anion-transporting polypeptide OATP1B1/SLCO1B1, OATP2B1/SLCO2B1, NTCP/SLC10A1, and OCT1/SLC22A1.

2.1.2. **Immortalized liver-derived cell lines**

To overcome the problems derived from using PHHs, cell lines established from hepatocellular carcinomas or immortalized liver cells are some valuable alternatives. These cells present several advantages, such as their availability, easy handling, stable phenotype and unlimited propagation potential, being useful tools for the study of the molecular and cellular mechanisms of liver injury. However, their specific use as predictive models of iDILI is limited due to the lack of some hepatocyte functions and low metabolism activity, since these cells show low endogenous levels of DMETs. Moreover, different batches of hepatoma-derived cells could contribute to inter-laboratory differences in the detection of hepatotoxicants.

Multiple hepatocyte-derived cellular carcinoma cell lines have been established over the years, such as HepG2, HepaRG and HuH7 (Table 1).

2.1.2.1. **HepG2 cells.**

HepG2 cell line was established from a liver tumor biopsy in 1979. It is the most frequently used hepatoma cell line in the testing of drugs and research of liver diseases, since these cells share the morphology of hepatocytes, retain some liver functions and secrete plasma proteins such as albumin and α-fetoprotein.

However, there are some drawbacks to using these cells as models. HepG2 cells exhibit low expression and activity levels of DMETs, although they may vary depending on the culture conditions and the assay procedures. Therefore, these cells may be acceptable for testing toxicity of parent drugs, but not drugs metabolically activated into toxic metabolites. This detriment can be partially overcome by transfecting with vectors containing relevant metabolic enzymes. Engineered HepG2 cells generated by adenoviral-mediated cytochrome P450 (CYP) transduction have also been proposed for hepatotoxicity studies, since adenoviral transduction allows the controlled expression of different CYP profiles in cells. However, it
is important to consider that overexpression of heterogeneous DMETs may distort the results of the assays.

2.1.2.2. HuH7 cells. HuH7 cell line was established from a hepatocellular carcinoma in 1982. As the other carcinoma-derived cell lines abovementioned, these cells retain liver functions, secrete plasma proteins and express DMETs, some of them at higher levels than other cell lines such as HepG2. The expression and activity levels of DMETs in these cells are elevated, although they may change depending on the culture conditions (e.g., DMSO exposition and cell confluency). HuH7 cells have been used to investigate the pathogenic mechanisms and possible therapies for different liver diseases, like metabolic associated fatty liver disease (MAFLD) and to study CYP-mediated drug metabolism.

2.1.2.3. Other hepatoma-derived cell lines. Hep3B and THLE are other hepatocellular carcinoma-derived cell lines that have not been widely used in toxicity testing, as they present low expression and activity levels of DMETs. However, THLE cells have been engineered to increase the expression of human CYP, allowing the exploration of the role of CYP-mediated metabolism in hepatotoxicity.

Other hepatocellular carcinoma-derived cell lines, such as BC2 cells and Fa2N-4 cells, have not been applied in drug toxicity testing yet, principally due to the need for further evaluation regarding the low expression of some DMETs, which compromises their application in iDILI research.

In short, immortalized cell lines in general are not an ideal option for modeling iDILI due to their limited metabolic capacities.

2.1.3. Hepatocyte-like cells (HLCs)

The use of HLCs derived from embryonic stem cells (ESCs), mesenchymal stem cells (MSCs) or human induced pluripotent stem cells (hiPSCs) represents a promising alternative to PHHs to model liver diseases in vitro and particularly iDILI (Table 1). They have been engineered to increase the expression of human CYP, allowing the exploration of the role of CYP-mediated metabolism in hepatotoxicity.

Figure 1  Current overview of idiosyncratic drug-induced liver injury general mechanisms. Considering that drug-related factors affect the responses that will occur within the host, some general mechanisms for idiosyncratic drug-induced liver injury have been proposed. First, the drug can be metabolized by hepatic enzymes, resulting in chemically reactive metabolites (CRMs). Parental or CRMs can directly damage hepatocytes at many subcellular levels, such as cytosol or mitochondria (resulting in oxidative stress), endoplasmic reticulum (ER, resulting in increased stress that leads to activation of the unfolded protein response, [UPR]) or lysosome (compromised liability). CRMs may also cause alterations in bile acids (BAs) homeostasis, which can lead to cholestatic injury development and eventually, to hepatocyte death. Cell damage can result in different outcomes. CRMs bind and alter several proteins, generating new drug-modified self-antigens that are probably released in exosomes. Damage-associated molecular patterns (DAMPs), such as high mobility group box 1 (HMGB1), are released into the intercellular space. Liver damage can also occur directly, as damaged hepatocytes can undergo a process of apoptosis/necrosis. On the other hand, pathogen-associated molecular patterns (PAMPs) may reach the liver as a consequence of an increase in intestinal permeability caused by alterations in intestinal microbiota. Innate immune cells, such as Kupffer cells or antigen presenting cells (APCs) process self-antigens and start an inflammatory response after DAMPs and PAMPs stimulation. The resulting inflammatory response may kill hepatocytes, causing liver injury. The environment created by the release of different factors recruits other immune cells, such as monocytes and neutrophils, which maintains the inflammation. APCs travel to lymph nodes and activate CD4+ helper T cells. Activation of CD4+ T cells can result in immune tolerance if the antigen binding is not strong enough or even in the resolution of the episode with a mild injury. If the hepatotoxic conditions are not restored, CD8+ cytotoxic cells are activated, and different populations of T effector cells are expanded, leading to an adaptive immune response (that might also be induced by pathways not involving upstream inflammation). CD8+ T cells may recognize self-antigens presented by the hepatocytes through major histocompatibility complex 1 (MHC-1) and kill them, thus contributing to the liver injury. These processes result in the massive release of DAMPs. By binding to receptors expressed by liver immune cells, DAMPs often exert potent immunostimulatory functions, hence aggravating the ongoing inflammatory response. In this setting, circulating immune cells keep being stimulated to secrete a wide panel of pro-inflammatory and hepatotoxic factors, resulting in a vicious cycle connecting inflammation and cell death that mediates severe hepatotoxic effects.

(This figure has been created using Biorender.com.)
| Model type                        | Configuration                                                                 | Advantage                                                                                           | Disadvantage                                                                                          | Endpoint                                                                 | Ref. |
|----------------------------------|-------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------|------|
| Single cell-type *in vitro* models | Primary hepatocytes                                                           | Direct study of the patient’s liver; ECM sandwich and spheroids culture allow long-term culture and maintain secretion of organic compounds, DMETs activities and secretion rates | Great variation between donors and difficult to obtain. In monolayer, do not survive over long periods of time | Cell death, drug metabolism, drug—drug interactions, cholestasis       | 20–22|
| Immortalized liver-derived cell lines | HepG2                                                                         | Availability and easily handling, hepatocyte morphology, liver functions, unlimited propagation potential, interdonor variability | Lack of some hepatocyte functions, low metabolism activity (↓ DMETs), genotypic instability, single donor information | Need for glucose manipulation, ↓ DMETs                                | 23–25|
|                                  | HepaRG                                                                        | Supports HBV infection, stable expression of many DMETs, can be differentiated into hepatocyte-like and biliary epithelial-like cells |                                          | Cell death, oxidative stress, mitochondrial damage, ER stress, cholestasis, phospholipidosis, steatosis, HCS | 26,27|
|                                  | HuH7                                                                           | Plasma proteins secretion and expression of some DMETs                                             | Highly heterogeneous                                                                         | Drug metabolism                                                       | 28,29|
|                                  | Hep3B                                                                          | Metabolically competent and with active CYPs                                                        | ↓ DMETs                                                                                 | Cell death, mitochondrial damage, ER stress                             | 30–32|
|                                  | THLE                                                                           | Support adenoviral-mediated CYP transduction                                                        | ↓ DMETs                                                                                 | Cell death, drug metabolism, mitochondrial function                   | 33,34|
| Hepatocyte-like cells            | HLCs derived from hESCs                                                        | Unlimited propagation potential, self-renewal potential, capacity for acquiring hepatocyte-like morphology | Capacity for urea synthesis, drug metabolism, lipid synthesis, albumin secretion              | Cell death and drug metabolism                                        | 35,36|
|                                  | HLCs derived from MSCs                                                          |                                                                                                     | Expression of DMETs, easy to gather from patients                                            | Metabolism, oxidative stress                                         | 37   |
|                                  | HLCs derived from hiPSCs                                                        |                                                                                                     | Allow the study of interindividual differences, avoidance of liver biopsies                  | Cell death, metabolism, cholestasis                                  | 38–40|

(continued on next page)
| Model type                  | Configuration                                             | Advantage                                                                 | Disadvantage                                                                 | Endpoint                                                                 | Ref.  |
|----------------------------|-----------------------------------------------------------|---------------------------------------------------------------------------|------------------------------------------------------------------------------|---------------------------------------------------------------------------|-------|
| Multiple-cell type in vitro models | 2D/3D, co-culture models, spheroids                      | Great representation of the phenotype and environment of liver, closer to in vivo conditions | Low availability of human liver NPCs cultures, difficult to expand cells in vitro, losing characteristic phenotypes in a few days, random distribution favors monolayer areas with suboptimal cell–cell interactions | Drug metabolism, hepatotoxicity mechanism, multicellular interactions on drug outcomes | 41–43 |
| Hepatocytes-fibroblasts co-cultures | 2D, co-culture models                                    | Extended cell viability (typically up to 6 weeks) with retention of in vivo-like hepatocyte functions in culture | Use of mouse fibroblasts as co-culture cell, may cause misunderstanding with immunologic studies that use only cells with human features | Cell death, drug–drug interactions, hepatotropic pathogens, study of DMETs | 44–46 |
| Precision-cut liver slices | 3D biological structure                                  | The closest representation of intact liver architecture; Contains all the relevant liver cell types and maintains cell–cell interactions; Expression ADME-related genes, allowing use as a toxicity model for the human liver | Low viability in culture and heterogeneous distribution of drugs through the different layers, not amenable to HCS | Drug behavior, drug-mediated CYP induction/inhibition assessment, study DMETs, iDILI mechanisms, fatty liver model, fibrosis model | 47,48 |
| 3D bioprinted liver        | 3D bioprinted biological structure                      | Prolonged culture periods with normal hepatocyte function and viability   | Difficult to precisely manipulate cells. Not reproduce the subtle anisotropy of the liver. Difficult to control medium changes | Liver fibrosis model, drug metabolism, toxicity testing Cell death, drug metabolism, drug-induced ROS formation and GSH depletion, drug toxicity | 49,50 |
| Liver microphysiological systems | 3D/chip (Integrated circuit)                      | Interaction between different tissues compartments, perfusion of cultures, round cell aggregates, in vivo-like medium to hepatic cells | Need validation to confirm its potential to predict iDILI better than the simpler in vitro models already established | Cell death, drug toxicity, Interactions between different tissues following drug exposure, drug toxicity | 51–53 |
| Multi-organ chips          | 3D/chip (integrated circuit)                            | Allow tissue–tissue interactions, supply fluid flow and recreate mechanical marks. Study different compartments of the organ | Can exhibit significant variation and inconsistency between different manufacturing batches in the same group |                                                                       | 54–56 |

2D, two-dimensional; 3D, three-dimensional; ADME, absorption, distribution, metabolism, and excretion; BSEP, bile salt export pump; CYP, cytochromes P450; iDILI, idiosyncratic drug-induced liver injury; DMETs, drug-metabolizing enzymes and transporters; ECM, extracellular matrix; ER, endoplasmic reticulum; GSH, glutathione; HBV, hepatitis B virus; HCS, high-content screening; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; HLCs, hepatocyte-like cells; MSCs, mesenchymal stem cells; NPCs, non-parenchymal cells; PHHs, primary human hepatocytes; ROS, reactive oxygen species; ↓, decreased.
contain the same genetic composition as the human donor cells, which makes them suitable for multiple applications, including the study of interindividual variations, personalized medicine and drug screening. 

2.1.3.1. HLCs derived from ESCs. Human embryonic stem cells (hESCs) are pluripotent cells that can be used as a cell source for transplantations and as a tool in human embryogenesis research. The first report demonstrating the ability of differentiation and isolation of HLCs from hESCs was published in 2003. Since then, HLCs derived from hESCs have gained importance as a possible new model to determine the metabolic and toxicological properties of drugs and different protocols have been established to obtain mature and clinically safe cells.

HLCs differentiated from hESCs have hepatic characteristics very similar to those of PHHs, such as their ability to synthesize urea, drug metabolism, lipid synthesis and albumin secretion. Despite the limited number of hESCs available and the ethical concerns in generation and usage, they have been widely used to model different liver diseases, such as HBV infection.

Nevertheless, great variability in DMETs activities has been reported by different laboratories; therefore, there is still much work to do to achieve stable functional cells for regular use in toxicity studies.

2.1.3.2. HLCs derived from mesenchymal stem cells (MSCs). MSCs are derived from human bone marrow, human adipose tissue or human umbilical cord tissue, and have been described as able to undergo hepatocyte differentiation despite its mesenchymal nature. Recently, Miranda’s laboratory established a hepatic differentiation protocol for deriving HLCs from human neonatal MSCs. This model showed levels of drug transporters and phase I and II enzymes expression and function comparable with primary hepatocytes and HepG2 cells. 3D models have improved human neonatal MSCs-HLCs biotransformation capacity, providing more reliable models for pathophysiology studies and more predictive systems for in vitro hepatotoxicity applications.

2.1.3.3. HLCs derived from iPSCs. iPSCs, like ESCs, can be differentiated into each of the three germ layers. They can be derived from adult somatic cells (blood, skin cells) through the ectopic expression of selected genes, using lentiviral vectors, nonintegrating episomal vectors, mRNAs, transient expression plasmids or transcription factors.

Notably, hiPSCs can be used to generate patient-derived HLCs through endoderm induction, hepatic specification, hepatoblast expansion and hepatic maturation, using specific growth factors. These patient-derived HLCs are being used to screen patient-tailored drugs with the advantage that no liver biopsies are needed. However, liver functions of HLCs derived from hiPSCs remain very low relative to adult PHHs and do not exhibit an adult-like range of CYP expression. The generation of a mature phenotype of these cells still needs to be improved. The use of HLCs in liver diseases modeling is also limited by the difficulty to produce large quantities of these cells and lack of validation. In fact, standardization of the production of HLCs derived from hiPSCs is still necessary to obtain cell lines suitable for comparing toxicological assays between laboratories. The generation and application of these HLCs should be reproducible and its pharmacological phenotype should be characterized using a defined panel of training compounds.

Despite the necessary validation, HLCs differentiated from hiPSCs have been used to model different liver diseases. In a recent work of Imagawa et al., human iPSCs were generated from two patients with BSEP-deficiency, and then differentiated into HLCs. The authors observed that in the BSEP-deficiency-iPSC-derived HLCs, BSEP was not expressed on the cell surface and the biliary excretion capacity was significantly impaired compared to control iPSC derived HLCs. These results suggested that patient-specific HLCs could be a promising model to study BSEP-deficiency and cholestasis in the future.

It has been recently observed that the iPSCs-derived HLCs obtained from two Alpers–Huttenlocher syndrome patients are more sensitive to valproic acid (VPA)-induced mitochondrial apoptosis than control iPSCs-derived HLCs. This increased sensitivity could explain the augmented risk of developing VPA toxicity in Alpers–Huttenlocher syndrome patients. With this work, Li et al. showed that patient-derived HLCs can be used as a toxicity model as well as drug/therapies screening platform.

2.2. Multiple-cell type in vitro models

In vitro assays focusing only on one cell type are unlikely to offer the optimal approach to study liver diseases pathophysiology. Hepatocytes cultures are not completely representative of in vivo cellular behavior, since the in vivo metabolism of drugs is poorly developed in cell lines, and commonly multiple cell types have a role in the development of adverse effects. Therefore, much progress has been made in cell cultures consisting of multiple cell types, such as liver non-parenchymal cells (NPCs) or fibroblasts or immune cells (Table 1).

2.2.1. HepaRG cells

HepaRG cell line was described for the first time in 2002, as a cell line exhibiting hepatocyte-like morphology, expressing specific hepatocyte functions and supporting hepatitis B virus infection. Two weeks after reaching confluency, HepaRG cell cultures form a monolayer of a mixed population of 2 cell types: hepatocyte-like colonies and epithelial cells corresponding to primitive biliary cells. When further cultured from this point in the presence of 1.7% DMSO, HepaRG cells undergo complete hepatic differentiation program, showing hepatocyte-like phenotype with elevated mRNA expression of many DMETs, which makes them a good model for hepatotoxicity studies. However, the presence of DMSO may alter the results of toxicological studies, since DMSO is a free radical scavenger, and therefore its use might alter results in situations where reactive oxygen radicals are produced during a cytotoxic response.

HepaRG cells show several advantages in comparison to other hepatocellular carcinoma cell lines: they show activity levels of phase II enzymes, phase III transporters and nuclear receptors comparable to PHHs. More interestingly, HepaRG cells show a stable expression of these proteins in time, which makes this model suitable to perform long-term studies, fitting very well in iDILI investigations.

HepaRG cell lines could be a valuable cell model to study the mechanism of action of drugs causing intrahepatic cholestasis (aberrations in the physiological flow of bile), as the cells maintain polarity, regulation of transporters expression and production of bile acids (BAs), as well as steatosis in vitro, since lipogenesis has been detected in HepaRG cells.
2.2.2. Hepatocytes-NPCs co-cultures
Interactions between hepatocytes and NPCs of the liver (liver sinusoidal endothelial cells [LSECs], Kupffer cells [KCs], hepatic stellate cells [HSCs] and cholangiocytes) are essential in hepatic differentiation and the correct function of the liver, playing a role in modulating hepatic functions and responses to several drugs\textsuperscript{105}.

Results from assays using co-cultures of hepatocytes with NPCs suggest that multiple-cell type models better show the phenotype and environment of liver, closer to \textit{in vivo} conditions\textsuperscript{48,49}. However, the culture techniques of NPCs are not as studied as those of PHHs: the availability of human liver NPCs cultures is still low and some of the cells, such as LSECs and HSCs, are difficult to expand \textit{in vitro}, losing their characteristic phenotypes in few days\textsuperscript{106}. The choice of a convenient cell type is important to enable high levels of liver function for longer time, as not all NPCs induce the same level of functions and stabilization in PHHs\textsuperscript{107}.

It is important to note that randomly distributed multicellular co-cultures contain areas of suboptimal monolayer cell—cell interactions, leading to instability and low levels of liver functions over long-term culture. In order to increase the area of interaction between cells, protocols have been designed to create heterogeneous plates with surfaces of varying sizes. This strategy is used in so-called micropatterned co-cultures (MPCCs), such as those formed by primary hepatocytes and NPCs, which have been used to study drug metabolism, among other applications\textsuperscript{41,108}.

Moreover, 3D spheroidal configurations of hepatocytes coculture with NPCs could facilitate the study of multicellular interactions on drug outcomes. For example, primary hepatocytes-NPCs spheroids have been used to detect hepatotoxicity of different drugs showing higher sensitivity than primary hepatocytes monolayer cultures\textsuperscript{109}.

2.2.3. Hepatocytes-fibroblasts co-cultures
HepatoPac\textsuperscript{2} is the most widely used micropatterned hepatocyte-fibroblast co-culture setup. In this system, human hepatocytes are supported by mouse embryonic 3T3 fibroblasts. It extends cell viability (typically up to 6 weeks) with retention of \textit{in vivo}-like hepatocyte functions in culture\textsuperscript{44}. However, the use of mouse fibroblasts as co-culture cells may interfere with immunologic studies that use only human cells with human features\textsuperscript{45}.

HepatoPac\textsuperscript{2} cultures have been used to test different drugs with known iDILI potential, obtaining a 66% sensitivity and 90% specificity. When testing the most iDILI concerning drugs, with at least two hepatocyte donors, the multicellular cultures displayed a sensitivity of 100%\textsuperscript{107}. This model has been also used to study the interplay of enzymes and transporters and the modeling of drug—drug interactions\textsuperscript{111}, and to predict the \textit{in vivo} clearance of commercially available compounds\textsuperscript{112}.

2.2.4. Precision-cut liver slices (PCLs)
PCLs are the closest representation of an intact liver architecture with all the relevant cell types of the liver. As an \textit{in vitro} model, PCLs maintain cell—cell interactions\textsuperscript{112} and show stable expression of drug absorption, distribution, metabolism and excretion (ADME)-related genes, allowing their use as a toxicity model for the human liver\textsuperscript{2}. Human PCLs can be used to study the consequences of inhibiting different cellular pathways (\textit{i.e.}, mitochondrial $\beta$ oxidation\textsuperscript{113}), to assess drug-mediated CYP induction/inhibition\textsuperscript{114}, hepatic transporters function\textsuperscript{115} and iDILI mechanisms related to inflammatory stress and to detect potential biomarkers\textsuperscript{116}.

Unfortunately, this technology shows low viability in culture and drugs are often heterogeneously distributed. Nevertheless, PCLs are thought to be the most physiologically complete \textit{in vitro} model for liver research\textsuperscript{116}.

2.3. Culture configurations

The diagram depicted in Fig. 2A illustrates the increasing complexity of the different cell culture configurations, as well as key features of the simplest and most complex setups.

2.3.1. 2D (conventional) cultures
Culture of PHHs and hepatoma-derived cell lines in conventional formats has been carried out for many years and has provided several advances in the field of liver diseases modeling (Fig. 2B).

The simplest culture configuration is a monolayer of hepatocytes plated on a rigid substratum pre-treated with ECM proteins, such as collagen, fibronectin or matrigel\textsuperscript{9}, showing a flattened morphology\textsuperscript{117}. Although PHHs in monolayer culture maintain key hepatic-specific functions, cells undergo rapid changes in their morphology, structure, polarity and bile canaliculi formation once they are plated, displaying a drastic loss of hepatic functionality over time. To prevent hepatocytes from going through some of these unwanted changes, they can also be displayed in a sandwich configuration, in which a confluent hepatocyte monolayer attached to collagen-1 is overlaid with another gelled ECM. This arrangement is used to study hepatobiliary drug transport, transport proteins regulation, drug—drug interactions and hepatotoxicity in long-term cultures, since it slows down the functional decline of hepatocytes compared to the simple monolayers, restores a similar polygonal morphology to that of \textit{in vivo} cells, enhances both secretion of organic compounds and DMETs activities, mimics \textit{in vivo} biliary excretion rates\textsuperscript{118} and enables the development of polarized cell surface domains over several days in culture\textsuperscript{119}.

Other parameter that can be modulated is how to plate the cells. In contrast to random seeded monolayer cultures, MPCCs have raised to control both cell—ECM and cell—cell interactions. For example, MPCCs have been observed to be beneficial to functionally mature HLCs derived from hiPSC and stabilize their phenotype\textsuperscript{120}.

2.3.2. 3D cultures
Morphology and 3D configuration are very important for hepatocytes function, due to their polygonal shape and multipolarization (showing at least two basolateral and two apical surfaces). However, conventional 2D cultures lack a suitable microenvironment capable of maintaining the physiological configuration of hepatocytes. Therefore, much effort is now being directed to develop different physiologically relevant \textit{in vitro} systems, such as 3D multicellular tissue chips, microfluidic systems and organ-on-a-chip systems (Fig. 2C).

3D configurations constitute an improvement over conventional systems for iDILI modelling, as cells can be maintained in culture for prolonged periods while remaining viable and retaining enhanced metabolic activity. This makes 3D cell cultures amenable to routine use and high-throughput adaptation. However, none of them are routinely used by the industry yet, likely due to the high complexity of these culture systems, which difficult the automatization of the cultures. In addition, 3D cultures are more expensive to maintain than 2D cultures, and imaging studies represent a challenge when dealing with 3D structures where the Z.
Figure 2  Overview of *in vitro* liver model configurations. (A) Flow diagram showing key features of *in vitro* hepatic cultures ranging from the simplest to the most complex. (B) Main 2D culture configurations used to study liver diseases include: 1) Suspension culture configuration, where hepatocytes are cultured in a liquid medium; 2) Monolayer configuration, where hepatocytes grow attached to a solid substratum pre-treated with ECM proteins; 3) Sandwich configuration, where confluent monolayer of hepatocytes cultured over a collagen-I matrix is covered with a gelled ECM layer; and 4) Micropatterned co-culture configuration, where hepatocytes are cultured over adsorbed collagen islands and fibroblasts are added to the plates. (C) Main 3D culture configurations used to study liver diseases include: 1) Spheroids, that can be cultured using different methods, *i.e.*, self-aggregation of hepatoma-derived cell lines or hanging drop system; 2) 3D bioprinted liver configuration, where hepatic stem cells, hepatocytes and NPCs are printed onto 3D scaffolds; 3) Liver-on-a-chip configuration, where the liver environment is simulated through molecules exchange between compartments, co-culture of different cell types and physiological oxygenation and shear stress reproduction; and 4) Multi-organ chip configuration, where several organs-on-a-chip are interconnected with a liver-on-a-chip. (This figure has been created using Biorender.com.)
axis must be taken into account. Also, studies aimed at assess the reproducibility of assays involving 3D cultures are still needed.

3D cultures range in complexity from spheroids to 3D scaffold systems and more advanced models using microfluidic in vitro systems (organ-on-a-chip models).

- **Spheroids**

  Hepatic cells can form small adherent spheroids when they are seeded directly on a gelled layer. Spheroids can be generated from self-aggregating hepatoma-derived cell lines, primary hepatocytes or HLCs derived from hiPSC with or without supporting NPCs.

  The simplest model for culturing spheroids is to seed primary hepatocytes and NPCs in 96-well plates, so spheroids will form by self-aggregation of the cells after centrifugation. This method allows obtaining hepatocyte spheroids similar to human liver hepatocytes based on their transcriptomic and proteomic signatures and their functionality. However, the random size distribution results in necrosis within the centers of spheroids due to accumulation of BAs and diffusion limitations of nutrients and oxygen. To overcome this issue, different bioreactors can be used to form uniform 3D spheroids, allowing an adequate oxygen diffusion between the hepatic cells, avoiding BAs accumulation.

  A different method to generate hepatocyte spheroids is the hanging drop system, in which gravitational force is used to allow cells to assemble into spheroids in a hanging droplet using specialized plates. The cells form the spheroids of controlled diameters, which produce ECM de novo. These spheroids can be transferred to different multi-well plates for drug screening or toxicity testing.

  Spheroids have been shown to promote higher functions in hepatocytes and increased sensitivity over liver toxicants compared to conventionally cultured hepatocytes, due to the establishment of homotypic cell–cell contacts and the presence of key ECM components within and around the aggregates. By using spheroids, Takayama et al. generated HLCs derived from hiPSC more sensitive to assess the hepatotoxicity of drugs than other conventional liver cellular models such as HepG2 cells.

  One of the advantages of spheroid systems is the possibility of establishing 3D co-cultures of human hepatocytes with NPCs, showing both polar hepatocyte structures and heterotypic cell–cell contacts. The purpose of this system is to obtain lobular structures with apical-basal polarization of hepatocytes. Kostadinova et al. established a human in vitro 3D liver co-culture system containing hepatocytes and NPCs, which maintained liver function and inducible CYP for up to 3 months, formed bile canaliculi-like structures and responded to inflammatory stimuli. Leite et al. have recently published a drug-induced fibrosis model using 3D spheroids consisting of HepaRG cells and primary HSCs. These hepatic organoids showed fibrotic features, such as HSC activation, collagen secretion and deposition after treating them with pro-fibrotic compounds and it has been the first model capable of detecting both hepatocyte-dependent and compound-induced HSC activation.

  Finally, hepatic spheroids can also be generated through isolation and expansion of stem and progenitor cells from hepatic stem cell niches from liver biopsies. Spheroids obtained through this method have shown indefinite capacity for self-renewal while maintaining physiological characteristics from the donor. However, this model requires collection of a liver biopsy from patients/donors, which is an invasive technique and therefore ethically concerning. Up to our knowledge, spheroids generated from human biopsies have not been used in drug-screening and toxicity assays.

- **3D bioprinted liver**

  3D printing is a process whereby biological materials are usually printed onto 3D scaffolds. In case of bioprinted 3D liver tissues, laser technology has been used to transfer hepatocytes and NPCs onto inserts of 24-well culture plates using specific media and matrix. The bioprinted livers maintain normal hepatocyte function and viability for long culture periods. In addition, 3D bioprinted liver tissues are able to detect toxicity more efficiently than 2D culture and conventionally used spheroids for a variety of drugs. The first commercial human liver tissue for assessment of drug hepatotoxicity was the exVive3D™ model, which is a bioprinted liver consisting of primary hepatocytes, HSCs and endothelial cells. exVive3D™ shows a similar structure and aceterminophen (APAP)-associated damage to native liver tissues. Other 3D bioprinted livers have also been used to model compound-induced fibrogenesis and have recently been proposed for chronic hepatotoxicity studies in vitro.

- **Liver-on-a-chip**

  Advances in microfluidic engineering have recently made possible to create miniaturized in vitro cell culture systems, known as organs-on-chips. In these chips, or so-called liver microphysiological systems (MPS), different tissue compartments can interact with each other through the sharing of secreted molecules. In comparison to static models, perfusion of cultures permits continuous nutrient exchange, better oxygen delivery and physiologic shear stress. These systems promote round cell aggregates that are more similar to in vivo morphology, increasing and maintaining liver-specific functions.

  Several groups have created modular livers-on-a-chip using primary hepatocytes, HLCs derived from iPSCs and HepG2 cells. Interestingly, Leclerc’s group has designed a liver-on-a-chip platform for evaluating drug metabolism in primary hepatocytes and investigating drug-induced reactive oxygen species (ROS) formation and glutathione (GSH) depletion in HepG2/HepC3a cells.

  Very recently, Rubiano et al. tested the reproducibility of drug toxicity assays using a liver MPS. Using trovafloxacin, the authors showed that the liver MPS was able to reproduce the hepatotoxict effects of the drug (increased lactate dehydrogenase [LDH] and reduced CYP3A4 activity), with different batches of KCS. Interestingly, this study showed that primary hepatocytes are more functionally stable in this configuration than in other platforms.

  Although the organs-on-a-chip have opened up the possibility to test the interaction between drugs and the host-specific factors in a complex, patient-specific model, in depth validation is needed to confirm its potential to predict iDILI more reliably than the simpler in vitro models already established.

- **Multi-organ chips (MOC)**

  MOC platforms with microfluidic perfusion are being developed to investigate interactions between different tissues following drug exposure. Viravaidya et al. designed a MOC...
model with cell lines derived from lung, liver and fat to investigate drug biodistribution. Bricks et al. have created a platform in which HepG2/HepC3A cells (liver unit) and Caco-2 cells (intestinal unit) are used to study transport and metabolism of drugs. Moreover, Wagner et al. designed a MOC with co-cultures of human artificial liver microtissues and skin biopsies, to study liver–skin interactions. Recently, Oleaga et al. have established a four-organ chip. This chip combined cardiac, muscle, neuronal and liver units and was used to evaluate the multiorgan toxicity response to different drugs. The key advantage of this approach is the use of the microfluidic technology, which mimics the blood flow, with the possibility to study several organs at the same time. Thus, involvement of other tissues functioning in addition to the liver can be explored, such as differences in drug metabolism, absorption or toxic effects. The novelty of MOCs, the high cost of platforms integrating these cultures and the complexity in scaling up to correctly modeling interactions between different human tissues are the main factors slowing down their extensive use in the field.

2.4. Highlights

- In vitro models are the most widely used models in the field of iDILI research.
- The single-cell models that best represent in vivo cellular behavior are PHHs cultures.
- Multiple-cell type models simulate the interactions between different cell types.
- In vitro models can be developed in 2D or 3D configurations.

3. Strategies and endpoints to study iDILI in vitro

iDILI is a leading cause of drug attrition during preclinical and clinical development, partly due to the lack of reliable in vitro and in vivo models. Current in vitro models for detecting hepatotoxic drugs are not sufficiently sensitive, as most are unable to withstand long-term studies (weeks to months). In addition, most of these models are only successful for screening compounds that injure hepatocytes in a dose-dependent manner, lacking interactions between other drug- and host-related factors, which are also crucial in the development of iDILI. One of the most important factors in iDILI’s development is the adaptive immune system, which involves different organs and locations, making it highly difficult to reproduce the human scenario using a unique in vitro test.

Although there is a need to develop new strategies to identify the liver toxicity a new drug may trigger, the pharmaceutical industries have devoted a lot of effort to this matter, and different approaches have evolved over the years to address this challenge. Some hypothesized pathophysiological mechanisms of iDILI are being studied in vitro using different cell models, and both the European Medicines Agency and the U.S. Food and Drug Administration (FDA) have developed guidelines for industries in order to make a premarketing clinical evaluation of iDILI. Since hepatocytes stress is often the first step in iDILI development, different cellular stress markers (endpoints) are used to detect potentially idiosyncratic drugs. Some of these endpoints are the formation of CRM, inhibition of hepatocyte transport proteins or metabolizing enzymes, mitochondrial damage, endoplasmic reticulum (ER) stress, oxidative stress or immune system activation. It is important to highlight that prior to treatment with any drugs, the phenotypes of the chosen cell type(s) are appraised using well-accepted markers towards providing the highest probability of obtaining a robust in vitro-to-in vivo correlation in the measured iDILI endpoints.

It is important to note that, to compare different in vitro systems, a standardization of drugs, endpoints and metrics are needed. Using different drug panels would have a huge impact on the predictive values of the test, as argued by McGill, thus a standard drug panel agreed by field experts. Likewise, using endpoints would make difficult to compare different in vitro systems to predict iDILI. Ingelman-Sundberg and Lauschke suggest that quantification of cellular ATP levels as the most appropriate due to its irreversible nature as a marker for cell viability, as opposed to other endpoints reflecting only reversible and potentially transient cellular stress. Finally, carefully interpretation of metrics such specificity, sensitivity and predictive values is needed, as previously argued.

Traditionally, in vitro systems to address iDILI have consisted of hepatoma-derived cell lines, but the use of primary hepatocytes and HLCs derived from hiPSCs or ESCs to study iDILI endpoints is gaining importance. Interestingly, HLCs derived from hiPSCs also allow the identification of iDILI-related mutations, since the cells are patient-derived. However, most of hiPSCs are derived from non-liver tissues (fibroblasts or blood), and these cells are less likely to show abnormalities expected to be in iDILI hepatocytes such as changes in genes expression or drug-induced mutations. Therefore, it will be necessary to obtain different sets of hiPSCs-derived HLCs from donors to identify significant changes in gene expression pathways involved in iDILI pathogenesis. In this section, we will review the different strategies and endpoints currently used to specifically study iDILI in vitro.

3.1. Cytotoxicity

To study iDILI potential of drugs, the simplest models are cellular systems that attempt to relate cytotoxicity with iDILI risk after a specific drug exposure. Cytotoxicity can be measured by direct cell count using brightfield microscopy or fluorescent staining of nuclei coupled to fluorescence microscopy or by indirect reactions, such as the tetrazolium assay. In most cases, it is also interesting to know how cells are dying, thus specific markers for discriminating apoptosis and necrosis are used, such as Annexin V-FITC/propidium iodide (PI) apoptosis detection kits or LDH release into the extracellular medium, to detect cell membrane integrity.

Hepatotoxicity assays should follow a low-concentration, long-term approach to mimic the characteristics of iDILI observed in patients. However, there is currently no consensus on how to select the concentrations to be tested and the time points for assessments. A practical approach is to use multiples of the maximum plasma concentration (Cmax) of the investigated drugs, since most hepatotoxic drugs show significant cytotoxicity within the 100-fold Cmax range.

Examples of widespread in vitro models for the determination of hepatic cytotoxicity of potentially iDILI drugs include engineered hepatoma-derived cell lines, such as HepG2 and THLE overexpressing different subtypes of CYP450. On the other hand, Vorrink et al. recently established 3D spheroid cultures of PHHs to evaluate the hepatotoxicity of drugs through cell viability measurement using ATP quantifications as the single endpoint. The authors were able to distinguish between hepatotoxic and
nontoxic structural analogues with 69% sensitivity and 100% specificity.

Although cytotoxicity testing is a simple, low-cost and rapid technique to detect potential hepatotoxic drugs, it may not distinguish all the molecular events that drive cellular injury in iDILI and even the interpretation of results can be variable; for example, ATP levels may decrease due to cell death or due to mitochondrial dysfunction. Also, some physicochemical properties of compounds, such as solubility or lipophilicity, may affect cytotoxicity measurements due to inefficient solubility in culture media. Finally, and following the “multiple determinant hypothesis”, iDILI is the result of several interacting factors, and most drugs that cause iDILI do not induce significant intrinsic hepatotoxicity in humans in vivo; therefore, it is not expected that hepatocyte cytotoxicity in cell culture would be able to predict iDILI risk by itself. Therefore, it is necessary to combine cytotoxicity with other endpoints related to cellular functions involved in iDILI pathophysiology.

3.2. Reactive metabolites production

A number of mechanisms related to the development of iDILI are associated with the hepatic metabolism of the drugs and the formation of CRM. In the liver, most detoxifications of drugs are catalyzed by hepatic DMETs, particularly the CYP family enzymes. However, DMETs also catalyze drugs metabolic activation, which sometimes produces high levels of CRM. These metabolites can induce iDILI by binding essential macromolecules and blocking their function, leading to acute cytotoxicity. They can also produce oxidative stress by depleting antioxidant molecules such as GSH, rendering the cells more susceptible to other environmental stresses. Moreover, CRM can covalently modify cellular proteins, which form adducts that trigger cytotoxicity through mitochondrial dysfunction, inhibition of BAs excretion and cellular stress. These events cause the release of damage-associated molecular patterns (DAMPs) that promote immune-mediated injury in susceptible individuals and initiate pathways to cell death (Fig. 1).

Currently, there are many different in vitro assays to detect CRM. Quantification of CRM formation by covalent binding assessment has been usually performed to distinguish compounds with iDILI potential from those non-hepatotoxic. This assay uses radiolabeled drugs and measures the amount of drug-related material covalently bound to microsomal proteins. Other strategy to detect CRM formation is called the trapping assay. Trapping agents such as N-acetylcysteine (NAC) and the soft nucleophile GSH are capable of scavenging electrophilic reactive metabolites producing an adduct that can be detected by mass spectrometry (MS).

To understand potential CRM forming metabolic pathways, in vitro metabolite identification studies are also using newer, more sensitive detection methods. For example, non-radiolabeled compounds are incubated in PHHs and metabolites are separated by high-performance liquid chromatography (HPLC) and identified using tandem mass spectrometry (MS/MS). However, to date, CRM assays have not been particularly adept at separating hepatotoxic drugs from those non-hepatotoxic.

3.3. Oxidative stress

Oxidative stress results from an imbalance between the generation of ROS and the antioxidant capacity of the cells. An excessive generation of ROS and other organic radicals and/or the inhibition of detoxification pathways have been associated with iDILI liability. It can occur as a consequence of mitochondrial impairment or due to the formation of CRM (Fig. 1).

Oxidative stress is commonly determined through direct quantification of ROS, assessment of lipid peroxidation, GSH depletion and/or the evaluation of stress response pathways activation, such as the nuclear factor erythroid 2-related factor 2 (NRF2) pathway. More comprehensive assays involve genetic engineering of HepG2 to express GFP-tagged sulfiredoxin-1 (SRXN1) and other stress response markers to measure oxidative stress increase and NRF2 response in real time. Using HepaRG cells, Antherieu et al. observed increased ROS levels shortly after chlorpromazine exposure, associated with an inhibition of taurocholic acid (TCA) efflux, indicating a major role of oxidative stress in chlorpromazine-induced cholestasis.

HLCs generated from ESCs have also been used to study oxidative stress in iDILI. Very recently, Cipriano et al. established an in vitro 3D spheroid model of HLCs derived from human neonatal MSCs to analyze DMETs and changes in the GSH net flux in nevirapine (NVP)-induced liver injury.

3.4. Mitochondrial damage and lysosomal dysfunction

Over the last years, mitochondrial dysfunction has been increasingly implicated in the etiology of iDILI caused by many drugs. This is attracting the attention of pharmaceutical companies, which may potentially use in vitro assays to detect mitochondrial damage during drug discovery phases. In a recent study of 124 drugs, 50%–60% of those that could elicit idiosyncratic toxicity caused mitochondrial dysfunction, whereas amongst non-iDILI drugs this fell to <5%.

There is a wide spectrum of different endpoints to assess drug-induced mitochondrial toxicity, and each one gives information about specific processes related to the drug’s mechanism of action. The most common endpoints measured to determine mitochondrial function are the mitochondrial membrane potential, glycolytic activity, oxidative phosphorylation (OXPHOS) capacity, activity of different complexes of the mitochondrial electron transport chain (ETC), fatty acids oxidation, mitochondrial DNA (mtDNA) levels, mitochondrial protein synthesis and mitochondrial oxidative stress.

However, mitochondrial bioenergetics assays using an extracellular flux analyzer and toxicity measurement in the glucose/galactose (Glu/Gal) assay are probably the most useful as predictor for iDILI involving mitochondrial dysfunction. In the Glu/Gal assay, cells are adapted to culture in either glucose or galactose-containing media over weeks and then exposed to investigated drugs for 24 h. A shift in cell death or ATP dose–response curves provide evidence of drug-induced mitochondrial dysfunction but these assays do not allow to distinguish the molecular mechanisms involved in it. On the other hand, bioenergetics assays based on the measurement of oxygen consumption rate can provide more information about the mechanisms of drug-induced mitochondrial dysfunction. Different commercial products can be used to measure the mitochondrial respiration and glycolytic activity of living cells in real time and detect parameters such as basal respiration, ATP-linked respiration, maximal and reserve capacities and non-mitochondrial respiration.

In terms of in vitro modelling, there is no consensus about which is the most useful cell model coupled to a specific endpoint measurement to assess mitochondrial dysfunction in...
iDILI. Primary hepatocytes are rarely used due to their low bioenergetic flexibility, which makes them unable to differentiate mitotoxicity from non-mitochondrial toxicity. Eakins et al. proposed the combination of two in vitro assays for the detection of drug-induced mitochondrial toxicity using HepG2 cells, which is the most widely used cell line in such analyses. The first assay measured cytotoxicity using the Glu/ Gal assay, and the second assay measured mitochondrial respiration, glycolysis and the reserve capacity. The authors tested 72 drugs known for their mitochondrial damage potential, obtaining a specificity, sensitivity and accuracy of 100%, 81% and 92%, respectively.

In order to choose the right model, it is also important to consider that mitochondrial toxicity often has a delayed presentation due to an apparent threshold effect, resulting from the multiplicity of mitochondria alongside various protective and compensatory mechanisms. To replicate long-term drug exposure in vitro and assess its chronic effects on mitochondrial function, the HepaRG model might be a more appropriate choice, since they rely on the OXPHOS machinery for survival more than other hepatic cell lines.

Other mitochondrial damage models include the Hep3B cells, which have been used in mechanistic studies about efavirenz (EFV)-iDILI, detecting an increased mitochondrial mass with defective morphology and mitochondrial autophagy (mitophagy) activation.

However, one important problem of mitochondrial damage assays is that the drug concentrations used are commonly much higher than the Cmax, and it is unlikely that the mechanism of toxicity at these concentrations parallels the mechanism of iDILI in vivo. Moreover, although mitochondrial injury could be involved in the mechanism of some iDILI events, it seems very unlikely that simple inhibition of the mitochondrial ETC would be a primary mechanism. Therefore, due to multifactorial nature of iDILI, it is expected that using only mitochondrial toxicity assays is not enough to predict clinical iDILI risk.

A strong relationship has been described between mitochondrial damage and lysosomal dysfunction. ATP is necessary to maintain lysosome function and seclude the cytosolic components. In cases of mitochondrial dysfunction (i.e., due to drug toxicity), ATP levels decrease, impairing correct lysosomal function and autophagosome–lysosome fusion, between other cellular pathways. In fact, lysosomal instability and release of lysosomal content into the cytosol have been proposed as a mechanism by which lysosomes can contribute to APAP-induced hepatotoxicity. Autophagic flux dysfunction due to lysosomal instability has also been described as a mechanism of diclofenac-iDILI, as observed in HepG2 cell cultures.

3.5. Endoplasmic reticulum (ER) stress

ER stress has been associated with various drug-induced liver lesions and drug-induced cholestasis. The unfolded protein response (UPR) activates to cope with stress when un-/misfolded proteins accumulate due to internal or external perturbations like aging and drug treatments. Activation of UPR can eventually promote inflammation, cell injury and steatosis. As a rule, when the cell is overwhelmed due to an increase in ER stress, pathways involving apoptotic death are activated to prevent harmful events at tissue level. Moreover, it has been observed that proinflammatory cytokines contribute to cell damage in vitro in part through activation of ER stress.

Hepatoma-derived cell lines have been widely used to investigate the drugs’ potential to cause ER stress, through measurement of components of the UPR pathways and/or the use of UPR inhibitors to examine the effects on drug-induced toxicity.

In HepG2 cells, endpoints frequently used to test drug-induced ER stress are the increased expression of proteins involved in UPR in HepG2 cells, as well as increased cytosolic free Ca2+ concentration and/or increased expression of calcium release-activated calcium channel protein 1 (CRAM) and stromal interaction molecule 1 (STIM1), which are key components of the store-operated calcium entry.

In a similar way, Hep3B cells have been used to study the role of ER stress in EFV-iDILI. Apostolova et al. observed an upregulation of different ER stress markers, such as the binding immunoglobulin protein (BiP), CCAAT-enhancer-binding protein homologous protein (CHOP), phosphorylated eukaryotic initiation factor 2 (p-eIF2A) and the spliced form of X-box binding protein 1 (XBP1) in EFV-treated cells. The authors also observed enhanced cytosolic Ca2+ content and morphological changes in the ER, suggesting the involvement of ER stress and UPR response in hepatotoxicity induced by EFV.

3.6. Alteration of bile acids (BAs) homeostasis

Disruption in BAs homeostasis is associated with alterations in bile formation and flow, which usually result in the accumulation of BAs in the liver. BAs are highly toxic to cells when present in abnormally high concentrations. Usually, the disruption of bile flow from the liver to the duodenum is referred to as cholestasis. However, cholestatic pattern of injury in iDILI is defined using a biochemical criterion (when ALP alone is elevated at least two times ULN, or the R value is at least 2). This indistinctive use of the term “cholestasis” may lead to confusion, since iDILI resulting from drug inhibition of BAs transport usually shows a hepatocellular pattern of injury. In this section, cholestasis is referred to alteration of BAs homeostasis.

The transport of BAs across the canalicular membrane is regulated by ATP-dependent canalicular transporters, especially the BSEP, a hepatic transport protein member of the ABC gene superfamily (codified by the ABCB11 gene), which is the major determinant of bile salt-dependent canalicular bile secretion. Its inhibition and/or repression have been proposed as key mechanisms for drug-induced cholestasis. Moreover, it has been observed that patients carrying the C allele in the ABCB11 1331T>C polymorphism are at increased risk of developing hepatocellular iDILI, when taking drugs containing a carbocyclic system with aromatic rings, suggesting that both ABCB11 polymorphisms and drug structure may affect BSEP function.

Currently, a number of in vitro methods are employed to assess the cholestatic potential of drugs. One of the most common assays uses vesicle systems expressing BSEP and other membrane transporters to measure the uptake of a TCA derivative after a treatment with the drug of interest. Despite this system provides useful information about the cholestatic potential of different drugs such as troglitazone, it is unable to assess the effect of transporters variability, since some compounds are able to inhibit multiple transporters. Therefore, the whole impact on bile salt uptake may be underestimated. However, this could be an advantage: membrane vesicles expressing a single transporter affords an opportunity to determine the drug effects on discrete hepatic transporters while eliminating possible confounding events.
Moreover, some drugs only trigger the accumulation of certain classes of BAs, thus the assessment of only cholestatic drugs could be misleading.

Endpoints frequently used in vitro to study BAs accumulation due to drugs include the inhibition of BSEP protein, disruption of BSEP protein cell surface expression and/or repression of transcription of the ABCC11 gene. Specifically, a commonly measured endpoint in drug-induced cholestasis involves the farnesoid X receptor (FXR) signaling pathway. In the case of bile salt transport inhibition, in vitro models have shown to be more sensitive than in vivo systems, since rodent models have differences in bile salt composition and compensatory mechanisms that make them less sensitive to cholestatic injury.

In recent years, evidence has been pointing to the fact that BSEP inhibitors, especially at high doses, are associated with a higher risk of iDILI. When BSEP inhibition by a specific drug is observed, the half maximum inhibitory concentration (IC₅₀) value should be determined. However, there are huge discrepancies between the BSEP IC₅₀ cutoff values determined by different researchers to test drugs as iDILI positive or negative. Therefore, it is necessary to standardize the methods and the reference drugs used to perform this type of assays. Furthermore, it remains impossible to predict whether BSEP inhibition due to a particular drug is sufficient to cause iDILI.

Primary hepatocytes are still the gold standard for evaluating drugs’ inhibitory effects on BSEP activity and BAs excretion, due to their functional polarization. However, PHHs in suspension are not widely used to evaluate canalicular BAs excretion.

On the contrary, sandwich-cultured hepatocytes are extensively used for evaluating BAs excretion and drugs capacity to inhibit BSEP function and cause iDILI, since they have proper localization of basolateral and canalicular transporters with functional bile networks. This system has been used to detect troglitazone and lopinavir repression of ABCC11. Troglitazone is an antidiabetic drug withdrawn from the market on 2000 due to high risk of iDILI through several mechanisms such as oxidative stress, cholestasis or mitochondrial disfunction. Interestingly, studies with troglitazone have served to demonstrate that drugs within the same therapeutic class (e.g., thiazolidinediones) may differ in terms of hepatic liability. Analyzing the gene expression on HepG2 systems, Gou et al. showed that troglitazone differentially expressed genes involved in necrosis, apoptosis and cell proliferation pathways compared to non-toxic rosiglitazone and pioglitazone, which confirms that physicochemical and toxicological drug properties affect iDILI risk.

Using the same strategy, a few years ago Pedersen et al. showed that drugs associated with severe iDILI significantly reduced the canalicular efflux of TCA, meanwhile drugs associated with less severe or no iDILI showed minimal effects on the canalicular efflux of TCA. Oorts et al. used different batches of PHHs in sandwich-culture to evaluate cholestasis induced by 14 compounds, obtaining data well correlated with clinical reports on drug-induced cholestasis.

Human hepatoma-derived cells have also been used to study drug-induced cholestasis. Different studies using HepG2 have shown that molecular mechanisms of nonsteroidal anti-inflammatory drugs-induced liver injury may be mediated through antagonism of FXR and that these cells could serve as a good model for evaluating the effects of drugs on the uptake of substrates and the potential for downstream cholestasis. HepaRG cells have also been used to investigate several mechanisms underlying drug-induced cholestasis by testing 12 potential cholestatic drugs and 6 non-cholestatic drugs.

3.7. Immune system activation

Liver is a highly immunological organ and dysregulation of its immune environment is thought to play a critical role in the initiation and progression of iDILI. The common delayed onset of symptoms after taking the drug and the rapid onset after rechallenge, the presence of modified drug-peptide antigens (hapten) and the presence of autoantibodies or sensitized T cells in serum are some of the factors that support the crucial role of the immune system in the pathogenesis of iDILI. The clear association of iDILI due to specific drugs to certain human leukocyte antigen (HLA) alleles also gives evidence that most iDILI is mediated by the adaptive immune system. Currently, the strongest association observed between an HLA allele and iDILI concerns HLA-B*57:01 and fluvoxacin. Other HLA alleles with a strong iDILI association are HLA DRB1*15:01-DQB1*06:02 for amoxicillin-clavulanic-associated hepatotoxicity and HLA-DRB1*07:01 for iDILI caused by ximelagatran and lapatinib associated iDILI.

However, adaptive immune activation first requires an innate immune response to activate APCs and produce inflammatory cytokines. Unlike the adaptive immune system activation, which requires specific immune cells receptors (idiodynnamic), the innate immune system previously activated by DAMPS, CRMs, etc. could be not idiodynamic. It has been observed that drugs can produce an innate immune response, although only a small proportion of patients develop an adaptive immune response and iDILI. Therefore, a new proposed approach for iDILI risk prediction could be the study of the potential of a suspected drug to activate the innate immune response.

Despite the general consensus about the critical role of immune system in iDILI pathogenesis, currently there is no standardized screening strategy to detect drug candidates that may cause immune-mediated iDILI.

Traditionally, the clinical diagnosis of drug hypersensitivity in vitro has been addressed using the enzyme-linked immunospot assay, which allows the identification of cells actively secreting cytokines, and the lymphocyte transformation test (LTT), which measures the proliferation of T cells to a chemical compound in vitro. However, this last technique exhibits limited sensitivity and specificity depending on the reaction and the responsible drug. A few years ago, a modified LTT measuring granzyme B and cytokines production was proposed to diagnose iDILI and determine the culprit drug by using human peripheral blood mononuclear cells (hPBMCs) from patients enrolled in the U.S. Drug-Induced Liver Injury Network (DILIN). However, this modified version of LTT could not reliably establish causality. A few years ago, a modified LTT measuring granzyme B and cytokines production was proposed to diagnose iDILI and determine the culprit drug by using human peripheral blood mononuclear cells (hPBMCs) from patients enrolled in the U.S. Drug-Induced Liver Injury Network (DILIN). However, this modified version of LTT could not reliably establish causality. A few years ago, a modified LTT measuring granzyme B and cytokines production was proposed to diagnose iDILI and determine the culprit drug by using human peripheral blood mononuclear cells (hPBMCs) from patients enrolled in the U.S. Drug-Induced Liver Injury Network (DILIN). However, this modified version of LTT could not reliably establish causality.

More recent attempts to study the role of the immune system in iDILI in vitro have developed cell culture methods aimed at characterizing primary T-cell responses to drugs. For example, Usui et al. developed a mechanistic study to observe the activation of T cells from a panel of 14 HLA-typed human donors after exposing them to different drugs, and they observed that the priming of T cells with certain drugs was skewed toward donors expressing specific HLA alleles.

A major gap in the current test systems for iDILI is a model which recapitulates the interactions of hepatic cells with immune cells. A common mechanism of immune activation involves stimulation of inflammmasomes by DAMPs, that can be released by...
hepatocytes after injury. However, since hepatocytes lack significant inflammasome activity, THP-1 cells, a macrophage cell line, can be incubated with the supernatant of hepatocytes treated with the drug of interest, to observe inflammasome activation. Using this strategy, Kato et al. \textsuperscript{11} observed that treating hepatocarcinoma cells with drugs associated with severe iDILI led to increased caspase-1 activity and production of interleukin (IL-)1β by THP-1 cells. Oda et al. \textsuperscript{220} found that IL-1β and IL-8 mRNA expression was significantly increased in human promyelocytic neutrophil-derived (HL-60) cells after exposure to the supernatant of human hepatoma cells treated with 96 drugs of interest.

In a similar way, freshly isolated PHHs have also been used to characterize drug-specific signaling between the liver and innate immune cells, observing that drug-treated PHHs released DAMPs, particularly the non-acetylated form high mobility group box 1 (HMGB1) protein during necrotic cell death. Interestingly, supernatant of these cells was able to stimulate dendritic cells to secrete pro-inflammatory cytokines \textsuperscript{221}.

To further investigate the role of inflammation in iDILI, Cosgrove et al. \textsuperscript{222} proposed an in vitro approach in which drugs were administered to hepatocytes together with lipopolysaccharides (LPS) and different cytokines such as tumor necrosis factor α (TNFa), interferon gamma (IFNy), IL-1α, and IL-6. The authors observed drug-cytokine hepatotoxicity synergies for multiple hepatotoxicants, suggesting that drug-cytokine co-treatment approach could be a useful preclinical tool for investigating inflammation-associated drug hepatotoxicity. Specifically, drugs such as sulindac, diclofenac and trovafloxacin have been shown to synergize with LPS and/or TNFa to kill hepatocytes in culture \textsuperscript{223–225}, suggesting an essential role of cytokine signaling in iDILI caused by these compounds. Very recently, Oda et al. \textsuperscript{226} established an in vitro system in which hPBMCs were used as the source of immune cells and were cocultured with HepG2 cells to predict the drugs’ potential to induce iDILI. However, some drugs were found to be false negatives or false positives due to differences between hPBMCs derived from different donors.

Other potential approach would be the use of engineered liver co-cultures with NPCs, such as LSECs, KCs and HSCs, as they are known to directly suffer drug toxicity and/or secrete molecules that regulate hepatocytes behavior \textsuperscript{227} and could help to determine drug-mediated changes in the innate immune response.

### 3.8. High content screening (HCS)

Individual endpoints are not enough to significantly predict iDILI, and considering that its mechanisms are complex and interlinked, a multiparametric approach is necessary \textsuperscript{20}. In these studies, several mechanistic endpoints (mitochondrial dysfunction, ROS accumulation, etc.) can be determined for one or more drugs simultaneously.

HCS has emerged as a powerful tool for predicting iDILI in the early phases of drug discovery, due to its higher sensitivity compared to conventional methods and its ability to study multiple cell parameters at the same time \textsuperscript{228}. HCS typically uses fluorescent probes to identify and quantify various biological processes, pathways, molecules, organelles and/or other cellular functions.

On the other hand, HCS has some limitations, as the need for good spectral separation in the fluorescent channels, the specificity of the probes or the ability of the imaging algorithms to accurately detect and quantify the relevant biological phenotypes.

Table 2 lists some of the main endpoints, fluorescent probes, cell models, examples of some of the drugs tested and the image analysis software used for HCS. A typical HCS toxicity detection assay includes four steps \textsuperscript{229}:

**3.8.1. Choice of a cellular model**

The cellular models normally chosen for HCS assays to assess iDILI risk are PHHs, hepatoma-derived cell lines and HLCs derived from hiPSC.

In addition to PHHs single-cell cultures, micropatterned PHHs-fibroblasts co-culture systems such as HepatoPac \textsuperscript{26} have also been used, measuring different endpoints after hepatotoxic drugs exposure, such as nuclear size and intensity, cell permeability or mitochondrial integrity \textsuperscript{230}.

Between the hepatoma-derived cell lines, HepaRG cells are a good model to perform HCS assays due to their normal expression levels of CYP enzymes. Also, in the case of cholestasis prediction, this cell model has demonstrated its value as a cell-based assay system for screening drug-induced liver steatosis.

Whereas HCS is relatively simple for 2D conventional cell cultures, it is technically challenging in 3D systems due to interlayer signal contamination. However, HCS is moving towards more complex cell models such as co-cultures and 3D systems, and there are hopes that the predictivity will further increase.

**3.8.2. Incubation with the drug of interest**

In this step, appropriate positive and negative controls should be properly selected to ensure that the assay allows the detection of the desired endpoints.

**3.8.3. Staining with fluorescent probes**

HCS applies multiparametric imaging of several endpoints after exposure of specific drugs, ranging from cell viability, ROS accumulation or ER damage to cholestasis, steatosis or phospholipidosis. These parameters can be measured by HCS imaging assays when stained with different fluorescent probes. The most usual ones are gathered by Tolosa et al. \textsuperscript{231}.

**3.8.4. Automated image acquisition and analysis**

After incubation with fluorescent probes, the automated acquisition of fluorescent images in separated channels is performed \textsuperscript{232}. Then, the images are processed automatically, and in this step, accurate cell segmentation is critical \textsuperscript{233}. Finally, data analysis of all images by image algorithms is essential, and a complex and fine-tuned system is required \textsuperscript{234}.

Although recent research shows HCS can be used by the pharmaceutical industry to screen candidate drugs for potential human liver toxicity, HCS should be used in combination with other higher sensitive approaches, such as transcriptomics or metabolomics strategies to ameliorate predictivity and sensitivity of the human iDILI potential for new and old drugs.

### 3.9. Omics technologies

iDILI is a multifactorial condition involving different organs. Therefore, there is not a single mechanism of action of a certain drug to induce iDILI. Because of that, -omics techniques that take into account one aspect of the cellular biology globally are needed...
to better understand the development of iDILI. Recently, new approaches to predict iDILI based on omics technologies are gaining attention. The rationale behind this fact is that, by leveraging omics-based measurements, there is potential to develop a more accurate predictive or prognostic model of iDILI. Transcriptomics and/or metabolomics are the most used in this field, as these techniques are able to account global cellular changes induced by specific drugs. Gene signatures of already studied compounds can help to categorize the mechanism of action of new experimental compounds. However, it is important to note that altered gene expression following exposure of cells to a drug is not always associated with mechanisms involving hepatocellular damage.

Only a few in vitro models have been developed for predicting human iDILI based on toxicogenomics data. Cha et al.249 analyzed differential gene expression profiles of hepatotoxic and non-hepatotoxic compounds using HepG2 cells in order to identify the classifier genes for hepatotoxicity prediction due to nonsteroidal anti-inflammatory drugs. The model was validated using 4 prototypical nonsteroidal anti-inflammatory drugs, obtaining a sensitivity and specificity of 100%. More recently, Ware et al.250 used PHHs-fibroblasts-based MPCCs to study the global gene expression patterns of primary hepatocytes when treated with hepatotoxic drugs and their non-liver-toxic analogs. The results showed that global gene expression profiles can be useful for detecting potentially iDILI drugs.

MicroRNAs (miRNAs) profiling has been widely investigated as a new biomarker of iDILI242. It has been observed that some miRNAs may be associated with iDILI development and progression243. miRNAs can be found in two different ways: free circulating or associated with extracellular vesicles (EVs)244. Due to their ubiquity and stability, EVs could be a promising source for studying transcriptomic profiles in iDILI and other liver diseases245, as some groups have proven for example a significantly increased of circulating miRNA-192 and miRNA-30 in patients with alcoholic hepatitis246 or alterations in the liver-specific RNA (albumin mRNA and miR-122) content of human primary hepatocytes-derived exosomes exposed at subtoxic doses of APAP247.

On the other hand, the first group to ever apply metabolomics to in vitro modeling of a human cell line were Ruiz-Aracama et al.248, who used HepG2 cells to study how the hepatotoxic drug 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) affected the cell metabolome. Very recently, Krajnc et al.249 performed an untargeted metabolomic approach by using HuH7 cells and primary mouse hepatocytes. The authors found that several pathways were being affected by the hepatotoxic drug nefazodone. They identify alterations in ATP production pathways, as gluconeogenesis, anaerobic glycolysis and OXPHOS when the cells were exposed to nefazodone, indicating that the drug has an inhibitory effect in both the mitochondrial-dependent and mitochondrial independent ATP production.

3.10. Combinations of in vitro assays

The combination of multiple in vitro assays with clinical and pharmacological information is a promising tool in iDILI research in the coming years, as recent publications in the field point out. Thompson et al.251 tested 36 drugs with varying degrees of iDILI concern in five in vitro assays using hepatic cells: multidrug resistance-associated protein 2 (MRP2)/BSEP vesicle transport assays, Glu/Gal mitochondrial function assay and cytotoxicity in THLE cell lines which differed only by CYP3A4 expression. Additional data, such as dose, fraction of drug absorbed and fraction of drug metabolized were included, allowing the categorization of compounds by iDILI severity. The results had a positive predictive value of 93% and a negative predictive value of 100%.

Shah et al.250 performed a retrospective analysis of several iDILI and non-iDILI drugs that were tested in three in vitro assays (hepatic cells cytotoxicity, BSEP inhibition and mitochondrial dysfunction). These endpoints were assessed independently and in the context of human Cmax data. The authors showed that cytotoxicity measurements only predicted iDILI in the presence of exposure data. However, BSEP inhibition and mitochondrial disruption showed IC50 threshold effects around 30 µmol/L. The predictive value was improved when Cmax was taken into account. In the same way, Aleo et al.251 investigated the inhibitory properties of 24 Most-iDILI-, 28 Less-iDILI-, and 20 No-iDILI-concern drugs. The authors observed that drugs with dual potency as mitochondrial and BSEP inhibitors were highly associated with more severe human iDILI and more restrictive product safety labeling related to liver injury.

Very recently, Aleo et al.252 designed a new strategy to ameliorate iDILI predictivity. The authors developed a scoring system called the hepatic risk matrix that mixed different in vitro risk factors (cytotoxicity, mitotoxicity and BSEP inhibition, all relative to Cmax) into a nonbinary scoring system based on exposure margins relative to in vitro activities. The combined scores were added to a physicochemical property score, based on properties of the compound of interest and it was used to predict iDILI risk potential in a data set of 200 Liver Toxicity Knowledge Base (LTKB) drugs. Hepatic risk matrix correctly identified most iDILI drugs with high sensitivity. The group further validated this strategy on 28 drugs whose clinical development program was stopped due to liver injury.

On the basis of the current research, it seems clear that the assessment of different in vitro assays combinations is a necessary approach to ameliorate iDILI predictivity in preclinical situation.

3.11. Highlights

- iDILI is a leading cause of drug withdrawal during preclinical and clinical development.
- To study iDILI potential of drugs, the simplest models are cellular systems that relate cytotoxicity after drug exposure with iDILI risk.
- Within hepatocytes, damage can occur at many intra and extracellular levels.
- Combining different in vitro assays seems a good approach to increase iDILI predictivity.

4. In vivo approaches to study iDILI

In vivo models usually are, together with in silico tools, the initial step in the study of the pharmacodynamics of a drug. As mentioned above, these models are also becoming increasingly promising for pre-clinical prediction of hepatotoxicity. However, in vitro systems lack some key properties that would be necessary to adequately unravel the mechanisms of iDILI, such as the contribution of an integrated immune system or cross-communication with other organs. For this reason, animal models are an essential preclinical tool to elucidate the pathophysiology and to assess new therapies and biomarkers in the diagnosis, prognosis and management of liver diseases. Different in vivo experimental models, such as rats, mice or zebrafish, have
| Parameter measured            | Probe                                  | Cell type                        | Example of drugs tested                                                                 | Image analysis software                                                                 | Ref.               |
|------------------------------|----------------------------------------|-----------------------------------|-----------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|--------------------|
| Cell number                  | Hoechst 33342                          | Primary human hepatocytes          | Rotenone, diclofenac, ibuprofen, aspirin, chlorpromazine, verapamil, acetaminophen, omeprazole, caffeine | ImagePro Plus software, Cellomics ArrayScan VTI platform, Developer XD, Compartmental Analysis v3 | 110,149,230        |
| Nuclear content              | DRAQ5                                  |                                   |                                                                                         |                                                                                        |                    |
| Cell viability               | Propidium iodide, WST-1 reagent, MTT colorimetric assay, Calcein AM, ATP-lite luminescence assay kit |                                   | Rotenone, diclofenac, ibuprofen, aspirin, chlorpromazine, verapamil, acetaminophen, omeprazole, caffeine |                                                                                        |                    |
| Oxidative stress (ROS generation) | CM-H2DCFDA, DHE, DCF, Carboxy-H2DCFDF |                                   | Rotenone, diclofenac, ibuprofen, aspirin, chlorpromazine, verapamil, acetaminophen, omeprazole, caffeine | ImagePro Plus software, Cellomics ArrayScan VTI platform, Developer XD, Compartmental Analysis v3 |                    |
| Mitochondrial membrane potential | TMRM, MitoTracker, JC-10              |                                   | Rotenone, diclofenac, ibuprofen, aspirin, chlorpromazine, verapamil, acetaminophen, omeprazole, caffeine | ImagePro Plus software, Cellomics ArrayScan VTI platform, Developer XD, Compartmental Analysis v3 |                    |
| Mitochondrial damage         | TMRE                                    |                                   | Rotenone, diclofenac, ibuprofen, aspirin, chlorpromazine, verapamil, acetaminophen, omeprazole, caffeine | ImagePro Plus software, Cellomics ArrayScan VTI platform, Developer XD, Compartmental Analysis v3 |                    |
| Intracellular GSH            | mBCI, GSH-GLO                           |                                   | Rotenone, diclofenac, ibuprofen, aspirin, chlorpromazine, verapamil, acetaminophen, omeprazole, caffeine | ImagePro Plus software, Cellomics ArrayScan VTI platform, Developer XD, Compartmental Analysis v3 |                    |
| Intracellular lipid/fat deposits | BODIPY 493/503, Steatosis Colorimetric Assay Kit | Hepatoma-derived cell lines | Amiodarone, chlorpromazine, cyclosporine A, flutamide, rotenone, tamoxifen, tetracycline, valproate, zidovudine | Scan®R, Kinetic-Scan HCS Reader, Compartmental Analysis V4 (Cellomics bioapplications), InCell Analyzer 1000, CellProfiler version 2.1.1, ArrayScan VTI platform, Operetta | 25,27,75,235       |
| Cellular ATP content         | ATPlTOX                                |                                   | Rotenone, diclofenac, ibuprofen, aspirin, chlorpromazine, verapamil, acetaminophen, omeprazole, caffeine | Scan®R, Kinetic-Scan HCS Reader, Compartmental Analysis V4 (Cellomics bioapplications), InCell Analyzer 1000, CellProfiler version 2.1.1, ArrayScan VTI platform, Operetta |                    |
| Caspase activity             | CaspaseGLO 3/7                          | Hepatoma-derived cell lines       | Amiodarone, chlorpromazine, cyclosporine A, flutamide, rotenone, tamoxifen, tetracycline, valproate, zidovudine | Scan®R, Kinetic-Scan HCS Reader, Compartmental Analysis V4 (Cellomics bioapplications), InCell Analyzer 1000, CellProfiler version 2.1.1, ArrayScan VTI platform, Operetta |                    |
| Endoplasmic reticulum status | ER Tracker, TOTO-3, YoYo-1              | Hepatoma-derived cell lines       | Amiodarone, chlorpromazine, cyclosporine A, flutamide, rotenone, tamoxifen, tetracycline, valproate, zidovudine | Scan®R, Kinetic-Scan HCS Reader, Compartmental Analysis V4 (Cellomics bioapplications), InCell Analyzer 1000, CellProfiler version 2.1.1, ArrayScan VTI platform, Operetta |                    |
| Membrane permeability        | Fluoro-4                                | Hepatoma-derived cell lines       | Amiodarone, chlorpromazine, cyclosporine A, flutamide, rotenone, tamoxifen, tetracycline, valproate, zidovudine | Scan®R, Kinetic-Scan HCS Reader, Compartmental Analysis V4 (Cellomics bioapplications), InCell Analyzer 1000, CellProfiler version 2.1.1, ArrayScan VTI platform, Operetta |                    |
| Calcium levels               | Cytochrome c                            | Hepatoma-derived cell lines       | Amiodarone, chlorpromazine, cyclosporine A, flutamide, rotenone, tamoxifen, tetracycline, valproate, zidovudine | Scan®R, Kinetic-Scan HCS Reader, Compartmental Analysis V4 (Cellomics bioapplications), InCell Analyzer 1000, CellProfiler version 2.1.1, ArrayScan VTI platform, Operetta |                    |
| Apoptosis                    |                                        |                                   | Rotenone, diclofenac, ibuprofen, aspirin, chlorpromazine, verapamil, acetaminophen, omeprazole, caffeine | ImageXpress Micro XL system, CellProfiler 2.1.1, HDF5, R, R studio                        | 236–239            |
| Lysosomal activation         | LysoTracker, BODIPY 665/676             | Hepatocyte-like cells derived from human stem cells | Acetazolamide, cyclophosphamide, hydroxyurea, mefenamic acid, phenacetin, phenylbutazone, pyrazinamide, quinine | ImageXpress Micro XL system, CellProfiler 2.1.1, HDF5, R, R studio                        | 236–239            |
| Lipid peroxidation           | LDH cytotoxicity Assay Kit              | Hepatocyte-like cells derived from human stem cells | Acetazolamide, cyclophosphamide, hydroxyurea, mefenamic acid, phenacetin, phenylbutazone, pyrazinamide, quinine | ImageXpress Micro XL system, CellProfiler 2.1.1, HDF5, R, R studio                        | 236–239            |
| LDH leakage                  | Albuwell kit                           | Hepatocyte-like cells derived from human stem cells | Acetazolamide, cyclophosphamide, hydroxyurea, mefenamic acid, phenacetin, phenylbutazone, pyrazinamide, quinine | ImageXpress Micro XL system, CellProfiler 2.1.1, HDF5, R, R studio                        | 236–239            |
| Albumin secretion            | Antibodies (primary or secondary) conjugated with different fluorochromes (i.e., GFP) | Hepatocyte-like cells derived from human stem cells | Acetazolamide, cyclophosphamide, hydroxyurea, mefenamic acid, phenacetin, phenylbutazone, pyrazinamide, quinine | ImageXpress Micro XL system, CellProfiler 2.1.1, HDF5, R, R studio                        | 236–239            |

ATP, adenosine triphosphate; CM-H2DCFDA, chloromethyl 2',7'-dichlorodihydrofluorescein diacetate; DCF, 2',7'-dichlorofluorescein; DHE, dihydroethidium; iDILI, drug-induced liver injury; GFP, green fluorescent protein; HCS, high-content screening; LDH, lactate dehydrogenase; MTT, tetrazolium; ROS, reactive oxygen species; TMRE, tetramethylrhodamine, ethyl ester; TMRM, tetramethylrhodamine, methyl ester.
been proposed for the study of chronic liver diseases such as alcohol-related fatty liver disease and MAFLD, alcohol-related and non-alcoholic steatohepatitis, and they have been extensively reviewed elsewhere.\textsuperscript{254}

Unfortunately, \textit{in vivo} models are not yet as widespread for the study of iDILI. In general, the safety of a drug is typically assessed in two pre-clinical animal species before the first dose is given to humans, being an unavoidable part of the pre-clinical drug development in majority of cases.\textsuperscript{255,256} It is a general belief that conventional animal toxicology models are of limited value for predicting the potential for human iDILI. These models commonly use small groups of inbred, healthy animals which are unable to reproduce the multifaceted characteristics of iDILI, making it difficult to establish a correlation between animal markers of hepatotoxicity and clinically significant iDILI.\textsuperscript{257} In fact, while animal toxicity studies can easily detect compounds with intrinsic toxicity, standard toxicology studies generally fail to identify drugs that produce iDILI.\textsuperscript{258} Failure to predict adverse drug reactions using \textit{in vivo} models is often also due to critical differences in drug ADME between species, e.g., lack of functional conservation of many CYPs, and difficulty of reproducing the disease unpredictability and host susceptibility factors. Therefore, it is crucial to consider these limitations when assessing the \textit{in vivo} detection of iDILI.

An ideal animal model of iDILI should show clinical and mechanistic resemblance, similar risk factors as human iDILI, as well as exhibit experimental conveniences, such as the ability to develop injury in days to weeks. Moreover, this ideal iDILI model should be capable of distinguishing between drugs that cause human iDILI and those that do not. In the case of drugs for which bioactivation may be a requisite for human iDILI, it would also be important that the chosen model produces CRM similarly to humans. To date, only a few potential animal models of iDILI meet these requirements and the most commonly used are small rodents, due to their affordable cost, large number of strains available and the low amount of compound needed for testing. In addition, improving transparency and reporting of toxicology studies involving animals is essential to reach high reproducibility.

Recently, the ARRIVE guidelines 2.0 have been published in order to provide recommendations for studies describing animal research.\textsuperscript{259}

Despite the \textit{in vivo} modeling of iDILI poses great challenges, several strategies have been suggested for the identification of drugs with the potential to induce iDILI, model its mechanisms and contribute to a better understanding of the pathophysiology of the disease. Table 3 summarizes the key features of the main \textit{in vivo} approaches in iDILI.

4.1. Conventional animal toxicity studies: Single or repetitive administration of the drug

Probably the most widespread way to study hepatotoxicity is simply to treat the animals with a large unique dose or repetitive doses of the drug to cause liver injury. The most illustrative example due to its clinical relevance and experimental convenience is the case of high dose administration of APAP in mice.\textsuperscript{260} APAP mechanisms of hepatotoxicity have been well documented\textsuperscript{261} and APAP overdose studies have allowed a further understanding of iDILI mechanisms such as mitochondrial dysfunction.

Other conventional animal models have used different compounds to induce an acute liver injury (Table 3), as reflected by mitochondrial injury, inflammation, steatosis, serum hepatic enzyme levels or hepatic necrosis and apoptosis.\textsuperscript{262} The choice of animal species depends primarily on similarities with the human metabolism and histopathology of the drug tested. For example, whereas mice are the preferred animal for isoniazid (IHN) or APAP intoxication studies\textsuperscript{263,264}, rats are likely the most used animals when using CCl\textsubscript{4}.\textsuperscript{265} Interestingly, low doses of CCl\textsubscript{4} given repeatedly cause persistent liver injury with inflammation and fibrosis, thus CCl\textsubscript{4} can also be used to model chronic liver diseases, such as alcoholic liver disease, MAFLD and non-alcoholic steatohepatitis.\textsuperscript{266} Co-administration of low doses of IHN and rifampicin (RIF) also resulted in liver injury.\textsuperscript{267}

It is important to note that most of the conventional models show acute liver injury shortly after the treatment with the drug of interest, which is different from the characteristics of iDILI in humans. One exception is a mouse model of amodiaquine-induced liver injury, in which the treatment with the drug led to a delayed-onset liver injury mediated by natural killer (NK) cells.\textsuperscript{268}

Another disadvantage of these models is that they are typically used in intrinsic iDILI studies. However, combined with omics techniques, these models could also provide valuable predictive data about iDILI. For example, transcriptomics data from rats treated with iDILI drugs or their pharmacologic comparators (non-iDILI compounds) revealed the activation of cellular pathways involved in mitochondrial injury, inflammation, and ER stress in the response to iDILI drugs but not negative controls.\textsuperscript{269} Metabolomic\textsuperscript{270} and proteomic\textsuperscript{271} approaches have also been used to study animals treated with iDILI compounds, observing elicited metabolite/proteomic profiles matching the mechanism of action patterns of various liver toxicities, which could provide useful information on the molecular basis of iDILI.

Contrary to the situation of animal models of intrinsic DILI, the study of iDILI \textit{in vivo} possesses greater technical challenges, since it is extremely difficult to develop an animal model able to recapitulate the specific clinical situation of individual iDILI patients. Some of the host-dependent or environmental risk factors can be modelled \textit{in vivo} using pre-treatments or genetic alterations designed to pre-dispose animals to injury. The major approaches that have been used to model and predict iDILI \textit{in vivo} are based on different hypothesized iDILI mechanisms, and involve 1) induction of inflammation, 2) suppression of immune tolerance, 3) genetic manipulation of mitochondrial function, 4) use of panels of inbred mice, 5) chimeric mice with humanized livers and 6) mouse models of immune-mediated iDILI.

4.2. The inflammagen model

The inflammagen model is based on the hypothesis that inflammatory stimulus can precipitate iDILI.\textsuperscript{1} A number of studies have demonstrated the role of circulating gut-derived endotoxins and inflammatory processes in liver injury outcome and development.\textsuperscript{272} The principal biologically active component of bacterial endotoxin is LPS, which binds to Toll-like receptors on mammalian cells, initiating signaling mechanisms that lead to a pro-inflammatory state.\textsuperscript{273}

Researchers have established several \textit{in vivo} models based on the inflammation hypothesis. In these models, animals are either pre-treated, co-treated or post-treated with bacterial LPS in order to induce inflammation. The first successful application of this model was obtained by Buchweitz et al.\textsuperscript{274} who reproduced the human chlorpromazine (CPZ) hepatotoxicity in rats after a pre-
### Table 3  Summary of the main *in vivo* models currently available to evaluate liver disease and drug toxicity.

| Model type                          | Description                                                                 | Advantage                                                                 | Disadvantage                                                                 | Example                  | Liver injury | Ref.          |
|-------------------------------------|-----------------------------------------------------------------------------|---------------------------------------------------------------------------|------------------------------------------------------------------------------|--------------------------|--------------|---------------|
| **Non-humanized models**            |                                                                             |                                                                           |                                                                              |                          |              |               |
| Conventional                        | Mice or rats supplied with high single or repetitive doses of drugs         | Most commonly used models, easy to reproduce, helpful in elucidating some iDILI mechanisms | Limited predictive capacity due to species-specific issues                  | APAP, CCl4, IHN, RIF, CsA, VPA, CBZ, TET, ADQ | ↑↑ plasma/serum liver enzymes, histopathology alterations, liver necrosis, immune cell infiltration | 258, 261–263, 265, 267 |
|                                    |                                                                             |                                                                           | Mostly intrinsic DILI, absence of delayed response                          |                          |              |               |
|                                    |                                                                             |                                                                           |                                                                              |                          |              |               |
| Inflammagen                         | Mice or rats co-treated with drug and LPS, which triggers inflammation     | First to mimic human iDILI pronounced liver injury, useful for inflammatory stress studies | Ignore other causal factors of iDILI                                        | CPZ, TVX, AMD, DIC, SUL, RAN | Timing of LPS exposure, absence of delayed response, difficult to replicate, loss of sensitivity to LPS, mainly innate immune response | Variable ↑ of serum markers, modest liver inflammation, moderate to severe liver lesions | 281, 283–285 |
|                                    |                                                                             |                                                                           |                                                                              |                          |              |               |
| Immune tolerance suppressed         | *Pd-1* KO mice co-treated with CTLA4 antibodies to break the immune tolerance | Mimic human iDILI immune activation, useful for predicting iDILI drugs     | Drug-immune system interactions are poorly understood, results are difficult to interpret, needs further validation | ADQ, HAL, IHN, NVP, TGZ, TOL | Drug-immune system interactions are poorly understood, results are difficult to interpret, needs further validation | Slight ↑ of ALT, 290–293 |
|                                    |                                                                             |                                                                           |                                                                              |                          |              |               |
| Mitochondrial dysfunction           | *Sod2*<sup>−/−</sup> mice with enhanced mitochondrial disfunction and oxidative stress | iDILI reproduced at therapeutic drug doses. Useful for detecting and studying drugs that enhance mitochondrial stress | Excessive mitochondrial oxidative stress in other organs, little extended use, contradictory or hard to replicate results | TGZ, FLT, Discrete ↑ TVX, NIM serum ALT activity, variable or delayed hepatocellular necrosis and/or apoptosis | Excessive mitochondrial oxidative stress in other organs, little extended use, contradictory or hard to replicate results | 294–296 |
|                                    |                                                                             |                                                                           |                                                                              |                          |              |               |
| GSH knockdown                       | GSH-depleted rats through inhibition of γ-GCS or by drug tolerance,        | Possibility of linking GSH to iDILI drugs causing                         | Drug tolerance, Variable plasma ▲ ALT and AST, (continued on next page) | APAP, DIC, FTL,         |              | 297–299       |
|                                    |                                                                             |                                                                           |                                                                              |                          |              |               |

(continued on next page)
| Model type             | Description                                                                 | Advantage                                      | Disadvantage                                                                 | Example | Liver injury                                                                 | Ref. |
|-----------------------|------------------------------------------------------------------------------|------------------------------------------------|--------------------------------------------------------------------------------|---------|-------------------------------------------------------------------------------|------|
| Table 3 (continued)   |                                                                              |                                                |                                                                                |         |                                                                               |      |
|                       | knocking down its expression                                                 | hepatotoxicity, high sensibility               | variability of GSH and GST levels between humans and rodents, drug dose difficult to extrapolate to humans | CBZ, ENA, LTG, MTZ, PHT | slight ↑ of bilirubin, modest subacute injuries, dose-variable necrosis      |      |
|                       | NRF2-luciferase                                                              | Real-time bioluminescence imaging of transgenic NRF2-luciferase reporter mice | Detection of organ specific drug-induced oxidative stress                      | APAP    | ↑ serum ALT, extensive centrilobular coagulative necrosis                     | 300  |
|                       | Multifactorial models                                                        | Multiple determinant                           | Consider more broadly the multifactorial origin and susceptibility factors of iDILI | HAL     | Variable ↑ of plasma/serum markers, histopathology alterations centrilobular necrosis, immune cell infiltration, steatosis | 301, 302 |
|                       | Panels of inbred mice                                                        | Panels of different mouse strains displaying genetic diversity |                                                                                | APAP    | Variable ↑ of serum markers, variable hepatic necrosis                       | 303  |
|                       | Humanized Tissue models                                                      | Single tissue humanization                     | Widespread use for studying idiosyncratic drug reactions (e.g., FXB-mouse<sup>6</sup>) | Extra-hepatic organs are not humanized, immunocompromised mice cannot mimic immune-mediated drug toxicities, reconstitution using human iPSCs is low | TGZ, FLT, FNA, THD, FIAU, BOS | Variable ↑ of serum markers, slight eosinophilic changes, immune cell infiltration, scattered single cell necrosis | 304–307 |
|                       | Mixed                                                                         | Inclusion of whole human HLA system provides the most complete | Limited to special research facilities, humanization is often heterogeneous, cannot be spread to offspring | Not found | Lack of validation in iDILI studies, mostly used for the study of viral infections, occasionally low hepatocyte replacement, thymic | 308–310 |

APAP: Variable ↓ of serum markers, variable hepatic necrosis
model to study human iDILI in vivo when high liver repopulation is achieved (e.g., FRGN mice mixed model).

Genetic humanization

| HLA alleles | Introduction of human genes into the mouse genome | Strains are easy to maintain, possibility to express human genes in a variety of organs | Useful for elucidating immune-mediated drug reactions and for studying risk factors | Unexpected regulation of human genes in mouse context, risk of compensatory gene expression changes in alternative mouse pathways | Genetic susceptibility is insufficient to reproduce human iDILI: does not consider the immune tolerance system or CYPs |
|-------------|-----------------------------------------------|----------------------------------------------------------------|----------------------------------------------------------------|----------------------------------------------------------------|----------------------------------------------------------------|
| Mice carrying single or multiple human HLA linked to drug reactions (e.g., HLA-B*57:01, HLA-B*15:02) | | | | | |

Other alleles

| Mice expressing CYPs, phase 2 enzymes or drug transporters (e.g., PXR) | May reliably reflect human drug metabolism situation | Does not consider the human rate of drug elimination and immune system contribution |
|-------------------|--------------------------------------------------|---------------------------------|

ABC, abacavir; ADQ, amodiaquine; AFC8, FK506 binding protein-caspase 8; AMD, amiodarone; APAP, acetaminophen; BOB, bosentan; CBZ, carbamazepine; CCl4, carbon tetrachloride; CPZ, chlorpromazine; CsA, cyclosporine A; CTLA4, cytotoxic T-lymphocyte-associated protein 4; CYP, cytochrome P450; DIC, diclofenac; iDILI, drug-induced liver injury; DMETs, drug metabolizing enzymes and transporters; ENA, enalapril; FAA, fumarylacetoacetate; FIAU, fialuridine; FK506, tacrolimus; FLT, flutamide; FNA, fenclozic acid; FRG, Fah–/–; Il2rg–/–; iDILI, FRGN mice on the NOD-strain background; γ-GCS, γ-glutamylcysteine synthetase; GSH, glutathione; GST, glutathione S-transferase; HAL, halothane; hiPSCs, human induced pluripotent stem cells; HLA, human leucocyte antigen; HLC, hepatocyte-like cells; HSPC, hematopoietic stem progenitor cells; IHN, isoniazid; KO, knock-out; LPS, lipopolysaccharide; LTG, lamotrigine; MTZ, metronidazole; NIM, nimesulide; NOG, NOD/Shi-scid/IL-2Rγ−/−; NRF2, nuclear factor erythroid 2-related factor 2; NVP, nevirapine; PD-1, programmed cell death protein 1; PHH, primary human hepatocytes; PHT, phenytoin; PXR, pregnane X receptor; RAN, ranitidine; RIF, rifampicin; SCID, severe immunodeficiency syndrome; SOD2, superoxide dismutase 2; SUL, sulindac; TET, tetracycline; TGZ, troglitazone; THD, thalidomide; TK, thymidine kinase; TOL, tolcapone; TVX, trovafloxacin; uPA, urokinase-type plasminogen activator; VPA, valproic acid; XMT, ximelagatran.
treatment with LPS. Since then, the murine inflammmogen models have been used to induce reproducible liver injury caused by different iDILI drugs at non-toxic doses (Table 3). However, this model has several limitations, such as the different timing of LPS exposure between studies, the acute response obtained rather than delayed, the difficulty to replicate the results and the rapid loss of sensitivity to LPS by Toll-like receptors. Moreover, this model commonly induces a neutrophil-mediated toxicity (innate immune response), and therefore does not consider the role of the adaptive immune system, which is also believed to be central in the progression of iDILI in humans, since increased activation of helper and cytotoxic T-cells has been detected in DILI patients.

Despite these disadvantages, models of LPS-drug interaction are the first to mimic the pronounced liver injury seen in iDILI patients, supporting the inflammatory stress hypothesis of iDILI.

4.3. The immune tolerance suppressed model

The immune tolerance suppressed model, or “Uetrecht-Pohl” model is based on the hypothesis that immune tolerance prevents progression to liver failure, and its breakage is necessary for iDILI to overcome. Metushi et al.290 developed an amodiaquine-induced liver injury using programmed cell death protein 1 (PD-1) KO female C57BL/6 mice co-treated with an antibody against cytoxic T-lymphocyte-associated protein 4 (CTLA4) in order to break the inhibition of cytotoxic T lymphocyte activation. Although the severity of the injury still does not fully mimic clinically significant iDILI, this model was a pioneer animal model for reproducing some histopathological and immune features occurring in human iDILI. Since then, the same hypothesis has been used to develop a halothane-291, IHN- and NVP-induced292 liver injury models. The “Uetrecht-Pohl” model has shown to be capable of distinguishing between hepatotoxic and non-hepatotoxic drugs of the same class and confirm an immune mechanism for troglitazone- and tolcapone-induced iDILI293. Although this model may be useful to predict which drug candidates are likely to be associated with an increased risk of causing iDILI, it is a relatively new approach and requires further validation with other iDILI drugs. For example, very recently, the “Uetrecht-Pohl” model was used to examine the effect of 1-methyl-α-tryptophan, an inhibitor of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO). Contrary to what was expected, cotreatment of amodiaquine with 1-methyl-α-tryptophan decreased liver injury. This shows that in vivo studies may reassert the complexity of the immune response and highlight that drug-immune system interactions are still difficult to model and predict.

4.4. The mitochondrial dysfunction model (Sod2+/− mice)

Animal models with previous mitochondrialopathies have been suggested as potential iDILI models. For example, mice with partial deficiency of the mitochondrial superoxide dismutase 2 (Sod2+/−) display an impaired mitochondrial function due to excessive mitochondrial oxidative stress. This renders the mice sensitive to drug-induced mitochondrial damage. This model has been used to study troglitazone-induced liver injury, observing both increased ALT levels and hepatic necrosis after drug treatment at similar doses to those used in humans294. In the same way, in Sod2−/− mice, flutamide, but not bicalutamide (a drug in the same pharmacologic class but without idiosyncratic liability) treatment caused oxidative stress in liver and decreased mitochondrial aconitate activity and complex I and III subunits expression295. Similar mitochondrial dysfunction was observed in Sod2−/− mice treated with trovafloxacin296. However, attempts to reproduce those results have had mixed success19 and this model is not commonly used. Recent discovered associations between Sod2 variants and risk of developing cholestatic/mixed iDILI297 and hepato cellular298 iDILI in humans may increase the interest in using mitochondrial dysfunction animal models in iDILI research.

4.5. The GSH knockdown model

Based on the hypothesis that CRM generation plays an important role in iDILI outcome, developing an animal model of liver injury in which the potential scavenger level is reduced could be an interesting strategy22.

The de novo synthesis of GSH is regulated in mammals by the enzyme γ-glutamylcysteine synthetase (γ-GCS). A GSH-depleted rat model was established by knocking down the expression of γ-GCS, showing a potentiated APAP-induced hepatotoxicity298 and significant ALT elevations after treatment with diclofenac and flutamide297. However, diclofenac- or flutamide-induced subacute hepatotoxicity was not detected, probably due to decreased responsiveness to toxicity (drug tolerance). Using a different approach, other researchers have developed several models of iDILI developed by administering l-buthionine-(S,R)-sulfoximine, an inhibitor of GSH synthesis23. In rats with coadministration of carbamazepine (CBZ) and l-buthionine-(S,R)-sulfoximine, an increase of the levels of ALT was observed, meanwhile rats treated only with CBZ did not show any sign of hepatotoxicity299,304. The same strategy has been used to establish an animal model of iDILI caused by other drugs (Table 3).

Despite the high sensitivity of this rat model, the activity and levels of hepatic glutathione S-transferase (GST) and GSH are higher in rodent than in humans, making it difficult to extrapolate drug dosage for studying human hepatotoxicity297.

4.6. The NRF2-luciferase reporter mouse

As many iDILI drugs induce oxidative stress via different mechanisms involving the generation of ROS, a model for the detection of drug-induced oxidative damage could be of great value. The transgenic NRF2-luciferase (NRF2-luc) reporter mouse has shown promise for detection of the NRF2 response to drug-induced oxidative stress at the body level, using real-time bioluminescence imaging305. This mouse model allows highly-localized cellular and organ specific drug-induced oxidative stress detection and could be employed in mechanistic investigations in preclinical drug development.

4.7. The “multiple determinant” mouse model

Consistent with the multiple determinant hypotheses, animal models that reproduce several human susceptibility factors would be useful to better understand the multifactorial origin of iDILI. Following this idea, an animal model of halothane-induced liver injury has been generated306,307. Clinical studies have revealed risk factors for halothane-induced liver injury, and these include female sex, adult age, and genetics as well as probable risk factors (fasting and inflammatory stress)325,326. Therefore, to generate an in vivo halothane-induced liver injury model, fasted, female,
BALB/c mice eight weeks of age were used. These mice responded to halothane with pronounced centrilobular necrosis and high plasma ALT levels. Interestingly, younger, male, or fed BALB/c mice and C57BL/6 mice were more resistant to the hepatotoxicity, confirming that age, genetics and sex are critical susceptibility factors for iDILI development.

4.8. Use of panels of inbred mice

This strategy is based on the hypothesis that using panels of different mouse strains displaying genetic diversity would increase the chances of detecting idiosyncratic reactions to drugs and finding specific susceptibility genetic polymorphisms. For example, 36 different inbred mouse strains were used to study differential susceptibility to APAP. Following the administration of a single dose of 300 mg/kg APAP, marked differences in liver toxicity were observed among the 36 mouse strains. The authors employed genome-wide association study and targeted sequencing and determined that polymorphisms in Cd44 (a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration) were associated with the magnitude of the increase in ALT activity after APAP exposure. Finally, the association between CD44 orthologous human gene with susceptibility to APAP was confirmed in two independent human cohorts. This study demonstrated that a diverse mouse population could be used to study iDILI mechanisms and identify genetic potential susceptibility biomarkers for the screening of human populations. More interestingly, Church et al. used omics technologies to analyze the effect of isoniazid on a panel of well-characterized, inbred mouse strains with genetic diversity comparable with that observed in humans. However, this study focused on IHN-induced steatosis and no serious liver injury (measured by microscopy and miR-122 levels) was reported in the animals used. Although iDILI was not detected, this work is another example of how diverse mouse panels can be used to identify transcriptional changes, metabolites, and gene variants that contribute to a specific liver response in genetically sensitive individuals. However, the use of this model in a screening assay for predicting iDILI during drug development has several disadvantages, such as its high cost and difficulty to implement. Besides, it has been assumed that, given the low incidence of iDILI, it would be necessary to perform toxicity testing in 30,000 animals for a single drug to predict iDILI and these panels of mice might not include sufficient biological diversity to elucidate iDILI responses confidently.

As we have pointed before, these animal models are hardly used for iDILI prediction, principally due to species-specific issues. In order to circumvent this problem, humanized animal models allow the detection of human-specific liver toxicity with the advantage of producing in vivo human-specific metabolites.

4.9. Humanized mouse models

Two different approaches have been proposed to generate humanized mouse models: (1) the engraftment of human cells into an immunodeficient host, resulting in tissue humanized animal models; and (2) the introduction of human genes into the mouse genome to generate genetically humanized mouse models (Fig. 3).

Figure 3  Main approaches for generating humanized mouse models. (a) Tissue humanized mice models can be generated in many different manners. These models have in common the use of immunosuppressed mice in which hepatocytes are eliminated and replaced with human hepatocytes. (b) Mixed humanized models combine the strategies to humanize mouse livers with the generation of a human-like immune system by the introduction of human hematopoietic progenitors. (c) The two main strategies for generating genetically modified mice include: 1) Gene-targeted transgene approach, where the transgene is inserted specifically into mice’s genome. DNA is introduced into embryonic stem cells (ESCs) that are positively selected and injected into early mouse blastocysts. These blastocysts are transferred into pseudopregnant females, that will deliver chimeric offspring. After crossbreeding with wild type mice, homozygous transgenic mice are obtained. 2) Standard transgene approach, where the transgene is inserted anywhere into the mice’s genome. Foreign DNA is injected into male pronucleus of fertilized mouse eggs that are cultured and implanted into pseudopregnant females. The offspring delivered is then screened for transgene presence. (This figure has been created using Biorender.com.)
4.9.1. Tissue humanization mouse models

In the tissue-humanized mouse models, the strategy consists on replacing rodent hepatocytes with human hepatocytes (PHHs or HLCs derived from hiPSC) so they can address some of the differences observed between animals and humans in liver pathways. These models can mimic human histologic and biochemical features of iDILI and human liver phenotype since they have humanized liver functions, including the synthesis of human DMETs at an appropriate level for pharmacological, biological and pathological studies. The cells’ sources for humanization are typically PHHs and HLCs, although there are also studies using immortalized cells such as HepaRG.

For humanization to be successful, host mice must have two key features: (1) exhibit a combination of severe immunodeficiency defects to accept xenogeneic cells when properly transplanted and (2) include a system for damaging endogenous murine liver cells. These two conditions prevent competition of host cells with human cells and facilitate an environment for the engraftment and proliferation of transplanted human liver cells into the mouse liver.

Immunodeficiency of these mice is usually achieved by following either a single or a combination of the following strategies: knocking out the recombinant activation gene-2 (Rag-2) or knocking out interleukin 2 receptor common gamma chain (Iγ2r) for example, NOG mouse is a highly immunodeficient non-obese diabetes (NOD) mouse strain, which harbors mutations inactivating both the innate (Iγ2r−/−) and adaptive (SCID) immune responses.

In terms of the mechanism for inducing the murine liver failure, there are also various approaches that can be used. The first studies of substantial repopulation of a mouse liver with human hepatocytes were published in 2001 by Dandri et al. and Mercer et al. and, in both studies, liver failure was induced by expression of urokinase genes controlled by an albumin promoter. This albumin-promoted urokinase-type plasminogen activator (Alb-uPA) leads to intracellular activation of plasminogen-activated plasmin, which induces proteolytic damage inside the hepatocytes. Another strategy consists of deleting the fumarylacetoacetate hydrolase gene (Fah−/−). Fah−/− mice accumulate the toxic metabolite fumarylacetoacetate, which induces chronic liver damage. However, the drug 2-(2-nitro-4-trifluoro-methylbenzoyl)1,3-cyclohexedion (NTBC) blocks the accumulation of the toxic metabolite and prevents liver damage, so animals can be maintained in a healthy state while taking the drug and selective pressure for repopulation of the liver with human donor cells can be applied by withdrawal of NTBC. Other options to destroy mouse liver cells are to transgenically express genes that, under the appropriate conditions, also lead to the death of murine hepatocytes. Some examples are the herpes simplex virus type 1 tyrosine kinase (HSVtk) and the diphtheria toxin receptor (DTR) under the control of the albumin promoter.

Humanized uPA/SCID mice were the first animal models able to reproduce the severe toxicity produced by troglitazone and have been used to study factors contributing to iDILI caused by this drug. Tateno et al. developed a variation of the uPA/SCID model, known as cDNA-uPA/SCID, since these mice express the cDNA of uPA instead of the whole gene. This model (PXB-mouse) has been extensively used in pharmacodynamic studies and to characterize in vivo idiosyncratic hepatotoxins such as troglitazone, flutamide and more recently, to determine the metabolic fate of fenoflaci, a potentially human iDILI toxin. PXB-mice have served to study the pharmacodynamics of 30 different drugs, obtaining a high correlation with humans. The uPA-NOG mouse model developed by Suemizu et al. has also been used to study iDILI by thalidomide. Moreover, a TK-NOG model has been used to introduce primary human hepatocytes or HLCs derived from hiPSC, achieving a humanized liver stable for more than 6 months, and allowing short- and long-term toxicity studies. This model has been employed to study fialuridine toxicity. Moreover, human liver chimeric TK-NOG mice have also been used to identify drugs that cause animal-specific hepatotoxicity and to model cholestatic liver toxicity induced by bosentan. Other cell types such as HepG2 and HepaRG have also been used to generate humanized models. However, the use of these cells usually results in a considerably lower percentage of chimerism than with primary human hepatocytes.

Although these examples suggest that chimeric mice with humanized livers could be used as experimental models to study iDILI, their use is quite limited to specialized research facilities and their translational value needs to be further validated, since human hepatocytes used to replace mouse’s ones are obtained from a small panel of human donors and represent a limited sample of human genetic diversity. Moreover, in chimeric mice, extra-hepatic organs are not humanized, and for studies of orally administered drugs, it would be desirable to develop model animals with humanized intestine. Furthermore, these animals are highly immunocompromised, thus they fail to mimic immune-mediated drug toxicities. Additionally, the humanization induced in these animals is often heterogeneous, cannot be spread to the offspring and the degree of reconstitution achieved when using hiPSCs is still low. However, in an attempt to minimize the effect of murine liver enzymes on drug metabolism, Barzi et al. generated a conditional knock-out of the NADPH-P450 oxidoreductase (Por) gene mouse model with a humanized liver. The authors were able to detect higher amounts of human metabolites after treatment with the anticancer drug gefitinib or the retroviral drug atazanavir. Additionally, the development of chimeric mice repopulated with PHHs from a range of individuals with a variety of DMET polymorphisms may be helpful for the prediction of the variability of human metabolites and its relationship with iDILI risk. An interesting approach consisting of hepatocytes from the immunized liver can also be used for in vitro studies and thus establish valuable in vitro–in vivo correlations. As we have mentioned before, certain iDILI risk factors can be modelled in vivo using pre-treatments or genetic alterations designed to pre-dispose the animals to injury, but human adaptive/innate immune system involvement is more difficult to adapt in animal models. Moreover, mouse immune system shows many differences in comparison to humans.

- Mixed humanized mouse models

The main reason animal models cannot be used as a surrogate for human immunological drug reactions is that they do not contain fully integrated human innate and adaptive immune function. Mouse models combining both humanized liver and immune system (mixed humanized mouse models) are emerging in order to detect any human-specific iDILI, which cannot be detected in mice only bearing humanized liver.

First attempts to develop mixed humanized mouse models were achieved in 2011, when Washburn et al. generated a
mouse engrafted with both human liver and immune cells to study hepatitis C virus infection. Using BALB/c/Rag2/C-null mice (which lack functional T, B and NK cells), the authors co-transplanted human CD34+ hematopoietic stem progenitor cells (HSPCs) and hepatocyte progenitors which led to efficient engraftment of human leukocytes and hepatocytes. A 40% repopulation rate of human liver cells in the mice was achieved by a drug-inducible suicidal activity in mouse liver cells. More recently, this model has been used to specifically study liver inflammation and cirrhosis during hepatitis C virus infection. However, the immature fetal liver cells used by Washburn et al. lack some critical features of adult drug metabolism and the degree of liver repopulation achieved was low. While low hepatocyte replacement indices can support some human liver infections, a high degree of liver chimerism is essential for any study that involves physiologic or xenobiotic metabolism, pharmacokinetics, drug–drug interactions, transport or toxicology.

To solve part of the limitations of the Washburn model, uPA-NOG mice were used for dual reconstitution with human liver and immune cells using mature hepatocytes (instead of fetal) and HSPCs after treosulfan conditioning. Similarly, Wilson et al. developed a model of dual hepatic and hematopoietic humanization. This model was generated on FRG mice on the NOD-strain background (FRGN) which was simultaneously co-transplanted with adult human hepatocytes and hematopoietic stem cells after busulfan and Ad:uPA pre-conditioning. The double-chimeric FRGN mice obtained liver repopulation rates of 80%, which makes it more suitable for preclinical drug testing. However, thymic epithelium was not humanized in these double-chimeric FRGN mice thus limiting their utility for studying human T-cell responses. In a recent study, Kim et al. used HepaRG as a source of hepatocytes for the generation of a mixed model. The authors achieved enhanced human hematopoietic reconstitution due to the immunomodulatory and hematopoietic properties of HepaRG. This model would also avoid the complexity and high costs of using fetal or primary hepatocytes for the generation of mixed models. Recently, Dagur et al. established a protocol for dual humanization of hepatocytes and HSPCs in TK-NOG mice for liver disease modelling, maintaining the chimeric mice for long periods after transplantation.

The introduction of a complete human HLA system to the liver-humanized models displays a more complete overview for prediction of iDILI and could contribute to further decipher the pathophysiology of the disease. However, to our knowledge these models have been primarily used to study viral diseases and have not yet been validated in enough iDILI studies despite being proposed for a wide variety of hepatotoxicity analyses.

4.9.2. Genetically humanized mouse models

Another approach to integrate human immune functions for modelling iDILI in animals consists in transgenic mice carrying human HLA alleles. The first model, a mouse carrying HLA-A2 (the most frequent HLA allele in humans) was generated by Matsunaga et al. in 1985. Since then, this approach has been used to generate several HLA-related disease models. A few years ago, HLA-DR3 transgenic mice were used to model autoimmune hepatitis (AIH), achieving a sustained elevation of ALT, development of autoantibodies, chronic immune cell infiltration and parenchymal fibrosis on liver histology. Recently, HLA-B*57:01-transgenic mice were generated to study HLA-linked skin reactions. Abacavir treatment was demonstrated to activate CD8+ T cells in these mice and induce inflammation in the skin. However, the drug was tolerated in vivo and the skin reactions did not fully mimic that observed in humans.

Song et al. attempted to reproduce abacavir-induced liver injury in HLA-B*57:01 transgenic mice. Oral administration of abacavir alone did not increase levels of ALT, but the cotreatment with abacavir and CpG oligodeoxynucleotide, a TLR9 agonist, resulted in a marked increase in ALT, pathological changes in liver, increased numbers of activated CD8+ T cells and tissue infiltration by immune cells in transgenic mice. In this model, the possible adaptive immune system-mediated liver injury might be a consequence of the T-cell homing led by an up-regulation of adhesion and costimulatory molecules on APCs, which is directly caused by the TLR9 agonist. These results showed that inflammatory reactions and/or innate immune activation are necessary for abacavir-induced HLA-mediated liver injury.

Although the use of HLA transgenic mice to investigate the mechanisms of immune-mediated drug induce toxicity is increasing worldwide, these models are not ideal, since iDILI is not developed in each individual of a given HLA model population when a hepatotoxic drug is taken. For example, no liver injury has been observed when ximelagatran was given to mice expressing HLA-DRB1*0701 and HLA-DRA*0102 or HLA-DQB1*0202 and HLA-DQA1*0201, showing that mimicking of genetic susceptibility in mice is not sufficient for reproducing the complex pathogenesis leading to iDILI in humans. Thus, other elements, such as the immune tolerance system, CYP metabolism of drugs and other genetic factors, should be considered. For example, CD14 variation and ER aminopeptidase 1 (ERAP1) haplotypes have been identified as factors that determine the susceptibility of HLA-B*57:01 to abacavir hypersensitivity. Moreover, the single nucleotide polymorphism rs2476601 in the protein tyrosine phosphatase non-receptor type 22 gene (PTPN22) doubles the risk of HLA-A*02:01 and DRB1*15:01-related amoxicillin-clavulanate-induced iDILI.

Another strategy for studying drug metabolism and toxicity is to genetically modify mice to express xenobiotic receptors involved in DMETs. For example, pregnane X receptor (PXR) is a transcription factor that regulates the metabolism of xenobiotics and endobiotics and has been extensively related to several iDILI reactions. Genetically humanized PXR-mice have been useful in unraveling iDILI mechanism for diclofenac, or for the cotreatment with Rif and IHN. Similarly, genetic humanization has also been performed for other receptors, such as the aryl hydrocarbon receptor and many individual CYP450 genes, including the families CYP1, CYP2, CYP3 and CYP4; and have continuously been proposed for drug toxicity analyses. The inclusion of multiple human genes instead of individual genes could also be a powerful strategy for predicting iDILI, as it is a more complete reflection of a real human drug metabolism system. For example, Scheer et al. deleted the constitutive androgen receptor, PXR and different P450 genes and exchanged them for their human counterpart.

An advantage of using genetically humanized models is that the strains are easier to maintain and there is a possibility to express human genes in a variety of organs. Despite being very widespread models in pharmacology for years, the major drawback of these humanized mouse models is the expression of human genes is still in the mouse context, which may lead to unexpected regulation that complicates data interpretation. Another limiting factor that applies to the genetically humanized but not tissue humanized mice is the risk of compensatory gene
expression changes in alternative mouse pathways as a consequence of the genetic modification.

4.10. Highlights

- In vivo systems are an essential part of the preclinical approach to IDILI.
- Ideal IDILI animal models have mechanistic resemblance, similar risk factors and experimental conveniences as humans.
- Non-humanized in vivo models have provided important insights into specific disease mechanisms.
- Humanized in vivo models are complex but may be the systems that most thoroughly attempt to encompass many of the factors involved in IDILI in humans.

5. In silico approaches to predict IDILI

An in silico approach is defined as the study of a specific topic through the use of computational analysis techniques based on the field of bioinformatics. Specifically, it refers to a computational model (i.e., a mathematical algorithm) and the specific organization of the related data that allow a subsequent computer-based analysis. In the case of iDILI studies, in silico models are used to predict the potential of different drugs to cause IDILI.

Since liver toxicity is a major issue for public health, pharmaceutical development and drug regulatory agencies, there is great worldwide interest in developing rapid and precise computational models to assess the IDILI risk early stages of drug development. While in vivo and in vitro approaches are expensive and time-consuming, currently available in silico techniques have been widely used to reduce the cost of IDILI risk assessment. Compared to other approaches, the most important advantage of in silico models is the ability to screen a large number of chemical compounds in short time, even before they are isolated or synthesized. Curiously, despite the clear advantages of these models, computational studies in iDILI field have only started to emerge in the last decade. In fact, to our knowledge, one of the first in silico models described was developed as recently as in 2003. The majority of available computational models for hepatotoxicity have been developed in recent times.

For some endpoints, such as mutagenicity, teratogenicity or skin sensitization, for which underlying mechanisms are enough understood, in silico models have been well developed. Nevertheless, for more complex endpoints, as the organ-level endpoints involved in IDILI, there is still a scarcity of well-validated computational models.

Both the characteristics of the drug and the individual factors of the patient (host factors) are important in the differential diagnosis of IDILI; therefore, it is necessary to take into account both of them when developing models for IDILI prediction.

Traditionally, in silico models for IDILI prediction have only been based on the chemical structure of the compounds. These methods have commonly been divided into two approaches: statistically-based, quantitative structure–activity relationships (QSARs) and qualitative expert-based systems. In addition, these models can be subdivided by i) the endpoint being modeled (overall hepatotoxicity or a iDILI specific endpoint, e.g., cholestasis or steatosis); ii) the type of algorithms used to develop the model; and iii) the type of data being modeled (in vivo or in vitro).

Statistical models are normally generated from a dataset of chemical structures, which defines the relationship between biological activity and chemical structure, together with associated toxicity data, which are used as training for an automatic algorithm. QSARs models can reach accuracies of 53%–84% in correctly predicting IDILI within the retrospective studies conducted, as reviewed. Frequently used algorithms in these type of models are Bayesian models, k-nearest neighbor quantitative structure (kNN-QSAR), random forest and artificial neural networks. On the other hand, expert-based systems take advantage of the expert-knowledge of toxicological mechanisms, toxicity of compounds and chemical reactivity and metabolism directly related to chemical structure and molecular features. These approaches are not usually based in statistics and result in the development of structural alerts or 3D pharmacophore models. One example of these models is eDISH software.

eDISH, which stands for “evaluation of drug-induced serious hepatotoxicity” was developed by the FDA in 2004, and it was a major breakthrough in assessing liver safety in clinical trials. This software (currently named “Hepatic Safety Explorer Chart” [https://safetygraphics.github.io/hep-explorer/]) plots peak serum values for ALT and TBIL in an x–y log–log graph for all subjects enrolled in clinical trials, and links this information to all liver chemistry values obtained for each subject. Subjects who reach moderate elevations of parameters after treatment with new drugs can be evaluated to distinguish between benign from malignant hepatic effects, preventing unnecessary stopping of safe drugs and facilitating these drugs reach the market. The authors have stated important new features are going to be included in eDISH, such as the parameter P_{ALT} (that includes both peak and area under the curve [AUC] ALT) and the new-R ratio.

Statistical-based models’ development is easier and faster than the experts-based systems, which is likely the reason why the majority of models developed to date are of this type. However, using models solely based on the physicochemical properties of the drug is insufficient, given the complexity of both liver biology and iDILI mechanisms. Because of this, the incorporation of biological characteristics of the patient in the models is essential.

Only a few models use statistical approaches to link existing data about the drug to clinical data of patients. These models combine both chemical and biological characteristics and are known as hybrid models. Recently, Gonzalez-Jimenez et al. have developed a prediction model to use in future iDILI phenotyping, by providing an estimated likelihood of hepatocellular versus cholestatic injury based on both properties of causal drug and host factors.

DILIsym software is also considered a hybrid model. Although it is a statistical-based model, it includes features of expert models and considers host factors. DILIsym is a platform created in the framework of DILIsym initiative, a public-private partnership, involving scientists from industry, academia and the FDA. The objective of the system is predicting IDILI events in humans. The model was initially built by analysis of data obtained from the literature, or experimentally, for example compounds that caused liver injury in vivo via a variety of mechanisms and chemically similar nontoxic compounds. This software simulates the mechanistic interactions and events from drug administration through the progression of liver injury and regeneration. It has been capable of modeling some aspects of CRM formation, inhibition of BAs efflux from hepatocytes, mitochondrial and BAs toxicity, mtDNA depletion, as well as innate immune responses.
DILIsym® has been already used to model iDILI events associated with troglitazone775, entacapone380, and acetaminophen81. One important asset of DILIsym model is that it is not limited to specific classes of drugs, but it is created by modeling both gene and protein interactions information from the literature. Therefore, it is hypothesized that this mechanistic computational model is likely to yield substantial improvements in the accuracy of predictions of iDILI. For example, MITOSym49 is an in silico tool developed in DILIsym87 to understand the mechanism of clinical mitochondrial hepatotoxicity882. It has been successfully used to retrospectively determine the role of mitochondria in tolvaptan883, macrolides884 and TAK-875 hepatotoxicity885.

During the initial development of drugs, computational models assessing hepatotoxic effects need to reach a high sensitivity and high specificity. Normally, in silico models have a slightly higher false positive rate than a false negative rate, to avoid the consequences of a hepatotoxic drug reaching clinical trials or the market886. Nevertheless, it is important for these models to be balanced, so that non-hepatotoxic drugs are not discarded during drug development stages.

All computational models developed to assess hepatotoxicity need to undergo internal and external validation to confirm their predictive power and reliability. This validation is considered the most accurate and correct method887. As the reader can infer, to achieve a good in silico predictive model, data becomes critical. The development of the predictive model and its validation are based on data, so the performance of the prediction algorithm will depend directly on the quantity and quality of data available during its development873. Regarding the characteristics of the drug, a multitude of databases are currently available on the market, both free and private-access, to consult different data on marketed drugs: drug metabolism, mechanisms of liver injury, toxicity, chemical structures, in vitro and in vivo bioassays, etc. For example, the LTKB874, the LiverTox website (https://www.LiverTox.nih.gov), Pharmapendium, ACToR (Aggregated Computational Toxicology Resource)888, PubChem (https://pubchem.ncbi.nlm.nih.gov) or Drug Induced Liver Injury Dataset (DILInd), etc. Together, these databases contain thousands of records of chemical structures and other interesting molecular features. Perhaps the greatest effort in obtaining this type of data is found in herbs, which have a more varied and complex composition.

There are also different databases focused on transcriptomics data. One example is “Open TG-GATEs” (http://toxico.nibiohn.go.jp/english/index.html), a toxicogenomics database which stores gene expression data from rat liver and kidney samples and from primary rat and human hepatocytes after treatment with 170 drugs and chemical compounds889. The database also contains associated toxicological data such as biochemical, blood and histopathological data. Open TG-GATEs are included in the open platform for predictive toxicology “Open Tox” (https://opentox.net).

However, obtaining the characteristics of patients who develop iDILI is highly complex. Although there are available data in medical records and literature, there are limited hepatotoxic data sets properly formatted for developing models and obtaining new hepatotoxicity data sets is becoming extremely challenging. Since there are few hepatotoxicity data sets available, they could have been used to develop different models that only differ in a few characteristics, such as the design of the algorithm itself, and, as a result, the variability of the models may be affected890. This absence of new data to support the models makes them less applicable in drug evaluation and less attractive to industry and researchers for developing hybrid models.

However, over the years, different initiatives have been developed to create databases that successfully collect suspected cases of iDILI and include both clinical and demographic characteristics useful in the diagnosis of iDILI. One of these, the followings may be of particular interest: in Spain, the Spanish iDILI Registry (RDI) founded in 1994, was the pioneer in the matter891, and in the US, the DILIN database founded in 2004 (https://dilin.org/). Also noteworthy, at the European level, the Prospective European Drug-induced Liver Injury Network (ProEuro iDILI Network) arising from an European Cooperation in Science and Technology (COST) action in 2018 (https://proeurodilinet.eu); at the Latin American level, the Latin American iDILI Registry (LAustinDILI network), founded in 2011892; and finally the incipient Indian Network for Drug-Induced Liver Injury (IN-iDILI), that has been collecting cases since 2013893. These databases provide very valuable and necessary information to develop iDILI prediction models in silico. Nevertheless, this area constantly demands more and more data and better classification systems. Building predictive models with the most up-to-date algorithms brings new challenges to find more data sets to validate the models.

Due to the complex nature of iDILI, future directions point to the combination of in vitro testing of drugs on human cell lines and in silico modeling of drug behavior and iDILI initiation/progression as the preferred approach to detect iDILI compounds. Cell-based in vitro systems would be first used to assess the effects of compounds on relevant stress pathways, and the information obtained by these assays would be used to optimize and validate in silico models, in order to predict, for example, the tolerable dose of a compound. In a recent study performed by Sankalp et al.894, in vivo data in combination with in silico predictions models, based on in vitro data, were used for modeling hepatic steatosis. This study represents an advance in the field and sets the guidelines to be followed in future studies.

Therefore, in the future, data generated both by in vitro and in vivo assays in combination with genetic and epigenetic information obtained from iDILI patients can be used for elaborating predictive in silico models to detect compounds with iDILI potential895.

5.1. Highlights

- In silico models allow the analysis of multiple chemical compounds at once.
- Validation of these computational models is needed for the study of complex endpoints.
- In silico prediction models are based on the patients’ and drugs’ characteristics.
- Lately, a great effort is being made to create more thorough iDILI registries.

6. Future perspectives

This section presents a series of future perspectives and goals, paths in which the research field of iDILI modeling could be directed according to the authors’ opinion.

6.1. Patient-derived cells and personalized medicine

The continuously growing evidence about iDILI mechanisms suggest that neither the drug-specific nor the patient-related
factors per se would typically pose a risk for IDILI; much more it is the combination of both which causes these rare events in patients leading to termination of drug development programs or withdrawal of drugs from the market (Fig. 4). Knowing patient-specific factors which could lead to IDILI in combination with a drug in development may allow for patient stratification and a more personalized development path.

Therefore, the prediction of IDILI requires a more precise understanding of individual risk factors, which should be included into in vitro test systems. The use of patient-derived cells in IDILI modeling would allow studying the impact of host factors, such as the individual’s genotype, on the pathogenesis of the disease. For example, in the next steps, external validation of new in vitro diagnostic test systems, such as MetaHeps™, which uses monocyte-derived hepatocyte-like (MH) cells from the affected patients, could help to identify the drug responsible for IDILI episodes in cases of polypharmacy and to identify potential individual susceptibility to IDILI due to specific drugs.

Some promising approaches include the use and characterization of patient-derived HLCs, obtained by differentiation of iPSCs (see Section 2.1.3) or by direct reprogramming of somatic cells; and the use of skin cells, since a relation between skin reactions and liver damage has been observed.

- **Induced hepatocytes (iHeps)**

The recent technology of direct cellular reprogramming of HLCs from somatic cells could overcome the shortage of liver donors, being an alternative source to generate hepatocytes.

The first successful direct conversion of murine fibroblasts into induced hepatocytes (iHeps) was achieved in 2011. The induced cells were generated via forced expression of Gata4, Hnf1a and FoxA3 in the context of P19 knockdown, or expression of Hnf4a plus FoxA1, FoxA2, or FoxA3.

Since then, new tools and strategies have been found to obtain murine and human iHeps (hiHeps) by direct reprogramming. This technique has several advantages compared to the generation of hepatocytes derived from hiPSCs, such as relative simplicity and short time requirements.

The generation of hiHeps by direct reprogramming of fibroblasts derived from IDILI patients may be a promising approach to understand the pathogenesis and biomarkers of IDILI, as well as to design strategies aimed at preventing liver injury. Moreover, panels of hiHeps with specific genetic polymorphisms may provide a way for evaluating patient-specific liver adaptation following cellular stress.

However, it is not known whether fibroblasts derived from IDILI patients can be directly reprogrammed into hiHeps that reflect the main pathological characteristics of IDILI. Therefore, comparison to freshly isolated PHHs will be necessary to assess whether hiHeps recapitulates the physiology of PHHs and liver tissue.

- **Skin cells**

Although liver injury due to hepatic cells damage is the most common phenotype in IDILI, this disease manifests in a variety of clinical presentations, including hypersensitivity features, which are present in a small proportion of patients. Indeed, in different IDILI registries, the prevalence of these hypersensitivity manifestations varies from 14% to 25%. The most frequent hypersensitivity symptoms in IDILI are skin reactions, usually related to a poor prognosis of the disease. On the other hand, severe cutaneous adverse reactions (SCARs) can manifest in a
spectrum of heterogeneous clinical presentations, including SJS, TEN and more commonly, drug reaction with eosinophilia and systemic symptoms (DRESS)408, in which the liver is the organ most frequently affected. A limited number of different causative drugs have been identified as responsible for iDILI associated with SCARs, such as anti-infective, anti-epileptics drugs and allopurinol419.

A relationship between dermatological manifestations and the severity and prognosis of the liver damage has been suggested410-412. In a prospective study with DRESS patients conducted at King’s College Hospital, London, UK, an association between the presence of erythema multiform-like eruptions and the development of severe hepatotoxicity in patients was identified412. More recently, a multicenter retrospective study showed that the most extensive necrosis (40% and 90%) was observed in two patients requiring liver transplantation, and 7 of 16 DRESS patients with ALF had activated lymphocytes with a cytotoxic phenotype in peripheral blood, skin, and liver411. In fact, the immune response could be an essential factor in the pathogenesis of iDILI and in other severe hypersensitivity reactions to drugs, such as DRESS413.

There are several HLA alleles considered as risk factors for the development of SCARs and iDILI produced by different drugs414. For example, the HLA-A*02:01 allele has been identified as a risk factor for both iDILI and CBZ-induced SCARs410.

Thus, an interdisciplinary approach would be useful to identify the risk factors and manage the hypersensitivity reactions affecting both the skin and the liver. In addition to the potential bioactivation of drugs in the skin415, recent studies suggest that keratinocytes and hepatocytes share a similar response to hepatotoxic drugs.

Different hepatotoxic compounds have been shown to induce the expression of genes related to the immune and inflammatory response, such as S100A8, S100A9, NALP3, IL-1β and RAGE in both hepatocytes417 and primary human keratinocytes418. Furthermore, it has been observed that the basal expression and induction of IL-1β in keratinocytes after exposure to hepatotoxic drugs can be a tool to identify individuals at high risk of iDILI418.

Therefore, skin cells, such as dermal fibroblasts and keratinocytes, could be involved in cytotoxicity mechanisms observed in iDILI. Human primary keratinocytes are metabolically and immunologically active, resulting in this cell type being the most used in cutaneous metabolic research when using a monolayer 2D system416. Furthermore, although dermal fibroblasts have non-immune capability, it is known that cross-talk between fibroblasts and keratinocytes maintains skin homeostasis, and they are two of the major cell types that respond to the inflammatory phase in the cutaneous repair/regeneration process419.

Hence, the use of primary dermal fibroblasts and keratinocytes derived from patients could be a useful tool to address the fundamental problems of iDILI research. iDILI mechanistic steps would be best analyzed using patients-derived samples with their unique genetic profile and other unknown risk factors instead of experimental models that lack these features.

6.2. In vivo models to study drug-induced cholestasis

Drug-induced cholestasis, as mentioned above, is the most common hepatic injury type in elderly, and much effort is still needed to obtain models that accurately reflect this phenomenon (Fig. 4). There are several in vitro models that try to approach cholestasis, and it seems that co-cultures and the mixed approaches, using high content screening, are getting closer to the real situation. Nevertheless, in order to implement a true-to-life model, drug-induced cholestasis prediction has to be approached in animal models. Although there are several animal models (mainly rodents) used to unravel cholestasis pathophysiological mechanisms, these animal models are not yet used for drug-induced cholestasis prediction, principally due to species-specific issues420. The composition of BAIs is different in rodents than in humans, and milder genetic BSEP deficiencies do not lead to serious liver injury, since there are several mechanisms to compensate for a decrease in BSEP activity421,422. All these factors complicate the development of a valid rodent model to study BSEP inhibition. Thus, much effort still needs to be made in order to develop animal models that represent cholestasis that is happening in iDILI patients’ livers.

6.3. Feasible in vivo models

In vivo modelling of iDILI is just as necessary as challenging. The difficulty in understanding and predicting iDILI has made animal models indispensable to consider the complexity of the disease from different approaches, such as cross-communication between organs. As discussed in this review, the in vivo systems that can most closely mirror what happens in human iDILI would be the mixed humanized models. However, there are still many issues to resolve that will mark the next steps in iDILI modelling using these animals, as the standardization of the procedure, the achievement of high liver repopulation rates and the validation of these models for iDILI research (Fig. 4).

In the growing trend towards personalized medicine, humanized models may also be of interest to identify potential patient-specific therapies, unravel disease mechanisms and improve drug safety and efficiency. Most chimeric mice used in hepatotoxicity testing were generated by transplanting PHHs. However, the use of iPSCs-derived HLCs to serve as hepatocyte sources for chimeric mice with a humanized liver is a promising alternative. Recently, Yuan et al.423 obtained a 40% of repopulation using HLC derived from hiPSC generated from human fibroblast on Fah−/− Rag2−/− Il2−Rγc−/− SCID (FRGS) mice. However, although improvements in the percentage of repopulation are being achieved using HLCs derived from iPSCs, the use of these cells may have a number of disadvantages for the study of bioenergetics in iDILI. Several studies have demonstrated that reprogramming into iPSCs resets cellular age424, and may cause mitochondrial rejuvenation and an improvement in the cellular energy production capacity upon differentiation425.

Therefore, exploring the use of patient-derived iPheps obtained by direct reprogramming to establish new humanized models could be an interesting approach in iDILI.

7. Discussion

Despite much effort and extensive research in the field, the prediction of iDILI using in vitro and in vivo models remains very unreliable, due to the complex genesis of iDILI itself because of the interactions among genetic, non-genetic and environmental factors.

It is clear from this review that no single system is fit for purpose as a universal test for the patient-specific, multifactorial process of iDILI. Therefore, a portfolio of robust and well-characterized predictive iDILI platforms with well-defined
purpose, and acceptable in a theoretical and practical sense to academic, industry and regulatory agencies is required. There is a need of consensus of in vitro systems to be used to detect iDILI risk and model the disease. However, it may not be possible to reach a consensus on the list of endpoints and models to measure hepatotoxicity in vitro in the near future. This is mainly due to using particular cell types and configurations to model different pathogenic mechanisms of iDILI. Furthermore, in addition to standard pre-clinical toxicology testing, innovative assays that address specific mechanisms such as the role of the immune system will more accurately identify the hepatotoxic potential of a drug.

As crosstalk between hepatocytes and immune cells is likely critical in determining the outcome of drug exposure, it will be necessary to develop a co-culture system using immune and induced pluripotent stem cell-derived hepatocyte-like cells from the same donors to explore the antigenic and polarizing signals released from hepatocytes, T cell activation and whether the activated T cells kill hepatocytes.

Moreover, there is also a need for consensus about the reference drugs to be used for the iDILI assay validations, including recommendations around range of concentrations to test as well as criteria for interpreting the data. However, establishing sets of reference drugs classified by their DILI concern has several difficulties. First, researchers have to consider and distinguish between intrinsic and idiosyncratic DILI, as current lists of DILI drugs used for the validation of in vitro models contain a combination of both. In the case of drugs causing iDILI, classification is particularly challenging due to their rarity and lack of knowledge of their toxicity potential due to the small number of affected patients and the heterogeneity of phenotypic presentation. Information on drug mechanisms of action, effects and severity could also be important to define reference drugs for iDILI. However, the variability in published iDILI annotations due to different resources used by researchers (i.e., literature, drug compendia, FDA reporting systems, etc.) is a major obstacle.

The establishment of reference drugs’ concentrations to be used in in vitro assays is even of more concern. Commonly, fixed concentrations of drugs (1, 100 μmol/L, etc.) and/or multiples of plasma C_{max} are used in majority of in vitro assays. This variety in the strategies in terms of concentration hinders the setting of a consensus concentration criteria to classify drugs as hepatotoxic. Moreover, although a C_{max}-based testing approach seems to be a reasonable strategy to differentiate safe versus hepatotoxic drugs, it does not take into account potential drug accumulation in the liver or protein binding. This limitation is an important consideration. Besides, the duration of treatment is relevant too. In addition, the length of the treatment does not affect to immortalized and primary cell lines in the same way, with the cell type chosen for screening being another variable to consider. All of these features make very difficult to reach a consensus about the reference drugs and the concentrations to be used in iDILI assays in vitro. An ongoing international collaboration (Cost Action 17112) is already working on this topic.

There is also a need to develop nonclinical iDILI guidance, at early stages of drug development, combining safety and pharmacokinetics disciplines in order to help the pharmaceutical industry to develop compounds with reduced risk of iDILI.

For all these reasons, in the last years, in silico strategies have become an interesting alternative approach for prediction of human iDILI risk. However, most of these models follow a statistical-quantitative approach based only on the physicochemical properties of the compounds, leaving out host factors. Regarding the complexity of iDILI events, previous models seem uncertain and present many limitations in their real applicability.

Therefore, a model that incorporates both structural and toxic properties of the drugs but also features of the patient who takes the drug and links them to in vivo and in vitro data seems to be the way to go.

From our point of view, the future strategy of iDILI modeling should be patient-based and combine in vivo, in vitro and in silico approaches, since each strategy has limitations itself, but taken together may prove illuminating. Very recently, Koido et al.\textsuperscript{426} proposed a ‘polygenicity-in-a-dish’ strategy to assess iDILI risk. A polygenic risk score (PRS) for iDILI was developed by aggregating effects of numerous genome-wide loci associated with iDILI risk previously identified. The utility of this new PRS was validated using genome-wide association study data obtained from an independent clinical trial of a hepatotoxic drug, as well as multiple donor-derived organoids and primary hepatocytes treated with a variety of hepatotoxic medications. This new PRS was also related to hepatocyte mechanisms leading to hepatotoxicity, such as UPR activation and oxidative stress. This approach suggests that in silico toxicity modeling, coupled with in vitro genomic and transcriptomic approaches, might identify compounds with potential for iDILI before entering clinical trials, demonstrating that genetic variations at the level of the hepatocyte contribute to iDILI susceptibility (Fig. 4).

iDILI animal models could be useful for the study of iDILI pathogenesis, and that knowledge should lead to cell-based assays adaptable to HCS of drug candidates. The combination of approaches is necessary, since animal models are more likely to capture the complexity of iDILI reactions but translating that information to humans without validating using human in vitro models is challenging due to interspecies variability. It would be necessary to also translate knowledge gained from patients to those cell-based systems in order to obtain clues about iDILI mechanisms of injury.

It is therefore highly recommended to establish multidisciplinary ‘iDILI teams’ with the support of external iDILI experts. In addition to many individual research groups throughout academia and pharmaceutical industry, several large consortia have been established aiming at advancing the research, tools and predictive approaches for iDILI. An integration of different expert knowledge is the key for success. Several national and international programs, such as Cost Action 17112 ProEuro iDILI Network, have been established to create a unique, cooperative, interdisciplinary European-based iDILI network of stakeholders, coordinating efforts in iDILI research, facilitating bidirectional exchange of discovered knowledge and promoting clinically impactful knowledge discovery as well as its translation into clinical practice. It is hoped that a more translational approach based on mechanisms established in humans will facilitate the design of animal and cell-based models with greater predictive value. All these efforts will improve our understanding of complex mechanisms of iDILI to create better testing strategies that will benefit patient safety.

Acknowledgments

This work was supported by grants of Instituto de Salud Carlos III cofounded by Fondo Europeo de Desarrollo Regional-FEDER (contract numbers: PI18/01804, PI19-00883, PT20/00127,
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UMA18-FEDERJA-194, PY18-3364, Spain) and grants of Consejería de Salud de Andalucia cofounded by FEDER (contract number: PEMP-0127-2020, Spain). M.V.P. holds a Sara Borrell (CD21/00198, Spain) research contract from ISCIII and Consejería de Salud de Andalucia. C.L.G. holds a Juan de la Cierva Incorporación (ICI-2017-31466, Spain) research contract from Ministerio de Ciencia del Gobierno de España. SCReN and CIBERehd are funded by ISCIII (Spain). This publication is based upon work from COST Action “CA17112—Prospective European Drug-Induced Liver Injury Network” supported by COST (European Cooperation in Science and Technology); www.cost.eu. The figures in this review were created with Biorender.com.

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Conflicts of interest
The authors declare no conflicts of interest.

References
1. Chen M, Suzuki A, Borlak J, Andrade RJ, Lucena MI. Drug-induced liver injury: interactions between drug properties and host factors. J Hepatol 2015;63:503–14.
2. Hoofnagle JH, Bjornsson ES. Drug-induced liver injury—types and phenotypes. N Engl J Med 2019;381:264–73.
3. 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;36:451–5.
4. de Abajo FJ, Montero D, Madurga M, Garcia Rodriguez LA. Acute and clinically relevant drug-induced liver injury: a population based case-control study. Br J Clin Pharmacol 2004;58:71–80.
5. De Valle MB, Av Klinteberg V, Alem N, Olsson R, Bjornsson E. Drug-induced liver injury in a Swedish University hospital outpatient hepatology clinic. Aliment Pharmacol Ther 2006;24:1187–95.
6. 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;144:1419–25.e3.
7. 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;66:1154–64.
8. Reuben A, Koch DG, Lee WM. Drug-induced acute liver failure: results of a U.S. multicenter, prospective study. Hepatology 2010;52:2065–76.
9. Kuna L, Bozic I, Kizivat T, Bojanic K, Mrso M, Kralj E, et al. Models of drug induced liver injury (DILI)—current issues and future perspectives. Curr Drug Metab 2018;19:830–8.
10. 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;147:109–118.e5.
11. Ashky K, Zhuang W, Gonzalez-Jimenez A, Alvarez-Alvarez I, Lucena MI, Andrade RJ, et al. Elevated bilirubin, alkaline phosphatase at onset, and drug metabolism are associated with prolonged recovery from DILI. J Hepatol 2021;75:333–41.
12. 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;89:806–15.
13. European Association for the Study of the Liver. EASL clinical practice guidelines: drug-induced liver injury. J Hepatol 2019;70:1222–61.
14. Lucena MI, Sanabria J, Garcia-Cortes M, Stephens C, Andrade RJ. Drug-induced liver injury in older people. Lancet Gastroenterol Hepatol 2020;5:862–74.
15. Danan G, Benichou C. Causality assessment of adverse reactions to drugs—I. A novel method based on the conclusions of international consensus meetings: application to drug-induced liver injuries. J Clin Epidemiol 1993;46:1323–30.
16. Ulrich RG. Idiosyncratic toxicity: a convergence of risk factors. Annu Rev Med 2007;58:17–34.
17. Gonzalez-Jimenez A, Meuwen K, Chen M, Suzuki A, Robles-Diaz M, Medina-Caliz I, et al. The influence of drug properties and host factors on delayed onset of symptoms in drug-induced liver injury. Liver Int 2019;39:401–10.
18. Macías-Rodríguez RU, Inzaugarat ME, Ruiz-Margain A, Nelson LJ, Trautwein C, Cubero FJ. Reclassifying hepatic cell death during liver damage: ferroptosis—a novel form of non-apoptotic cell death?. Int J Mol Sci 2020;21:1651.
19. O’Brien PJ, Chan K, Silber PM. Human and animal hepatocytes in vitro with extrapolation in vivo. Chem Biol Interact 2004;150:97–114.
20. Hendriks DF, Fredriksson Puigvert L, Messner S, Mortiz W, Ingelman-Sundberg M. Hepatic 3D spheroid models for the detection and study of compounds with cholesterol liability. Sci Rep 2016;6:35434.
21. Richet 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;34:870–9.
22. Vorrink SU, 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;163:655–65.
23. Kamalian L, Chadwick AE, Bayliss M, French NS, Monshouwer M, Snoeys J, et al. The utility of HepG2 cells to identify direct mitochondrial dysfunction in the absence of cell death. Toxicol Vitro 2015;29:732–40.
24. Xuan J, Chen S, Ning B, Tolleson WH, Guo L. Development of HepG2-derived cells expressing cytochrome P450s for assessing metabolism-associated drug-induced liver toxicity. Chem Biol Interact 2015;255:63–73.
25. Wink S, Hiemstra SW, Huppelschoten S, Klip JE, van de Water B. Dynamic imaging of adaptive stress response pathway activation for prediction of drug induced liver injury. Arch Toxicol 2018;92:1797–814.
26. Antherieu S, Chesne C, Li R, Guguen-Guillouzo C, Guillouzo A, Trautwein C, Cubero FJ. Reclassifying hepatic cell death during liver damage: ferroptosis—a novel form of non-apoptotic cell death?. Int J Mol Sci 2020;21:1651.
27. Choi S, Sainz Jr B, Corcoran P, Uprichard S, Jeong H. Characterization of increased drug metabolism activity in dimethyl sulfoxide (DMSO)-treated HuH7 hepatoma cells. Xenobiotica 2009;39:205–17.
28. Chu CC, Pan KL, Yao HT, Hsu JT. Development of a whole-cell screening system for evaluation of the human CYP1A2-mediated metabolism. Biotechnol Bioeng 2011;108:2932–40.
antiretroviral drug efavirenz induces cell survival-promoting autophagy. *Hepatology* 2011;54:1009–19.

31. Apostolova N, Gonzalez-Saucerquia LJ, Alegre F, Funes HA, Víctor VM, Barrachina MD, et al. ER stress in human hepatocellular treated with Efavirenz: mitochondria again. *J Hepatol* 2013;59:780–9.

32. Polo M, Alegre F, Funes HA, Blas-García A, Víctor VM, Esplugues JV, et al. Mitochondrial (dis)function—a factor underlying the variability of efavirenz-induced hepatotoxicity?. *Br J Pharmacol* 2015;172:1713–27.

33. Thompson RA, Isn EM, Li Y, Weidolf L, Page K, Wilson I, et al. In vitro approach to assess the potential for risk of idiosyncratic adverse reactions caused by candidate drugs. *Chem Res Toxicol* 2012;25:1616–32.

34. Gustafsson F, Foster AJ, Sarna 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. *Toxicol Sci* 2014;137:189–211.

35. Yıldırımman R, Brolen G, Vilardell M, Eriksson G, Synnergren J, Thompson RA, Isin EM, Li Y, Weidolf L, Page K, Wilson I, et al. Patient-specific hepatocyte-like cells derived from human embryonic stem cell lines. *Toxicology* 2017;387:1–9.

36. Cipriano M, Pinheiro PF, Sequeira CO, Rodrigues JS, Oliveira NG, Antunes AMM, et al. Nevirapine biotransformation insights: an integrated in vitro approach unveils the biocompetence and glutathiolomic profile of a human hepatocyte-like cell 3D model. *Int J Mol Sci* 2020;21:3998.

37. Choudhury Y, Toh YC, Xing J, Qu Y, Poh J, Li H, et al. Model of hepatic drug metabolism and responses in CYP2C19 poor metabolizer using genetically manipulated human iPSCs. *Drug Metab Dispos* 2019;47:652–9.

38. Novik E, Maguire TJ, Chao P, Cheng KC, Yarmush ML. A microfluidic hepatic coculture platform for cell-based drug metabolism studies. *Biochem Pharmacol* 2010;79:1036–44.

39. Nguyen TV, Ukairo O, Khetani SR, McMvay 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;43:774–85.

40. Baze A, Parmentier C, Hendriks DFG, Hurrell T, Heyd B, Bacheller P, et al. Three-dimensional spheroid primary human hepatocytes in monoculture and coculture with nonparenchymal cells. *Tissue Eng Part C Methods* 2018;24:534–45.

41. Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. *Nat Biotechnol* 2008;26:120–6.

42. 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;10:2027–53.

43. 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* 2019;47:58–66.

44. Efferink MG, Olinga P, van Leeuwen EM, Bauerschmidt S, Polman J, Schoonen WG, et al. Gene expression analysis of precision-cut human liver slices indicates stable expression of ADME-Tox related genes. *Toxicol Appl Pharmacol* 2011;253:57–69.

45. Hadi M, Westra IM, Starokozhko V, Dragovic S, Merema MT, Groothuis GM. Human precision-cut liver slices as an ex vivo model to study idiosyncratic drug-induced liver injury. *Chem Res Toxicol* 2013;26:710–20.

46. Ma L, Wu Y, Li Y, Aazmi A, Zhou H, Zhang B, et al. Current advances on 3D-bioprinted liver tissue models. *Adv Healthc Mater* 2020;9:e2001517.

47. Serras AS, Rodrigues JS, Cipriano M, Rodrigues AV, Oliveira NG, Miranda JP. A critical perspective on 3D liver models for drug metabolism and toxicology studies. *Front Cell Dev Biol* 2021;9:626805.

48. Bdourin R, Prot JM, Nicolas G, Brocheton J, Brochot C, Legallais C, et al. Evaluation of seven drug metabolisms and clearances by cryo-preserved human primary hepatocytes cultivated in microfluidic biochips. *Xenobiotica* 2013;43:140–52.

49. Choucha Snouber L, Bunescu A, Naudot M, Legallais C, Brochot C, Dumas ME, et al. Metabolomics-on-a-chip of hepatotoxicity induced by anticancer drug flutamide and its active metabolite hydroxy-flutamide using HepG2/C3a microfluidic biochips. *Toxicol Sci* 2013;132:8–20.

50. Rubiano A, Indapurkar A, Yokosawa R, Miedzik A, Rosenzweig B, Arefin A, et al. Characterizing the reproducibility in using a liver microphysiological system for assaying drug toxicity, metabolism, and accumulation. *Clin Transl Sci* 2021;14:1049–61.

51. Wagner I, Materne EM, Brincker S, Sussbier U, Fradrich D, Busch M, et al. A dynamic multi-organ-chip for long-term cultivation and substance testing proven by 3D human liver and skin tissue coculture. *Lab Chip* 2013;13:5538–47.

52. Jardine SR, Legendre A, Fleury MJ, Zeller P, Merlier F, et al. Human embryonic stem cell-derived hepatocytes like cells as a tool for in vitro hazard assessment of chemical carcinogenicity. *Toxicol Sci* 2011;124:278–90.

53. Kim DE, Jang MJ, Kim YR, Lee JY, Cho EB, Kim E, et al. Prediction of drug-induced immune-mediated hepatotoxicity using hepatocyte-like cells derived from human embryonic stem cells. *Toxicology* 2017;387:1–9.

54. Wagner I, Materne EM, Brincker S, Sussbier U, Fradrich D, Busch M, et al. A dynamic multi-organ-chip for long-term cultivation and substance testing proven by 3D human liver and skin tissue coculture. *Lab Chip* 2013;13:5538–47.

55. Bricks T, Paullier P, Legendre A, Fleury MJ, Zeller P, Merlier F, et al. Development of a new microfluidic platform integrating co-cultures of intestinal and liver cell lines. *Toxicol Vitro* 2014;28:885–95.

56. Oleaga C, Bernabini C, Smith AS, Srinivasan B, Jackson M, McLamb W, et al. Multi-organ toxicity demonstration in a functional human in vitro system composed of four organs. *Sci Rep* 2016;6:20030.

57. Zeilingher K, Fayer N, Damm G, Seehofer K, Knoespel F, Cell sources for in vitro human liver cell culture models. *Exp Biol Med (Maywood)* 2016;241:1684–98.

58. Lin C, Ballinger KR, Khetani SR. The application of engineered liver tissues for novel drug discovery. *Expert Opin Drug Discov* 2015;10:619–40.

59. 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 detoxification. *Toxicol Sci* 2015;147:412–24.

60. 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;91:1385–400.

61. den Braver-Sewradj SP, den Braver MW, Vermeulen NP, Commandeur JN, Richert L, Vos JC. Inter-donor variability of phase I/II metabolism of three reference drugs in cryopreserved human liver-derived single cell culture models reveals significant variation in the capacity for drug disposition, bioactivation, and detoxification. *Toxicol Vitro* 2016;33:71–9.

62. Inoue C, Yamamoto H, Nakamura T, Ichihara A, Okamoto H, Nicotinamide prolongs survival of primary cultured hepatocytes without involving loss of hepatocyte-specific functions. *J Biol Chem* 1989;264:4747–50.

63. Kost DP, Michalopoulos GK. Effect of 2% dimethyl sulfoxide on the mitogenic properties of epidermal growth factor and hepatocyte growth factor in primary hepatocyte culture. *J Cell Physiol* 1991;147:274–80.

64. Tolosa L, Gomez-Lechon MJ, Lopez S, Guzman C, Castell JV, Donato MT, et al. Human upcyte hepatocytes: characterization of the
hepatic phenotype and evaluation for acute and long-term hepatotoxicity routine testing. Toxicol Sci 2016;152:214–29.
65. Tolosa L, Jimenez N, Pelecha M, Castell JV, Gomez-Lechon MJ, Donato MT. Long-term and mechanistic evaluation of drug-induced liver injury in Upcycled human hepatocytes. Arch Toxicol 2019;93:519–32.
66. Schulz C, Kammerer S, Kupper JP. NADPH-cytochrome P450 reductase expression and enzymatic activity in primary-like human hepatocytes and HepG2 cells for in vitro biotransformation studies. Clin Hemorheol Microcirc 2019;73:249–60.
67. Schaefer M, Morinaga G, Matsui A, Schanzle G, Bischoff D, Sussmuth RD. Quantitative expression of hepatobiliary transporters and functional uptake of substrates in hepatic two-dimensional sandwich cultures: a comparative evaluation of upcyle and primary human hepatocytes. Drug Metab Dispos 2018;46:166–77.
68. Guo L, Dial S, Shi L, Branhman W, Liu J, Fang JL, et al. Similarities and differences in the expression of drug-metabolizing enzymes between human hepatic cell lines and primary human hepatocytes. Drug Metab Dispos 2011;39:528–38.
69. Gerets HH, Tilmant K, Gerin B, Chanteux H, Depelchin BO, van Esch R, et al. Characterization of human primary 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;28:69–87.
70. Aden DP, Fogel A, Plotkin S, Damjanov I, Knowles BB. Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature 1979;282:615–6.
71. Costantini S, Di Bernardo G, Cammarota M, Castello G, Colonna G. Gene expression signature of human HepG2 cell line. Gene 2013; 518:335–45.
72. Doostdar H, Dartie SJ, Burke MD, Melvin WT, Grant MH. The influence of culture medium composition on drug metabolising enzyme activities of the human liver derived Hep G2 cell line. FEBS Lett 1988;241:15–8.
73. Tyakht AV, Ilina EN, Alexeev DG, Ischenko DS, Gorbachev AV, Semashko TA, et al. RNA-Seq gene expression profiling of HepG2 cells: the influence of experimental factors and comparison with liver tissue. BMC Genom 2014;15:1108.
74. Tolosa L, Jimenez N, Perez G, Castell JV, Gomez-Lechon MJ, Donato MT. Customised in vitro model to detect human metabolism-dependent idiosyncratic drug-induced liver injury. Arch Toxicol 2018;92:383–99.
75. Tolosa L, Gomez-Lechon MJ, Perez-Cataldo G, Castell JV, Donato MT. HepG2 cells simultaneously expressing five P450 enzymes for the screening of hepatotoxicity: identification of bioactive drugs and the potential mechanism of toxicity involved. Arch Toxicol 2013;87:1115–27.
76. Donato MT, Jover R, Gomez-Lechon MJ. Hepatic cell lines for drug hepatotoxicity testing: limitations and strategies to upgrade their metabolic competence by gene engineering. Curr Drug Metab 2013;14:946–68.
77. Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res 1982;42:3858–63.
78. Louisa M, Syatuna FD, Wamandi SI, Ash P, Syafriuddin D. Differential expression of several drug transporter genes in HepG2 and Huh-7 cell lines. Adv Biomed Res 2016;5:104.
79. Siverstris L, Edchert I, Palmertz MP, Ingelman-Sundberg M, Neve EP. Induced CYP3A4 expression in confluent Huh7 hepatoma cells as a result of decreased cell proliferation and subsequent pregnane X receptor activation. Mol Pharmacol 2013;83:659–70.
80. Khamphaya T, Chukjirungrat N, Saengsirisuwan V, Mitchell-Richards KA, Robert ME, Mennone A, et al. Nonalcoholic fatty liver disease impairs expression of the type II inositol 1,4,5-trisphosphate receptor. Hepatology 2018;67:560–74.
81. Mace K, Aguilar F, Wang JS, Vautravers P, Gomez-Lechon M, Gonzalez FJ, et al. Aflatoxin B1-induced DNA adduct formation and p53 mutations in CYP450-expressing human liver cell lines. Carcinogenesis 1997;18:1291–7.
82. Glaise D, Iyin GP, Loyer P, Cariou S, Bilodeau M, Lucas J, et al. Cell cycle gene regulation in reversibly differentiated new human hepatoma cell lines. Cell Growth Differ 1998;9:165–76.
83. Mills JB, Rose KA, Sadagopan N, Sahi J, de Morais SM. Induction of drug metabolism enzymes and MDR1 using a novel human hepatocyte cell line. J Pharmacol Exp Ther 2004;309:303–9.
84. Scott CW, Peters MF, Dragan YP. Human induced pluripotent stem cells and their use in drug discovery for toxicity testing. Toxicol Lett 2013;219:49–58.
85. Rambhatla L, Chiu CP, Kundu P, Peng Y, Carpenter MK. Generation of hepatocyte-like cells from human embryonic stem cells. Cell Transplant 2003;12:11–15.
86. Lavon N, Yanuka O, Benvenisty N. Differentiation and isolation of hepatic-like cells from human embryonic stem cells. Differentiation 2004;72:230–8.
87. Schwartz RE, Linehan JL, Pinschbak MS, Hu WS, Verfaillie CM, Kaufman DS. Defined conditions for development of functional hepatic cells from human embryonic stem cells. Stem Cells Dev 2005; 14:643–55.
88. Takayama K, Inamura M, Kawabata K, Katayama K, Higuchi M, Tashiro K, et al. Efficient generation of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4alpha transduction. Mol Ther 2012;20:127–37.
89. Li Z, Wu J, Wang L, Han W, Yu J, Liu X, et al. Generation of qualified clinical-grade functional hepatocytes from human embryonic stem cells in chemically defined conditions. Cell Death Dis 2019;10:763.
90. Xia Y, Carpentier A, Cheng X, Block PD, Zhao Y, Zhang Z, et al. Human stem cell-derived hepatocytes as a model for hepatitis B virus infection, spreading and virus-host interactions. J Hepatol 2017;66:494–503.
91. Lee HJ, Jung J, Cho KJ, Lee CK, Hwang SG, Kim GJ. Comparison of in vitro hepaticogenic differentiation potential between various placenta-derived stem cells and other adult stem cells as an alternative source of functional hepatocytes. Differentiation 2012;84:223–31.
92. Cipriano M, Correia JC, Camoes SP, Oliveira NG, Cruz P, Cruz H, et al. The role of epigenetic modifiers in extended cultures of functional hepatocyte-like cells derived from human neonatal mesenchymal stem cells. Arch Toxicol 2017;91:2469–89.
93. Moon JH, Heo JS, Kim JS, Jun EK, Lee JH, Kim A, et al. Reprogramming fibroblasts into induced pluripotent stem cells with Bml1. Cell Res 2011;21:1305–15.
94. Si-Tayeb K, Noto FK, Nagaoa M, Li J, Battle MA, Duris C, et al. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 2010;51:297–305.
95. Takata A, Otsuka M, Kogiso T, Koijima K, Yoshikawa T, Tateishi R, et al. Direct differentiation of hepatic cells from human induced pluripotent stem cells using a limited number of cytokines. Hepatol Int 2011;5:890–8.
96. Nakamura N, Saeki K, Mitsumoto M, Matsuyama S, Nishio M, Saecki K, et al. Feeder-free and serum-free production of hepatocytes, cholangiocytes, and their proliferating progenitors from human pluripotent stem cells: application to liver-specific functional and cytotoxic assays. Cell Reprograming 2012;14:171–85.
97. Schwartz RE, Fleming HE, Khentani SR, Bhatia SN. Pluripotent stem cell-derived hepatocyte-like cells. Biotechnol Adv 2014;32:504–13.
98. 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;61:1730–9.
culturalization of human hepatocytes and non-parenchymal liver cells. 

Exp Biol Med (Maywood) 2015;240:645–56.

100. Granitzny A, Knebel J, Muller M, Braun A, Steinberg P, Dassenbrock C, et al. Evaluation of a human in vitro hepatocyte-NPC co-culture model for the prediction of idiopathic drug-induced liver injury: a pilot study. Toxicol Rep 2017;4:89–103.

101. Gripin P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, et al. Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl Acad Sci U S A 2009;106:15655–60.

102. Le Vee M, Gouan J, Goan E, Stieger B, Fardel O. Polarized expression of drug transporters in differentiated human hepatoma HepaRG cells. Toxicol Vitro 2013;27:979–86.

103. Josse R, Annat C, Glaise D, Dumont J, Fessard V, Morel F, et al. Long-term functional stability of human HepaRG hepatocytes and use for chronic toxicity and genotoxicity studies. Drug Metab Dispos 2008;36:1111–8.

104. Antherieu S, Rogue A, Fromenty B, Guillouzo A, Robin MA. Induction of vesicular steatosis by amiiodarone and tetracycline is associated with up-regulation of lipogenic genes in HepaRG cells. Hepatology 2011;53:1895–905.

105. Jenne CN, Kubes P. Immune surveillance by the liver. Nat Immunol 2013;14:996–1006.

106. Olsen AL, Bloomer SA, Chan EP, Gaca MD, Georges PC, Sackey B, et al. Hepatic stellate cells require a stiff environment for myofibroblast differentiation. Am J Physiol Gastrointest Liver Physiol 2011;301:G110–8.

107. Ware BR, Durham MJ, Monckton CP, Khetani SR. A cell culture platform to maintain long-term phenotype of primary human hepatocytes and endothelial cells. Cell Mol Gastroenterol Hepatol 2018;5:187–207.

108. Khetani SR, Berger DR, Ballinger KR, Davidson MD, Lin C, Ware BR. Microengineered liver tissues for drug testing. J Lab Automat 2015;20:216–50.

109. 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 Virology 2019;171:2949–63.

110. 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;132:107–17.

111. Lin C, Shi J, Moore A, Khetani SR. Prediction of drug clearance and drug–drug interactions in microscale cultures of human hepatocytes. Drug Metab Dispos 2016;44:127–36.

112. Graaf IA, Grootenhuis GM, Olinga P, Precision-cut tissue slices as a method for determining drug-induced hepatotoxicity. Toxicology and applied pharmacology. 2016;34:368–81.

113. Vickers AE, Bentley P, Fisher RL. Consequences of mitochondrial injury induced by pharmaceutical fatty acid oxidation inhibitors is characterized in human and rat liver slices. Toxicol Vitro 2006;20:1173–82.

114. Edwards RJ, Price RJ, Watts PS, Renwick AB, Tredger JM, Boobis AR, et al. Induction of cytochrome P450 enzymes in cultured precision-cut human liver slices. Drug Metab Dispos 2003;31:282–8.

115. Starokozhko V, Vatakat S, Schievink B, Merema MT, Asplund A, Symmangen J, et al. Maintenance of drug metabolism and transport functions in human precision-cut liver slices during prolonged incubation for 5 days. Arch Virology 2017;162:2079–92.

116. Paola E, Doornbal B, Chokshi S. Precision-cut liver slices: a versatile tool to advance liver research. Hepatol Int 2019;13:S1–7.

117. Gomez-Lechon MJ, Tolosa L, Conte I, Donato MT. Competency of different cell models to predict human hepatotoxic drugs. Expert OpinDrugMetab Toxicol 2014;10:1553–68.

118. De Bruyn T, Chatterjee S, Fattah S, Keemink J, Nicolai J, Augustjons P, et al. Sandwich-cultured hepatocytes: utility for in vitro exploration of hepatobiary drug disposition and drug-induced hepatotoxicity. Expert Opin Drug Metab Toxicol 2013;9:589–616.

119. Hewitt NJ, Lechon MJ, Houston JB, Hallifax D, Brown HS, Maurel P, et al. Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Metab Rev 2007;39:159–234.

120. 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;61:1370–81.

121. Takahashi Y, Hori Y, Yamamoto T, Urashima T, Ohara Y, Tanaka H. 3D spheroid cultures improve the metabolic gene expression profiles of HepaRG cells. Biosci Rep 2015;35:e00208.

122. Bell CC, Hendriks DF, Moro SM, Ellis E, Walsh J, Renblom A, et al. Characterization of primary human hepatocyte spheroids as a model system for drug-induced liver injury, liver function and disease. Sci Rep 2016;6:25187.

123. Sirekou O, Hancock MK, Hesley J, Hong D, Cohen A, Gentry J, et al. Phenotypic characterization of toxic compound effects on liver spheroids derived from iPSC using confocal imaging and three-dimensional image analysis. Assay Drug Dev Technol 2016;14:381–94.

124. Andria B, Bracco A, Cirino G, Chamuleau RA. Liver cell culture devices. Cell Med 2010;1:55–70.

125. Kostadnina R, Booes F, Applegate D, Suter L, Weiser T, Singer T, et al. A long-term three dimensional liver co-culture system for improved prediction of clinically relevant drug-induced hepatotoxicity. Toxicol Appl Pharmacol 2013;281:1–16.

126. Ohkura T, Ohta K, Togashi K, Kusumoto K, Koeda A, Ueda T, et al. Evaluation of human hepatocytes cultured by three-dimensional spheroid systems for drug metabolism. Drug Metab Pharmacokinet 2014;29:373–8.

127. Takayama K, Kowahara K, Nagamoto Y, Kishimoto K, Tashiro K, Kusakari F, et al. 3D spheroid culture of HESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing. Biomaterials 2013;34:1781–9.

128. Leite SB, Roosens T, El Taghdouini A, Mannaerts I, Smout AJ, Najimi A, et al. Novel human hepatic organoid model enables testing of drug-induced liver fibrosis in vitro. Biomaterials 2016;78:1–10.

129. Broulier T, Andersson-Rolf A, Hindley CJ, Boi SF, Clevers H, Koo BK, et al. Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation. Nat Protoc 2016;11:1724–43.

130. 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:e0158674.

131. Ide I, Nagao E, Kajiyama S, Mizoguchi N. A novel evaluation method for determining drug-induced hepatotoxicity using 3D bioprinted human liver tissue. Toxicol Mech Methods 2020;30:189–96.

132. Wang JZ, Xiong NY, Zhao LZ, Hu JT, Kong DC, Yuan JY. Review fantastic medical implications of 3D-printing in liver surgeries, liver regeneration, liver transplantation and drug hepatotoxicity testing: a review. Int J Surg 2018;56:1–6.

133. Norona LM, Nguyen DG, Gerber DA, Presnell SC, LeCluyse EL, El Taghdouini A. Editor’s highlight: modeling compound-induced fibrogenesis in vitro using three-dimensional bioprinted human liver tissues. Toxicol Sci 2016;154:534–67.

134. Schmidt K, Berg J, Roehrs V, Kurreck J, Al-Zeer MA. 3D bioprinted HepaRG cultures as a model for testing long term aflatoxin B1 toxicity in vitro. Toxicol Rep 2020;7:1578–87.

135. Peck RW, Hinojosa CD, Hamilton GA. Organs-on-chips in clinical pharmacology: putting the patient into the center of treatment selection and drug development. Clin Pharmacol Ther 2020;107:181–5.

136. Viravaidya K, Sin A, Shuler ML. Development of a microscale cell culture analog to probe naphthalene toxicity. Biotechnol Prog 2004;20:316–23.

137. Regev A. Drug-induced liver injury and drug development: industry perspective. Semin Liver Dis 2014;34:227–39.
138. Watkins PB. Drug safety sciences and the bottleneck in drug development. Clin Pharmacol Ther 2011;89:788–90.

139. Gerassi A, Natalini A, Antonangeli F, Mancuso C, Agostinetti E, Barisani D, et al. Immune-mediated drug-induced liver injury: immunogenetic and experimental models. Int J Mol Sci 2021;22:4557.

140. Walker PA, Ryder S, Lavado A, Dilworth C, Riley RJ. The evolution of strategies to minimise the risk of human drug-induced liver injury (DILI) in drug discovery and development. Arch Toxicol 2020;94:2559–85.

141. Weaver RJ, Blomme EA, Chadwick AE, Copple IM, Gerets HHJ, Hewitt PG, et al. Test systems in drug discovery for hazard identification and risk assessment of human drug-induced liver injury: a roadmap for the development and deployment of preclinical predictive models. Nat Rev Drug Discov 2020;19:131–48.

142. European Medicines Agency. Reflection paper on non-clinical evaluation of drug-induced liver injury (DILI), 2010. Published [19/07/2010]. Updated [19/07/2010]. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/reflection-paper-non-clinical-evaluation-drug-induced-liver-injury-dili_en.pdf. [Accessed 4 November 2021].

143. US Food and Drug Administration. Guidance for industry: drug-induced liver injury: premarketing clinical evaluation. 2009. Published [01/07/2009]. Updated [17/10/2019]. Available from: https://www.fda.gov/media/116737/download. [Accessed 4 November 2021].

144. Weaver RJ, Betts C, Blomme EAG, Gerets HHJ, Gjervig Jensen K, Hewitt PG, et al. Test systems in drug discovery for hazard identification and risk assessment of human drug-induced liver injury. Expert Opin Drug Metab Toxicol 2017;13:767–82.

145. McGill MR. The problem with predictive values: are we using the right metrics for preclinical prediction of drug hepatotoxicity?. Toxicol Sci 2018;165:3–4.

146. Ingelman-Sundberg M, Lauschke VM. Current statistical metrics are pragmatic measures to compare the predictive quality of preclinical assays. Toxicol Sci 2018;165:4–5.

147. Gilbert DF, Friedrich O. Cell viability assays: methods and protocols. Methods in molecular biology. New York: Humana Press; 2017.

148. Atienzar FA, Blomme EA, Chen M, Hewitt P, Kenna JG, Labbe G, et al. Key challenges and opportunities associated with the use of in vitro models to detect human DILI: integrated risk assessment and mitigation plans. BioMed Res Int 2016;2016:9737920.

149. Xu JJ, Henstock PV, Dunn MC, Smith AR, Chabot JR, de Graaf D. Cellular imaging predictions of clinical drug-induced liver injury. Curr Opin Drug Metab Toxicol 2008;10:97–105.

150. Thompson RA, Isin EM, Ogose MO, Mettet JL, Williams DP. Reactive metabolites: current and emerging risk and hazard assessments. Chem Res Toxicol 2016;29:505–33.

151. Iorga A, Dara L, Kaplowitz N. Drug-induced liver injury: cascade of events leading to cell death, apoptosis or necrosis. Int J Mol Sci 2017;18:1018.

152. Nakayama S, Atsumi T, Takakusa H, Kobayashi Y, Kurihara A, Nagei Y, et al. A zone classification system for risk assessment of idiosyncratic drug toxicity using daily dose and coherent binding. Drug Metab Dispos 2009;37:1970–7.

153. Norman BH. Drug induced liver injury (DILI). Mechanisms and medicinal chemistry avoidance/mitigation strategies. J Med Chem 2008;51:11397–419.

154. Ramachandran A, Jaeschke H. Oxidative stress and acute hepatic injury. Curr Opin Toxicol 2018;8:17–21.

155. Brink A, Palher A, Funk C, Schuler F, Schadt S. Minimizing the risk of chemically reactive metabolism formation of new drug candidates: implications for preclinical drug design. Drug Discov Today 2017;22:751–6.

156. Chen S, Zhang Z, Qing T, Ren Z, Yu D, Couch L, et al. Activation of the Nrf2 signaling pathway in usnic acid-induced toxicity in HepG2 cells. Arch Toxicol 2017;91:1293–307.

157. Anthériaux S, Bachour-El Azzi P, Dumont J, Abdel-Razzak Z, Guguen-Guilhou C, Fromenty B, et al. Oxidative stress plays a major role in chlorpromazine-induced cholestasis in human HepaRG cells. Hepatology 2013;57:1518–29.

158. Han D, Dara L, Win S, Than TA, Yuan L, Abbas SQ, et al. Regulation of drug-induced liver injury by signal transduction pathways: critical role of mitochondria. Trends Pharmacol Sci 2013;34:243–53.

159. Pessayre D, Fromenty B, Berson A, Robin MA, Letteron P, Moreau R, et al. Central role of mitochondria in drug-induced liver injury. Drug Metab Rev 2012;44:34–87.

160. Boelsterli UA, Lim PL. Mitochondrial abnormalities—a link to idiosyncratic drug hepatotoxicity?. Toxicol Appl Pharmacol 2007;220:92–107.

161. Labbe G, Pessayre D, Fromenty B. Drug-induced liver injury through mitochondrial dysfunction: mechanisms and detection during preclinical safety studies. Fundam Clin Pharmacol 2008;22:335–53.

162. Beigrache K, Massart J, Robin MA, Borgne-Sanchez A, Fromenty B. Drug-induced toxicity on mitochondria and lipid metabolism: mechanistic diversity and deleterious consequences for the liver. J Hepatol 2011;54:773–94.

163. Pessayre D, Mansouri A, Berson A, Fromenty B. Mitochondrial involvement in drug-induced liver injury. Handb Exp Pharmacol 2010:311–65.

164. Porceddu M, Buron N, Roussel C, Labbe G, Fromenty B, Borgne-Sanchez A. Prediction of liver injury induced by chemicals in human with a multiparametric assay on isolated mouse liver mitochondria. Toxicol Sci 2012;129:332–45.

165. Porceddu M, Buron N, Rustin P, Fromenty B, Borgne-Sanchez A. In vitro assessment of mitochondrial toxicity to predict drug-induced liver injury. In: Chen M, Will Y, editors. Drug-induced liver toxicity. New York: Springer New York; 2018. p. 283–300.

166. Marroquin LD, Hynes J, Dykens IA, Jamieson JD, Will Y. Circumventing the Crabtree effect: replacing media glucose with galactose increases susceptibility of HepG2 cells to mitochondrial toxicants. Toxicol Sci 2007;97:539–47.

167. Rana P, Aleo MD, Gosink M, Will Y. Evaluation of in vitro mitochondrial toxicity assays and physicochemical properties for prediction of organ toxicity using 228 pharmaceutical drugs. Chem Res Toxicol 2019;32:56–67.

168. Nadanaciva S, Rana P, Beeeson GC, Chen D, Ferrick DA, Beeeson CC, et al. Assessment of drug-induced mitochondrial dysfunction via altered cellular respiration and acidification measured in a 96-well platform. J Biob Energy Biomembr 2012;44:421–37.

169. Eakins J, Bauch C, Woodhouse H, Park B, Bevan S, Dilworth C, et al. A combined in vitro approach to improve the prediction of mitochondrial toxicants. Toxicol Vitro 2016;34:161–70.

170. Timmenstein MA, Hu CX, Gales TL, Maleeff BE, Narayanan PK, Kurali E, et al. Effects of troglitazone on HepG2 viability and mitochondrial function. Toxicol Sci 2002;69:131–8.

171. Dykens IA, Jamieson JD, Marroquin LD, Nadanaciva S, Xu JI, Dunn MC, et al. In vitro assessment of mitochondrial dysfunction and cytotoxicity of nefazodone, trazodone, and buspirone. Toxicol Sci 2008;103:335–45.

172. Hynes J, Nadanaciva S, Swiss R, Carey C, Kirwan S, Will Y. A high-throughput dual parameter assay for assessing drug-induced mitochondrial dysfunction provides additional predictivity over two established mitochondrial toxicity assays. Toxicol Vitro 2015;27:560–9.

173. Moran M, Delmira A, Blazquez A, Ugalde C, Arenas J, Martin MA. Bulk autophagy, but not mitophagy, is increased in cellular model of mitochondrial disease. Biochim Biophys Acta 2014;1842:1059–70.

174. Coton D, Cordero MD, Garrido-Maraver J, Oropesa-Avila M, Rodriguez-Hernandez A, Gomez Izquierdo L, et al. Secondary coenzyme Q10 deficiency triggers mitochondria degradation by mitophagy in MELAS fibroblasts. FASEB J 2011;25:2669–87.

175. Moles A, Torres S, Bauties A, Garcia-Ruiz C, Fernandez-Checa JC. Mitochondrial—lyosomal axis in acetaminophen hepatotoxicity. Front Pharmacol 2018;9:453.

176. Jung SH, Lee W, Park SH, Lee KY, Choi YJ, Choi S, et al. Diclofenac impairs autophagic flux via oxidative stress and lysosomal damage and alters mitochondrial function. Biochim Biophys Acta 2014;1842:1059–70.
185. Ulzurrun E, Stephens C, Crespo E, Ruiz-Cabello F, Ruiz-Nunez J, Garzel B, Yang H, Zhang L, Huang SM, Polli JE, Wang H. The role of Mosedale M, Watkins PB. Drug-induced liver injury: advances in the pathogenesis of liver ischemia and reperfusion injury. *Transplantation* 2012;94:211–7.

184. Ren Z, Chen S, Zhang J, Doshi U, Li AP, Guo L. Endoplasmic reticulum stress induction and ERK1/2 activation contribute to nefazodone-induced toxicity in hepatic cells. *Toxicol Sci* 2016;154:368–80.

183. Chen S, Zhang Z, Wu Y, Shi Q, Yan H, Mei N, et al. Endoplasmic reticulum stress and store-operated calcium entry contribute to usnic acid-induced toxicity in hepatic cells. *Toxicol Sci* 2015;146:116–26.

182. Mosedale M, Watkins PB. Drug-induced liver injury: advances in mechanistic understanding that will inform risk management. *Clin Pharmacol Ther* 2017;101:469–90.

181. Yang K, Kock K, Sedýk A, Tropsa A, Brouwer KL. An updated review on drug-induced cholestasis: mechanisms and investigation of physicochemical properties and pharmacokinetic parameters. *J Pharm Sci* 2013;102:3037–57.

180. Garzel B, Yang H, Zhang L, Huang SM, Polli JE, Wang H. The role of bile salt export pump gene repression in drug-induced cholestatic liver toxicity. *Drug Metab Dispos* 2014;42:318–22.

179. Ulzurrun E, Stephens C, Crespo E, Ruiz-Cabello F, Ruiz-Nunez J, Saenz-Lopez P, et al. Role of chemical structures and the 1331T polymorphism in idiosyncratic drug-induced toxicity. *Drug Metab Dispos* 2014;42:318–22.

178. Morgan RE, Trauner M, van Staden CJ, Lee PH, Ramachandran B, Eschenberg M, et al. Interference with bile salt export pump function is a susceptibility factor for human liver injury in drug development. *Toxicol Sci* 2010;116:485–500.

177. Jennitz K, Veres Z, Verczeyky L. Contribution of high basolateral bile salt efflux to the lack of hepatotoxicity in rat in response to drugs inducing cholestasis in human. *Toxicol Sci* 2010;115:80–8.

176. Ellis EC, Nilsson LM. The use of human hepatocytes to investigate bile acid synthesis. *Methods Mol Biol* 2010;640:417–30.

175. Yang K, Guo C, Woodhead JL, St Claire 3rd RL, Watkins PB, Siler SQ, et al. Sandwich-cultured hepatocytes as a tool to study drug disposition and drug-induced liver injury. *J Pharm Sci* 2016;105:443–59.

174. Guo L, Zhang L, Sun Y, Mushkelishvili L, Blazi E, Dial S, et al. Differences in hepatotoxicity and gene expression profiles by anti-diabetic PPAR gamma agonists on rat primary hepatocytes and human HepG2 cells. *Mol Divers* 2006;10:349–60.

173. Pedersen JM, Matsson P, Bergström CA, Hoogstraate J, Noren A, LeCluyse EL, et al. Early identification of clinically relevant drug interactions with the human bile salt export pump (BSEP/ABC11). *Toxicol Sci* 2013;136:528–43.

172. Oorts M, Baze A, Bachellier P, Heyd B, Zacharias T, Annaert P, et al. Drug-induced cholestasis risk assessment in sandwich-cultured human hepatocytes. *Toxicol Vitro* 2016;34:179–86.

171. Lu W, Cheng F, Jiang J, Zhang C, Deng X, Xu Z, et al. FXR antagonism of NSAIDs contributes to drug-induced liver injury identified by systems pharmacology approach. *Sci Rep* 2015;5:8114.

170. Burbank MG, Burban A, Sharanek A, Weaver RJ, Guaguen-Guillouzo C, Guillouzo A. Early alterations of bile canaliculi dynamics and the Rho kinase/myosin light chain kinase pathway are characteristics of drug-induced intrahepatic cholestasis. *Drug Metab Dispos* 2016;44:1780–93.

169. Racanelli V, Rehermann B. The liver as an immunological organ. *Hepatology* 2006;43:554–62.

168. Robinson MW, Harmon C, O’Farrellly C. Liver immunology and its role in inflammation and homeostasis. *Cell Mol Immunol* 2016;13:267–76.

167. Papay JI, Clines D, Rafi R, Yuen N, Britt SD, Walsh JS, et al. Drug-induced liver injury following positive drug challenge. *Regul Toxicol Pharmacol* 2009;54:84–90.

166. Ju C, Reilly T. Role of immune reactions in drug-induced liver injury (DILI). *Drug Metab Rev* 2012;44:107–15.

165. 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;16:258–64.

164. Daly AK, Donaldson PT, Bhattachar P, Shen Y, Pe’er I, Floratos A, et al. HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to fluvoxacinil. *Nat Genet* 2009;41:816–9.

163. Monshi MM, Faulkner L, Gibson A, Jenkins RE, Farrell J, Earnshaw CJ, et al. Human leucocyte antigen (HLA)-B*57:01 restricted activation of drug-specific T cells provides the immunological basis for fluvoxacinil-induced liver injury. *Hepatology* 2013;57:727–39.

162. Nicoletti P, Aithal GP, Chamberlain TC, Coulthard S, Alshabeeb M, Grove JI, et al. Drug-induced liver injury due to fluvoxacinil: relevance of multiple human leucocyte antigen alleles. *Clin Pharmacol Ther* 2019;106:245–53.

161. Hautekeete ML, Horsmans Y, Van Waeyenberge C, Demanet C, Guillouzo C, Guillouzo A. Early alterations of bile canaliculi dysfunction: implications for hepatotoxicity. *Hepatology* 2013;57:552–63.

160. Kato R, Uetrecht J. Supernatant from hepatocyte cultures with drugs suggests an underlying immune pathogenesis. *Chem Res Toxicol* 2017;30:1327–32.

159. Kim SW, Olsson LA. Liver immunology and its role in inflammation and homeostasis. *Cell Mol Immunol* 2016;13:267–76.

158. Parham LR, Briley LP, Li L, Shen J, Newcombe PJ, King KS, et al. Comprehensive genome-wide evaluation of lapatinib-induced liver injury yields a single genetic signal centered on known risk allele HLA-DRB1*07:01. *Pharmacogenomics J* 2016;16:180–5.

157. Pollmacher T, Hinze-Selch D, Mullington J. Effects of clozapine on plasma cytokine and soluble cytokine receptor levels. *J Clin Psychopharmacol* 1996;16:403–9.

156. Kato R, Uetrecht J. Supernatant from hepatocyte cultures with drugs suggests an underlying immune pathogenesis. *Chem Res Toxicol* 2017;30:1327–32.

155. Jee A, Sernoskie SC, Uetrecht J. Idiosyncratic drug-induced liver injury: mechanistic and clinical challenges. *Int J Mol Sci* 2021;22:2954.

154. Kouwenhoven M, Ozenci V, Teleshova N, Hussein Y, Huang YM, Eusebio A, et al. Enzyme-linked immunospot assays provide a...
sensitive tool for detection of cytokine secretion by monocytes. *Clin Diagn Lab Immunol* 2001;8:1248–57.

216. Pichler WJ, Tilich J. The lymphocyte transformation test in the diagnosis of drug hypersensitivity. *Allergy* 2004;59:809–20.

217. Rive CM, Bourke J, Phillips EJ. Testing for drug hypersensitivity syndromes. *Clin Biochem Rev* 2013;34:15–38.

218. Whiten J, Ko M, Zong Q, Wang J, Tartaro K, Schneider P, et al. Development of a modified lymphocyte transformation test for diagnosing drug-induced liver injury associated with an adaptive immune response. *J Immunotoxicol* 2017;14:31–8.

219. Usui T, Faulkner L, Farrell J, French NS, Alfirevic A, Pirmohamed M, et al. Application of in vitro T cell assay using human leukocyte antigen-typed healthy donors for the assessment of drug immunogenicity. *Chem Rev Toxicol* 2018;31:165–7.

220. Oda S, Matsuo K, Nakajima A, Yokoi T. A novel cell-based assay for the evaluation of immune- and inflammatory-related gene expression as biomarkers for the risk assessment of drug-induced liver injury. *Toxicol Lett* 2016;241:60–70.

221. Ogese MO, Faulkner L, Jenkins RE, French NS, Copple IM, Oda S, Uchida Y, Aleo MD, Koza-Taylor PH, Matsui Y, Hizue M, Beggs KM, Maiuri AR, Fullerton AM, Poulsen KL, Breier AB, Fredriksson L, Herpers B, Benedetti G, Matadin Q, Puigvert JC, de Momen-Heravi F, Saha B, Kodys K, Catalano D, Satishchandran A, Koberle V, Pleli T, Schmithals C, Augusto Alonso E, Haupenthal J, Bonig H, et al. Differential stability of cell-free circulating microRNAs: implications for their utilization as biomarkers. *PLoS One* 2013;8:e75184.

222. Sato K, Meng F, Glaser S, Alpini G. Exosomes in liver pathology. *J Hepatol* 2016;65:213–21.

223. Momen-Heravi F, Saha B, Kodys K, Catalano D, Satoshichandran A, Szabo G. Increased number of circulating exosomes and their microRNA cargos are potential novel biomarkers in alcoholic hepatitis. *Clin Biochem Rev* 2015;36:141–9.

224. LeCluyse EL, Witek RP, Andersen ME, Powers MJ. Organotypic coculture system of human peripheral blood mononuclear cells with hepatocellular carcinoma-derived cells for predicting drug-induced liver injury. *Arch Toxicol* 2021;95:149–68.

225. LeCluyse EL, Witek RP, Andersen ME, Powers MJ. Organotypic coculture system of human peripheral blood mononuclear cells with hepatocellular carcinoma-derived cells for predicting drug-induced liver injury. *Arch Toxicol* 2021;95:149–68.

226. LeCluyse EL, Witek RP, Andersen ME, Powers MJ. Organotypic coculture system of human peripheral blood mononuclear cells with hepatocellular carcinoma-derived cells for predicting drug-induced liver injury. *Arch Toxicol* 2021;95:149–68.

227. LeCluyse EL, Witek RP, Andersen ME, Powers MJ. Organotypic coculture system of human peripheral blood mononuclear cells with hepatocellular carcinoma-derived cells for predicting drug-induced liver injury. *Arch Toxicol* 2021;95:149–68.

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228. Ware BR, Berger DR, Khetani SR. Prediction of drug-induced liver injury in micropatterned co-cultures containing iPSC-derived human hepatocytes. *Toxicol Sci* 2015;145:252–62.

229. Hiemstra S, Ramaiahgari SC, Wink S, Callegaro G, Coonen M, Meerman J, et al. High-throughput confocal imaging of differentiated 3D liver-like spheroid cellular stress response reporters for identification of drug-induced liver injury liability. *Arch Toxicol* 2019;93:2895–911.

230. Cha HJ, Ko MJ, Ahn SM, Ahn JI, Shin HJ, Jeong HS, et al. Identification of classifier genes for hepatotoxicity prediction in non steroid anti inflammatory drugs. *Mol Cell Toxicol* 2010;6:247–53.

231. Oda S, Uchida Y, Aleo MD, Koza-Taylor PH, Matsui Y, Hizue M, Beggs KM, Maiuri AR, Fullerton AM, Poulsen KL, Breier AB, Fredriksson L, Herpers B, Benedetti G, Matadin Q, Puigvert JC, de Momen-Heravi F, Saha B, Kodys K, Catalano D, Satishchandran A, Koberle V, Pleli T, Schmithals C, Augusto Alonso E, Haupenthal J, Bonig H, et al. Differential stability of cell-free circulating microRNAs: implications for their utilization as biomarkers. *PLoS One* 2013;8:e75184.

232. Sato K, Meng F, Glaser S, Alpini G. Exosomes in liver pathology. *J Hepatol* 2016;65:213–21.

233. Momen-Heravi F, Saha B, Kodys K, Catalano D, Satoshichandran A, Szabo G. Increased number of circulating exosomes and their microRNA cargos are potential novel biomarkers in alcoholic hepatitis. *Clin Biochem Rev* 2015;36:141–9.

234. LeCluyse EL, Witek RP, Andersen ME, Powers MJ. Organotypic coculture system of human peripheral blood mononuclear cells with hepatocellular carcinoma-derived cells for predicting drug-induced liver injury. *Arch Toxicol* 2021;95:149–68.

235. Persson M, Lore AF, Mow T, Hornberg JJ. A high content screening assay to predict human drug-induced liver injury during drug discovery. *J Pharmacol Toxicol Methods* 2013;68:302–13.

236. Lu J, Einhorn S, Venkatarangan L, Miller M, Dann MA, Watkins PB, et al. Morphological and functional characterization and assessment of iPSC-derived hepatocytes for in vitro toxicity testing. *Toxicol Sci* 2015;147:39–54.

237. Pradip A, Steel D, Jacobsson S, Holmgren G, Ingelman-Sundberg M, Sartipy P, et al. High content analysis of human pluripotent stem cell derived hepatocytes reveals drug induced steatosis and phospholipidosis. *Stem Cells Int* 2016;2016:2475631.

238. Ware BR, Berger DR, Khetani SR. Prediction of drug-induced liver injury in micropatterned co-cultures containing iPSC-derived human hepatocytes. *Toxicol Sci* 2015;145:252–62.

239. Hiemstra S, Ramaiahgari SC, Wink S, Callegaro G, Coonen M, Meerman J, et al. High-throughput confocal imaging of differentiated 3D liver-like spheroid cellular stress response reporters for identification of drug-induced liver injury liability. *Arch Toxicol* 2019;93:2895–911.
253. Morgan SJ, Elangbam CS. Animal models of disease for future toxicity predictions. In: Will Y, McCaffie JE, Olahaski AJ, Jeffy BD, editors. Drug discovery toxicology: from target assessment to translational biomarkers. Chichester: John Wiley & Sons; 2016. p. 261–97.

254. 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. Regul Toxicol Pharmacol 2000;32:56–67.

255. Lutkewitte AJ, Schweitzer GG, Kennon-McGill S, Clemens MM, James LP, Chiew A, Abdel-Rahman SM, Letzig L, Graudins A, Day P, Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, et al. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. PLoS Biol 2020;18:e3000410.

256. Lukwewitte AJ, Schweitzer GG, Kennon-McGill S, Clemens MM, James LP, Jaeschke H, et al. Lipin deactivation after acetaminophen overdose causes phosphatidic acid accumulation in liver and plasma in mice and humans and enhances liver regeneration. Food Chem Toxicol 2018;115:573–83.

257. Ramachandran A, Jaeschke H. Mechanisms of acetaminophen hepatotoxicity and their translation to the human pathophysiology. J Clin Transl Res 2017;3:157–69.

258. James LP, Chiew A, Abdel-Rahman SM, Letzig L, Graudins A, Day P, et al. Acetaminophen protein adduct formation following low-dose acetaminophen exposure: comparison of immediate-release vs. extended-release formulations. Eur J Clin Pharmacol 2013;69:851–7.

259. Thrall KD, Vuclelic ME, Gies RA, Zangar RC, Weitz KK, Poet TS, et al. Comparative metabolism of carbon tetrachloride in rats, mice, and hamsters using gas uptake and PBPK modeling. J Toxicol Environ Health A 2000;60:521–48.

260. Metushi IG, Nakagawa T, Uetrecht J. Direct oxidation and covalent binding of isoazidaz to rodent liver and human hepatic microsomes: humans are more like mice than rats. Chem Res Toxicol 2012;25:2567–76.

261. Korolczuk A, Caban K, Amarowicz M, Czechowska G, Irla-Miduch J. Oxidative stress and liver morphology in experimental cyclosporine A-induced hepatotoxicity. BioMed Res Int 2016:2016: 5823271.

262. Hagar HH. The protective effect of taurine against cyclosporine A-induced oxidative stress and hepatotoxicity in rats. Toxicol Lett 2004;131:355–43.

263. Abdel-Dayem MA, Elmarakby AA, Abdel-Aziz AA, Pye C, Said SA, El-Mowafy AM. Valproate-induced liver injury: modulation by the omega-3 fatty acid DHA proposes a novel anticonvulsant regimen. Drugs R D 2014;14:855–94.

264. Sokmen BB, Tunali S, Yanardag R. Effects of vitamin U (metabolomics) of rat plasma. Toxicol Appl Pharmacol 2014;264:387–94.

265. Metushi IG, Nakagawa T, Uetrecht J. Acetaminophen-induced liver injury from animal models to humans. J Clin Transl Hepatol 2014;2:153–61.

266. Hall PD, Plummer JL, Ilesley AH, Cousins MJ. Hepatic fibrosis and cirrhosis after chronic administration of alcohol and "low-dose" carbon tetrachloride vapor in the rat. Hepatology 1991;13:815–9.

267. Tsuchida T, Lee YA, Fujiwara N, Ybanez M, Allen B, Martins S, et al. A simple diet- and chemical-induced murine NASH model with rapid progression of steatohepatitis, fibrosis and liver cancer. J Hepatol 2018;69:385–95.

268. Chen X, Xu J, Zhang C, Yu T, Wang H, Zhao M, et al. The protective effects of ursodeoxycholic acid on isoniazid plus rifampicin induced liver injury in mice. Eur J Pharmocol 2011;659:53–60.

269. Metushi IG, Cai P, Dervovic D, Liu F, Lobach A, Nakagawa T, et al. Development of a novel mouse model of amodiaquine-induced liver injury with a delayed onset. J Immunotoxicol 2015;12:247–60.

270. Laienfeld D, Qiu L, Swiss R, Park J, Macorittio M, Will Y, et al. Utilization of causal reasoning of hepatic gene expression in rats to identify molecular pathways of idiosyncratic drug-induced liver injury. Toxicol Sci 2014;137:234–48.

271. Watt W, Davis K, Fabian E, Greenhaw J, Herold M, Looser R, et al. Detection of hepatotoxicity potential with metabolite profiling (metabolomics) of rat plasma. Toxicol Lett 2014;230:467–78.

272. Eum JW, Bae HJ, Shin Q, Park SJ, Kim HS, Shin WC, et al. Characteristic molecular and proteomic signatures of drug-induced liver injury in a rat model. J Appl Toxicol 2015;35:152–64.

273. Nolan JP. The role of intestinal endotoxin in liver injury: a long and evolving history. Hepatology 2010;52:1829–35.

274. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell 2010;140:805–20.

275. Buchweitz JP, Ganey PE, Bursian SJ, Roth RA. Underlying endotoxemia augments toxic responses to chlorpromazine: is there a relationship to drug idiosyncrasy?. J Pharmaco Exp Ther 2002:300: 460–7.

276. Shaw PJ, Hopfensperger MJ, Ganey PE, Roth RA. Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor-alpha. Toxicol Sci 2007;100:559–66.

277. Lauf J, Jones AD, Harkema JR, Roth RA, Ganey PE. Amiodarone exposure during modest inflammation induces idiosyncrasy-like liver injury in rats: role of tumor necrosis factor-alpha. Toxicol Sci 2012; 125:126–33.

278. Deng X, Stachlewitz RF, Liguori MJ, Blomme EA, Waring JF, Luyendyk JP, et al. Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. J Pharmaco Exp Ther 2006;319:1191–9.

279. Waring JF, Liguori MJ, Luyendyk JP, Maddox JF, Ganey PE, Stachlewitz RF, et al. Microarray analysis of lipopolysaccharide potentiation of trovafloxacin-induced liver injury in rats suggests a role for proinflammatory chemokines and neutrophils. J Pharmaco Exp Ther 2006;316:1080–7.

280. Zhou W, Devi SS, Spakenbaugh E, Younis HS, Roth RA, Ganey PE. D Hepatotoxic interaction of sulindac with lipopolysaccharide: role of the hemostatic system. Toxicol Sci 2009;108:184–93.

281. Luyendyk JP, Maddox JF, Cosma GN, Ganey PE, Cockrell GL, Roth RA. Ranitidine treatment during a modest inflammatory response precipitates idiosyncrasy-like liver injury in rats. J Pharmaco Exp Ther 2003;307:9–16.

282. Heidari R, Almahdi F, Rahimi HR, Azapira N, Hosseinzadeh M, Najibi A, et al. Exacerbated liver injury of antithyroid drugs in endotoxin-treated mice. Drug Chem Toxicol 2019;42:615–23.

283. Tukov FF, Luyendyk JP, Ganey PE, Roth RA. The role of tumor necrosis factor alpha in lipopolysaccharide/ranitidine-induced inflammatory liver injury. Toxicol Sci 2007;100:267–80.

284. Cueto-Sanchez A, Niu H, Del Campo-Herrera E, Robles-Diaz M, Sanabria-Cabrera J, Ortega-Alonso A, et al. Lymphocyte profile and immune checkpoint expression in drug-induced liver injury: an immunophenotyping study. Clin Pharmacol Ther 2021. Available from: https://doi.org/10.1002/cpt.2423.

285. Metushi IG, Hayes MA, Uetrecht J. Treatment of PD-1+/− mice with amiodarone and anti-CTLA4 leads to liver injury similar to idiosyncratic liver injury in patients. Hepatology 2015;61: 1332–42.

286. Chakraborty M, Fullerton AM, Semple K, Chea LS, Proctor WR, Bourdi M, et al. Drug-induced allergic hepatitis develops in mice when myeloid-derived suppressor cells are depleted prior to halothane treatment. Hepatology 2015;62:546–57.
309. Gutti TL, Knibbe JS, Makarov E, Zhang J, Yannam GR, Ito K, et al. 
Gutierrez H, Suemizu H, Igaya S, Shimizu M, Shibata N, 
Bility MT, Nio K, Li F, McGivern DR, Lemon SM, Feeney ER, et al. 
Harrill AH, Watkins PB, Su S, Ross PK, Harbourt DE, Stylianou IM, 
Dugan CM, MacDonald AE, Roth RA, Ganey PE. A mouse model of 
Preclinical models of iDILI 3723

297. Morita M, Akai S, Hosomi H, Tsuneyama K, Nakajima M, Yokoi T. 
Ong MM, Latchoumycandane C, Boelsterli UA. Troglitazone- 
Wilson ID. The metabolic fate of fenclozic acid in chimeric mice 
Toxicol 2011; 38: 204–13.

294. Ong MM, Latchoumycandane C, Boelsterli UA. Troglitazone- 
FRGN mice.

293. Morita M, Akai S, Hosomi H, Tsuneyama K, Nakajima M, Yoko T. 
Drug-induced hepatotoxicity test using gamma-glutamylcysteine 
synthetase knockdown rat. Toxicol Lett 2009; 189: 159–65.

291. Aki S, Hosomi H, Minami K, Tsuneyama K, Katoh M, Nakajima M, et al. Knock down of gamma-glutamylcysteine synthetase in rat causes acetaminophen-induced hepatotoxicity. J Biol Chem 2007; 282: 23996–4003.

292. Iida A, Sasaki E, Yano A, Tsuneyama K, Fukami T, Nakajima M, et al. Carbamazepine-induced liver injury requires CYP2E1-mediated metabolism and glutathione depletion in rats. Drug Metab Dispos 2015; 43: 958–68.

290. Forootan SS, Mutter FE, Kipar A, Iwawaki T, Francis B, Goldberg CE, et al. Real-time in vivo imaging reveals localized Nr2f2 stress responses associated with direct and metabolism-dependent drug toxicity. Sci Rep 2017; 7: 16084.

289. Dugan CM, MacDonald AE, Roth RA, Ganey PE. A mouse model of severe halothane hepatitis based on human risk factors. J Pharmacol Exp Ther 2010; 333: 364–72.

288. You Q, Cheng L, Reilly TP, Wegmann D, Ju C. Role of neutrophils in a mouse model of halothane-induced liver injury. Hepatology 2006; 44: 1421–31.

287. Harrill AH, Watkins PB, Su S, Ross PK, Harbouret DE, Stylianou IM, et al. Mouse population-aged sequencing reveals that variants in CD44 contribute to acetaminophen-induced liver injury in humans. Genome Res 2009; 19: 1507–15.

286. Nakani M, Morii T, Matsuo K, Katoh Y, Nakajima M, Tatemoto C, et al. Chimeric mice with a humanized liver as an animal model of trilostolate-induced liver injury. Toxicol Lett 2012; 214: 9–18.

285. Ek dahal A, Weidolf L, Baginski M, Morikawa Y, Thompson RA, Wilson ID. The metabolic fate of fenclofic acid in chimeric mice with a humanized liver. Arch Toxicol 2018; 92: 2819–28.

284. Yamazaki H, Suemizu H, Igaya S, Shimizu M, Shibata N, Nakamura M, et al. In vivo formation of a glutathione conjugate derived from thioldamidine in humanized uPA-NOG mice. Chem Res Toxicol 2011; 24: 287–9.

283. Xu D, Wu M, Nishiuma S, Nishiuma T, Mishie SA, Zheng M, et al. Chimeric TK-NOG mice: a predictive model for cholestatic human liver toxicity. J Pharmacol Exp Ther 2015; 352: 274–80.

282. Biltiy MT, Nio K, Li F, McGivern DR, Lemon SM, Feeney DR, et al. Chronic hepatocite C infection-induced liver fibrogenesis is associated with M2 macrophage activation. Sci Rep 2016; 6: 39520.

281. Gitti TL, Knibbe JS, Makarov E, Zhang J, Yannam GR, Gorantla S, et al. Human hepatocytes and hematolymphoid dual reconstitution in treosulfan-conditioned uPA-NOG mice. Am J Pathol 2014; 184: 101–9.

280. Wilson EM, Bial J, Tarlow B, Bial G, Jensen B, Greiner DL, et al. Extensive double humanization of both liver and hematopoiesis in FRGN mice. Stem Cell Res 2013; 14: 404–12.

279. Song B, Aoki S, Liu C, Susukida T, Ito K. An animal model of abacavir-induced HLA-mediated liver injury. Toxicol Sci 2018; 162: 713–23.
332. Sanoh S, Ohta S. Chimeric mice transplanted with human hepatocytes as a model for prediction of human drug metabolism and pharmacokinetics. Biopharm Drug Dispos 2014;35:71–86.

333. Yuan L, Liu X, Zhang L, Zhang Y, Chen Y, Li X, et al. Optimized HepaRG is a suitable cell source to generate the human liver chimeric mouse model for the chronic hepatitis B virus infection. Emerg Microbes Infect 2018;7:144.

334. Foster JR, Lund G, Sapelnikova S, Tyrell DL, Kneteman NM. Chimeric rodents with humanized liver: bridging the pre-clinical/clinical trial gap in ADME/toxicity studies. Xenotransplant 2014;44:109–22.

335. Dandri M, Burda MR, Torok E, Pollok JM, Iwanska A, Sommer G, et al. Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. Hepatology 2001;33:981–8.

336. Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, et al. Hepatitis C virus replication in mice with chimeric human livers. Nat Med 2001;7:927–33.

337. Sueizumi H, Hasegawa M, Kawai K, Taniguchi K, Monnai M, Wakui M, et al. Establishment of a humanized model of liver using NOD/Shi-scid IL2rgnull mice. Biochem Biophys Res Commun 2008;377:248–52.

338. Azuma H, Paulk N, Ranade A, Dorrell C, Al-Dhalimi M, Ellis E, et al. Robust expansion of human hepatocytes in Fath /Rag2 /Il2rg × Alb-TRECK/SCID mouse. Nat Biotechnol 2007;25:903–10.

339. Hasegawa M, Kawai K, Mitsuji T, Taniguchi K, Monnai M, Wakui M, et al. The reconstituted ‘humanized’ liver in TK-NOG mice is mature and functional. Biochem Biophys Res Commun 2011;405:405–10.

340. Zhang RR, Zheng YW, Li B, Tsuchida T, Ueno Y, Nie YZ, et al. Human hepatic stem cells transplanted into a fulminant hepatic failure Alb-TRECK/SCID mouse model exhibit liver reconstitution and drug metabolism capabilities. Stem Cell Res Ther 2015;6:49.

341. Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamazaki S, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 2004;165:901–12.

342. Tateno C, Kawase Y, Tobita Y, Hamamura S, Oshita H, Yokomichi H, et al. Generation of novel chimeric mice with humanized livers by using hemizygous CDNA-uPA/SCID mice. PLoS One 2015;10:e012145.

343. Miyamoto M, Kosugi Y, Iwasaki S, Chisaki I, Nakagawa S, Amano N, et al. Characterization of plasma protein binding in two mouse models of humanized liver, FXB mouse and humanized TK-NOG mouse. Xenotransplant 2021;5:51–60.

344. Yamanaka H, Karibayashi S, Inoue T, Honda T, Tateno C, Oofusa K, et al. Zone analysis by two-dimensional electrophoresis with accelerator mass spectrometry of in vivo protein bindings of idiosyncratic drug-induced liver injury. Xenobiotica 2011;41:479–91.

345. Takebe T, Sekine K, Enomura M, Koike H, Kimura M, Ogaeri T, et al. A humanized mouse model to study hepatitis C virus infection, immune response, and liver disease. Gastroenterology 2011;140:1334–44.

346. Kim I, Ryu B, Kim U, Kim CH, Hur GH, Kim CY, et al. Improved human hematopoietic reconstitution in HepaRG co-transplanted humanized NSG mice. MBMB Rep 2020;53:466–71.

347. Dagar RS, Wang W, Makarov E, Sun Y, Poluektova LY. Establishment of the dual humanized TK-NOG mouse model for HIV-associated liver pathogenesis. J Vis Exp 2019;151:e58645.

348. Matsunaga T, Cabily-Horesh O, Terz D, Lee A, Christian L, Ohno S, et al. Use of transgenic mice with human MHC class 1 gene (HLA-A2). In: Klaus GGB, editor. Microenvironments in the lymphoid system. Boston: Springer; 1985. p. 341–7.

349. Yuksel M, Wang Y, Tai N, Peng J, Guo J, Beland K, et al. A novel “humanized mouse” model for autoimmune hepatitis and the association of gut microbiota with liver inflammation. Hepatology 2015;62:1536–50.

350. Susakida T, Aoki S, Kogo K, Fujimori S, Song B, Liu C, et al. Evaluation of immune-mediated idiosyncratic drug toxicity using chimeric HLA transgenic mice. Arch Toxicol 2018;92:1177–88.

351. Cardone M, Garcia K, Tilahun ME, Boyd LF, Gebreyohannes S, Yano M, et al. A transgenic mouse model for HLA-B*57:01-linked abacavir drug tolerance and reactivity. J Clin Invest 2018;128:2819–32.

352. Lundgren H, Martinsson K, Cederbrant K, Jirholt J, Mucs D, Madesky-Bengtson K, et al. HLA-DR7 and HLA-DQ2 transgenic mouse strains tested as a model for xenelagatan hepatotoxicity. PLoS One 2017;12:e0184744.

353. Pavlos R, Deshpande P, Chopra A, Leary S, Strautins K, Nolan D, et al. New genetic predictors for abacavir tolerance in HLA-B*57:01 positive individuals. Hum Immunol 2020;81:300–4.

354. Cirulli ET, Nicoletti P, Abramson K, Andrade RJ, Bjornsson ES, Yano M, et al. A transgenic mouse model for HLA-B*57:01-linked abacavir drug tolerance and reactivity. J Clin Invest 2018;128:2819–32.

355. Wang J, Bwaiy M, Gee RRF, Chen T. PXR-mediated idiosyncratic drug-induced liver injury: mechanistic insights and targeting approaches. Expert Opin Drug Metab Toxicol 2020;16:711–22.

356. Bissig KD, Han W, Barzi M, Kovalchuk N, Ding L, Fan X, et al. P450-humanized and human liver chimeric mouse models for studying xenobiotic metabolism and toxicity. Drug Metab Dispos 2018;46:1734–44.

357. Li X, Chen Y, Song X, Zhang Y, Li H, Zhao Y. The development and application of in silico models for drug induced liver injury. RSC Adv 2018;8:8101–11.

358. Cheng A, Dixon SL. In silico models for the prediction of dose-dependent hepatotoxicity. J Comput Aided Mol Des 2003; 17:811–23.

359. Greene N, Fisk L, Naven RT, Note RR, Patel ML, Pelletier DJ. Developing structure–activity relationships for the prediction of hepatotoxicity. Chem Res Toxicol 2010;23:1215–22.

360. Hewitt M, Przybylak K. In silico models for hepatotoxicity. Methods Mol Biol 2016;1425:201–36.

361. Woodhead JL, Watkins PB, Howell BA, Siler SQ, Shoda LKM. The role of quantitative systems pharmacology modeling in the prediction and explanation of idiosyncratic drug-induced liver injury. Drug Metab Pharmacokinet 2017;32:40–5.

362. Marchant CA, Fisk L, Note RR, Patel ML, Suarez D. An expert systems approach to the assessment of hepatotoxic potential. Chem Biodivers 2009;6:2107–14.

363. Chen M, Bissig H, Tong L, Hong H, Fang H, Borlak J, et al. Toward predictive models for drug-induced liver injury in humans: are we there yet?. Biomark Med 2014;8:201–13.

364. Williams DP, Lazic SE, Foster AJ, Semenova E, Morgan P. Predicting drug-induced liver injury with bayesian machine learning. Chem Res Toxicol 2020;33:239–48.

365. Li T, Tong W, Roberts R, Liu Z, Thakkar S. Deep learning on high-throughput transcriptomics to predict drug-induced liver injury. Front Bioeng Biotechnol 2020;8:562677.
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371. Hong H, Thakkar S, Chen M, Tong W. Development of decision forest models for prediction of drug-induced liver injury in humans using a large set of FDA-approved drugs. Sci Rep 2017; 7:17311.

372. Schöning V, Krähenbühl S, Drewe J. The hepatotoxic potential of protein kinase inhibitors predicted with Random Forest and Artificial Neural Networks. Toxicol Lett 2016; 299:145–8.

373. Przybylak KR, Cronin MT. In silico models for drug-induced liver injury—current status. Expert Opin Drug Metab Toxicol 2012; 8:201–17.

374. Zhu XW, Sedykh A, Liu SS. Hybrid in silico models for drug-induced liver injury using chemical descriptors and in vitro cell-imaging information. J Appl Toxicol 2014; 34:281–8.

375. Liu R, Yu X, Wallqvist A. Data-driven identification of structural alerts for mitigating the risk of drug-induced human liver injuries. J Cheminform 2015; 7:4.

376. Gonzalez-Tijerina A, Suzuki A, Chen M, Alvarez-Alvarez I, Andrade RJ, et al. Drug properties and host factors contribute to biochemical presentation of drug-induced liver injury: a prediction model from a machine learning approach. Arch Toxicol 2021; 95:1793–803.

377. Shoda LK, Woodhead JL, Siler SQ, Watkins PB, Howell BA. Linking physiology to toxicity using DILIsym®, a mechanistic mathematical model of drug-induced liver injury. Biopharm Drug Dispos 2014; 35:33–49.

378. Howell BA, Yang Y, Kumar R, Woodhead JL, Harrill AH, Clewell HJ 3rd, et al. In vitro to in vivo extrapolation and species response comparisons for drug-induced liver injury (DILI) using DILIsym: a mechanistic, mathematical model of DILI. J Pharmacokinet Pharmacodyn 2012; 39:527–41.

379. Yang K, Woodhead JL, Watkins PB, Howell BA, Brouwer KL. Systems pharmacology modeling predicts delayed presentation and species differences in bile acid-mediated triglyceride hepatotoxicity. Clin Pharmacol Ther 2014; 96:589–98.

380. Longo DM, Yang Y, Watkins PB, Howell BA, Siler SQ. Elucidating differences in the hepatotoxic potential of tolcapone and entacapone with DILIsym®, a mechanistic model of drug-induced liver injury. J Pharmacometr Pharmacodyn 2012; 39:527–41.

381. Howell BA, Siler SQ, Watkins PB. Use of a systems model of drug-induced liver injury (DILIsym®) to elucidate the mechanistic differences between acetaminophen and its less-toxic isomer, AMAP, in mice. Toxicol Lett 2014; 242:163–72.

382. Yang Y, Nadanaciva S, Will Y, Woodhead JL, Howell BA, Watkins PB, et al. MITOsym®, a mechanistic, mathematical model of hepatocellular respiration and bioenergetics. Pharm Res 2015; 32:1975–92.

383. Woodhead JL, Brock WJ, Roth SE, Shoaf SE, Brouwer KL, Church R, et al. Application of a mechanistic model to evaluate putative mechanisms of tolvanap drug-induced liver injury and identify patient susceptibility factors. Toxicol Sci 2017; 155:61–74.

384. Woodhead JL, Yang K, Oldach D, MacLauchlin C, Fernandes P, Watkins PB, et al. Analyzing the mechanisms behind macrolide antibiotic-induced liver injury using quantitative systems toxicology modeling. Pharm Res 2019; 36:48.

385. Longo DM, Woodhead JL, Walker P, Heredi-Szabo K, Mogyorosi K, Woleński FS, et al. Quantitative systems toxicology analysis of in vitro mechanistic assays reveals importance of bile acid accumulation and mitochondrial dysfunction in TAK-875-induced liver injury. Toxicol Sci 2019; 167:458–67.

386. Cruz-Monteagudo M, Cordeiro MN, Borges F. Computational chemistry approach for the early detection of drug-induced idiosyncratic liver toxicity. J Comput Chem 2008; 29:533–49.

387. Thakkar S, Chen M, Fang H, Liu Z, Roberts R, Tong W. The Liver Toxicity Knowledge Base (LKTB) and drug-induced liver injury (DILI) classification for assessment of human liver injury. Expert Rev Gastroenterol Hepatol 2018; 12:31–8.

388. Judson R, Richard A, Dix D, Houck K, Elloumi F, Martin M, et al. ACToR—a aggregated computational toxicoology resource. Toxicol Appl Pharmacol 2008; 233:7–13.

389. Igarashi Y, Nakatsu N, Yamashita T, Ono A, Ohno Y, Urushidani T, et al. Open TG-GATEs: a large-scale toxicogenomics database. Nucleic Acids Res 2015; 43:D921–7.

390. Zuo XW, Li SJ. In silico prediction of drug-induced liver injury based on adverse drug reaction reports. Toxicol Sci 2017; 158:391–400.

391. Stephens C, Robles-Diaz M, Medina-Caliz I, Garcia-Cortes M, Ortega-Alonso A, Sanabria-Cabrera J, et al. Comprehensive analysis and insights gained from long-term experience of the Spanish DILI Registry. J Hepatol 2021; 75:86–97.

392. Bessone F, Hernandez N, Lucena MI, Andrade RJ, Network OboTLD, Registry SD. The Latin American DILI registry experience: a successful ongoing collaborative strategic initiative. Int J Mol Sci 2016; 17:313.

393. Devarbhavi H, Joseph T, Sunil Kumar N, Rathi C, Thomas V, Prasad Singh S, et al. The Indian network of drug-induced liver injury: etiology, clinical features, outcome and prognostic markers in 1288 patients. J Clin Exp Hepatol 2021; 11:288–98.

394. Jain S, Norinder U, Escher SE, Zdrazil B. Combining in vivo data with in silico predictions for modeling hepatic steatosis by using stratified bagging and conformal prediction. Chem Res Toxicol 2021; 34:656–68.

395. Sanz F, Carrio P, Lopez O, Capoferri L, Kooi DP, Vermeulen NP, et al. Integrative modeling strategies for predicting drug toxicities at the eTOX project. Mol Inform 2015; 34:477–84.

396. Benesic A, Leifl A, Gerbes AL. Monocyte-derived hepatocyte-like cells for causality assessment of idiosyncratic drug-induced liver injury. Gut 2016; 65:1555–63.

397. Benesic A, Rotter J, Dragoi D, Weber S, Leifl A, Buchholtz ML, et al. Development and validation of a test to identify drugs that cause idiosyncratic drug-induced liver injury. Clin Gastroenterol Hepatol 2018; 16:1488–14894 e5.

398. Dragoi D, Benesic A, Pichler G, Kulak NA, Bartsch HS, Gerbes AL. Proteomics analysis of monocyte-derived hepatocyte-like cells identifies integrin beta 3 as a specific biomarker for drug-induced liver injury by dicrofenc. Front Pharmacol 2018; 9:699.

399. Huang P, He Z, Ji S, Sun H, Xiang D, Liu C, et al. Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. Nature 2011; 475:386–9.

400. Sekiya S, Suzuki A. Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. Nature 2011; 475:390–3.

401. Simeonov KP, Uppal H. Direct reprogramming of human fibroblasts to hepatocyte-like cells by synthetic modified mRNAs. PLoS One 2014; 9:e100134.

402. Huang P, Zhang L, Gao Y, He Z, Yao D, Wu Z, et al. Direct reprogramming of human fibroblasts to functional and expandable hepatocytes. Cell Stem Cell 2014; 14:370–84.

403. Du Y, Wang J, Jia J, Song N, Xiang C, Xu J, et al. Human hepatocytes with drug metabolic function induced from fibroblasts by lineage reprogramming. Cell Stem Cell 2014; 14:394–403.

404. Nakamori D, Akamine H, Takayama K, Sakurai F, Mizuguchi H. Direct conversion of human fibroblasts into hepatocyte-like cells by ATFS, PROXI, FOXAX2, FOXAX3, and HNF4A transduction. Sci Rep 2017; 7:16675.

405. Ballester M, Bolonio M, Santamaría R, Castell JV, Ribes-Koninckx C, Bort R. Direct conversion of human fibroblast to hepatocytes using a single inducible polycistic vector. Stem Cell Res Ther 2019; 10:317.

406. Vallier L. Hepes with pep: direct reprogramming into human hepa-tocytes. Cell Stem Cell 2014; 14:267–9.

407. 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; 148:1340–13452 e7.

408. Sanabria-Cabrera J, Medina-Caliz I, Stankevicute S, Rodriguez-Nicolás A, Almarza-Torres M, Lucena MI, et al. Drug-induced liver injury associated with severe cutaneous hypersensitivity reactions: a
complex entity in need of a multidisciplinary approach. *Curr Pharm Des* 2019;25:3855–71.

409. Lin IC, Yang HC, Strong C, Yang CW, Cho YT, Chen KL, et al. Liver injury in patients with DRESS: a clinical study of 72 cases. *J Am Acad Dermatol* 2015;72:984–91.

410. Devarbhavi H, Raj S, Aradya VH, Rangegowda VT, Veeranna GP, Singh R, et al. Drug-induced liver injury associated with Stevens–Johnson syndrome/toxic epidermal necrolysis: patient characteristics, causes, and outcome in 36 cases. *Hepatology* 2016;63:993–9.

411. Ichai P, Laurent-Bellue A, Saliba F, Moreau D, Besch C, Francoz C, et al. Acute liver failure/injury related to drug reaction with eosinophilia and systemic symptoms: outcomes and prognostic factors. *Transplantation* 2017;101:1830–7.

412. Walsh S, Diaz-Cano S, Higgins E, Morris-Jones R, Bashir S, Bernal W, et al. Drug reaction with eosinophilia and systemic symptoms: is cutaneous phenotype a prognostic marker for outcome? A review of clinicopathological features of 27 cases. *Br J Dermatol* 2013;168:391–401.

413. Illing PT, Purcell AW, McCluskey J. The role of HLA genes in pharmacogenomics: unravelling HLA associated adverse drug reactions. *Immunogenetics* 2017;69:617–30.

414. Sousa-Pinto B, Correia C, Gomes L, Gil-Mata S, Araujo L, Correia O, et al. HLA and delayed drug-induced hypersensitivity. *Int Arch Allergy Immunol* 2016;170:165–79.

415. Nicolletti P, Barrett S, McEvoy L, Daly AK, Aithal G, Lucena MI, et al. Shared genetic risk factors across carbamazepine-induced hypersensitivity reactions. *Clin Pharmacol Ther* 2019;106:1028–36.

416. Sharma AM, Uetrecht J. Bioactivation of drugs in the skin: relationship to cutaneous adverse drug reactions. *Drug Metab Rev* 2014;46:1–18.

417. Yano A, Oda S, Fukami T, Nakajima M, Yokoi T. Development of a cell-based assay system considering drug metabolism and immune- and inflammatory-related factors for the risk assessment of drug-induced liver injury. *Toxicol Lett* 2014;228:13–24.

418. Hirashima R, Itoh T, Takey RH, Fujiwara R. Prediction of drug-induced liver injury using keratinocytes. *J Appl Toxicol* 2017;37:863–72.

419. Wojtowicz AM, Oliveira S, Carlson MW, Zawadzka A, Rousseau CF, Baksh D. The importance of both fibroblasts and keratinocytes in a bilayered living cellular construct used in wound healing. *Wound Repair Regen* 2014;22:246–55.

420. Petrov PD, Fernandez-Murga ML, Lopez-Riera M, Gomez-Lechon MJ, Castell JV, Jover R. Predicting drug-induced cholestasis: preclinical models. *Expert Opin Drug Metab Toxicol* 2018;14:721–38.

421. Cheng Y, Chen S, Freed C, Chen W, Zhang Y, Abraham P, et al. Bile salt homeostasis in normal and Bsep gene knockout rats with single and repeated doses of troglitazone. *J Pharmacol Exp Ther* 2017;362:385–94.

422. Lam P, Soroka CJ, Boyer IL. The bile salt export pump: clinical and experimental aspects of genetic and acquired cholestatic liver disease. *Semin Liver Dis* 2010;30:125–33.

423. Yuan L, Liu X, Zhang L, Li X, Zhang Y, Wu K, et al. A chimeric humanized mouse model by engrafting the human induced pluripotent stem cell-derived hepatocyte-like cell for the chronic hepatitis B virus infection. *Front Microbiol* 2018;9:908.

424. Hsu YC, Chen CT, Wei YH. Mitochondrial resetting and metabolic reprogramming in induced pluripotent stem cells and mitochondrial disease modeling. *Biochim Biophys Acta* 2016;1860:686–93.

425. Suhr ST, Chang EA, Tjong J, Alcasid N, Perkins GA, Goissis MD, et al. Mitochondrial rejuvenation after induced pluripotency. *PLoS One* 2010;5:e14095.

426. Koido M, Kawakami E, Fukumura J, Noguchi Y, Ohori M, Nio Y, et al. Polygenic architecture informs potential vulnerability to drug-induced liver injury. *Nat Med* 2020;26:1541–8.