Acta Pharmaceutica Sinica B 2021;11(12):3685−3726

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

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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

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[9].

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[10]. Some of these patients will require a liver transplantation or ultimately died, 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 DILI onset are associated with the time course for DILI[11].

Biochemical signs of iDILI mainly include increased levels of liver enzymes measured in blood, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), ALP and TBIL. Clinically, iDILI is defined when one of the three following thresholds are met[12,13]: 1) ALT or AST elevation $\geq$5 times the upper limit of normal (ULN); 2) ALP $\geq$2 $\times$ ULN; or 3) the combination of ALT or AST $\geq$3 $\times$ ULN with simultaneous elevation of TBIL exceeding 2 $\times$ ULN. Depending on the extent of increase in those enzymes and the R-ratio of ALT/ALP or AST/ALP activity at iDILI presentation, iDILI is roughly classified as hepatocellular when ALT or AST $\geq$5 $\times$ ULN alone or when R-ratio is $\geq$5, cholestatic when ALP $\geq$2 $\times$ ULN alone or if R-ratio is $\leq$ 2, and 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-exposition. 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 ($\leq$0 excluded; 1−2 unlikely; 3−5 possible; 6−8 probable; and $\geq$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[20–56] 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[57]. 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)[58]. 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[59]. 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}[60]. 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[61]. 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[21] 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[62] and dimethyl sulfoxide (DMSO) together with different growth factors[63].

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[64]. 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[65]. 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[66], 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 canalicular 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[67].

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[68,69]. 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[70]. 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[71].

However, there are some drawbacks to using these cells as models. HepG2 cells exhibit low expression and activity levels of DMETs[59], although they may vary depending on the culture conditions[72] and the assay procedures[73]. 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[74]. Engineered HepG2 cells generated by adenoviral-mediated cytochrome P450 (CYP) transduction have also been proposed for hepatotoxicity studies[75], since adenoviral transduction allows the controlled expression of different CYP profiles in cells[76]. However, it

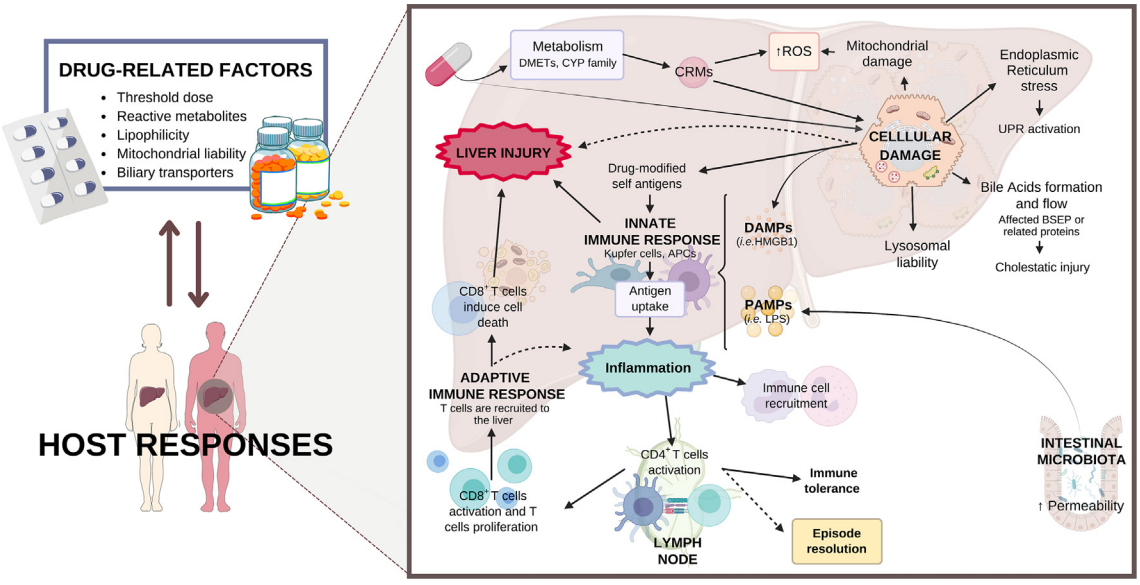

Figure 1 Current overview of idiosyncratic drug-induced liver injury general mechanisms. Considering that drug-related factors affect the responses that will occur within the host, some general mechanisms for idiosyncratic drug-induced liver injury have been proposed. First, the drug can be metabolized by hepatic enzymes, resulting in chemically reactive metabolites (CRMs). Parental drug 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.)

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[77]. 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[78]. The expression and activity levels of DMETs in these cells are elevated, although they may change depending on the culture conditions (*i.e.*, DMSO exposition[28] and cell confluency[79]).

HuH7 cells have been used to investigate the pathogenic mechanisms and possible therapies for different liver diseases, like the metabolic associated fatty liver disease (MAFLD)[80] and to study CYP-mediated drug metabolism[29].

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[68,81]. 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[34].

Other hepatocellular carcinoma-derived cell lines, such as BC2 cells[82] and Fa2N-4 cells[83], 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

Table 1 *In vitro* models to evaluate liver pathophysiologies and drug toxicity.

Model type			Configuration	Advantage		Disadvantage		Endpoint	Ref.
Single cell-type *in vitro* models	Primary hepatocytes		Monolayer, ECM sandwich, spheroids, liver-on-a-chip	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	2D, spheroids, microencapsulation, microfluidic chips	Availability and easily handling, hepatocyte morphology, liver functions, unlimited propagation potential, interdonor variability	Plasma proteins secretion, supports adenoviral-mediated CYP transduction	Lack of some hepatocyte functions, low metabolism activity (↓ DMETs), genotypic instability, single donor information	Need for glucose manipulation, ↓ DMETs	Cell death, oxidative stress, mitochondrial damage, ER stress, cholestasis, phospholipidosis, steatosis, HCS	23−25
		HepaRG	2D, spheroids		Supports HBV infection, stable expression of many DMETs, can be differentiated into hepatocyte-like and biliary epithelial-like cells		BSEP, CYP2D6 and CYP2E1 levels lower that PHHs	Cell death, drug metabolism, oxidative stress, mitochondrial damage, cholestasis, steatosis, HCS	26,27
		HuH7	2D, spheroids, 3D bioreactor		Plasma proteins secretion and expression of some DMETs		Highly heterogeneous	Drug metabolism	28,29
		Hep3B	2D, spheroids, 3D microarray		Metabolically competent and with active CYPs		↓ DMETs	Cell death, mitochondrial damage, ER stress	30−32
		THLE	2D, spheroids		Support adenoviral-mediated CYP transduction		↓ DMETs	Cell death, drug metabolism, mitochondrial function	33,34
	Hepatocyte-like cells	HLCs derived from hESCs	2D, spheroids	Unlimited propagation potential, self-renewal potential, capacity for acquiring hepatocyte-like morphology	Capacity for urea synthesis, drug metabolism, lipid synthesis, albumin secretion	Limited number of hESCs available, ethical concerns in generation and usage, great variability in DMETs activity		Cell death and drug metabolism	35,36
		HLCs derived from MSCs	2D, spheroids, 3D bioreactor		Expression of DMETs, easy to gather from patients	Dynamic and complex environment needed to differentiate MSCs into HLCs, variation due to the MSCs origin		Metabolism, oxidative stress	37
		HLCs derived from hiPSCs	2D, co-culture models, spheroids, bioprinted, microfluidics, liver on a chip		Allow the study of interindividual differences, avoidance of liver biopsies	Low liver functions compared to PHHs, low expression of CYPs, lack of full maturity, interlaboratory differences		Cell death, metabolism, cholestasis	38−40

(*continued on next page*)

Table 1 (*continued*)

Model type		Configuration	Advantage	Disadvantage	Endpoint	Ref.
Multiple-cell type *in vitro* models	Hepatocytes-NPCs co-cultures	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	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 metabolism, drug-induced ROS formation and GSH depletion, 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	Interactions between different tissues following drug exposure, drug toxicity	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[84].

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[85]. 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[86–89].

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[90].

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[91]. Recently, Miranda's laboratory[92] 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[93].

Notably, hiPSCs can be used to generate patient-derived HLCs through endoderm induction, hepatic specification, hepatoblast expansion and hepatic maturation, using specific growth factors[94–96]. 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[97]. 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.[39], 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-dependent apoptosis than control iPSCs-derived HLCs[98]. This increased sensitivity could explain the augmented risk of developing VPA toxicity in Alpers–Huttenlocher syndrome patients. With this work, Li et al.[98] 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)[99], fibroblasts[44] or immune cells[100] (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[101]. 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 hepatocyte differentiation program, showing hepatocyte-like phenotype with elevated mRNA expression of many DMETs[26], 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[102]. More interestingly, HepaRG cells show a stable expression of these proteins in time[103], 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)[102], as well as steatosis *in vitro*, since lipogenesis has been detected in HepaRG cells[104].

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[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 *in vivo* conditions[42,43]. 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 *in vitro*, losing their characteristic phenotypes in few days[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[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[41,108].

Moreover, 3D spheroidal configurations of hepatocytes co-culture 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[109].

2.2.3. *Hepatocytes-fibroblasts co-cultures*

HepatoPac® 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 *in vivo*-like hepatocyte functions in culture[44]. However, the use of mouse fibroblasts as co-culture cells may interfere with immunologic studies that use only human cells with human features[45].

HepatoPac® 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%[110]. This model has been also used to study the interplay of enzymes and transporters and the modeling of drug−drug interactions[111], and to predict the *in vivo* clearance of commercially available compounds[46].

2.2.4. *Precision-cut liver slices (PCLSs)*

PCLSs are the closest representation of an intact liver architecture with all the relevant cell types of the liver. As an *in vitro* model, PCLSs maintain cell−cell interactions[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[47]. Human PCLSs can be used to study the consequences of inhibiting different cellular pathways (*i.e.*, mitochondrial β oxidation[113]), to assess drug-mediated CYP induction/inhibition[114], hepatic transporters function[115] and iDILI mechanisms related to inflammatory stress and to detect potential biomarkers[48].

Unfortunately, this technology shows low viability in culture and drugs are often heterogeneously distributed. Nevertheless, PCLSs are thought to be the most physiologically complete *in vitro* model for liver research[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®, showing a flattened morphology[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-I 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 *in vivo* cells, enhances both secretion of organic compounds and DMETs activities, mimics *in vivo* biliary excretion rates[118] and enables the development of polarized cell surface domains over several days in culture[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[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 *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

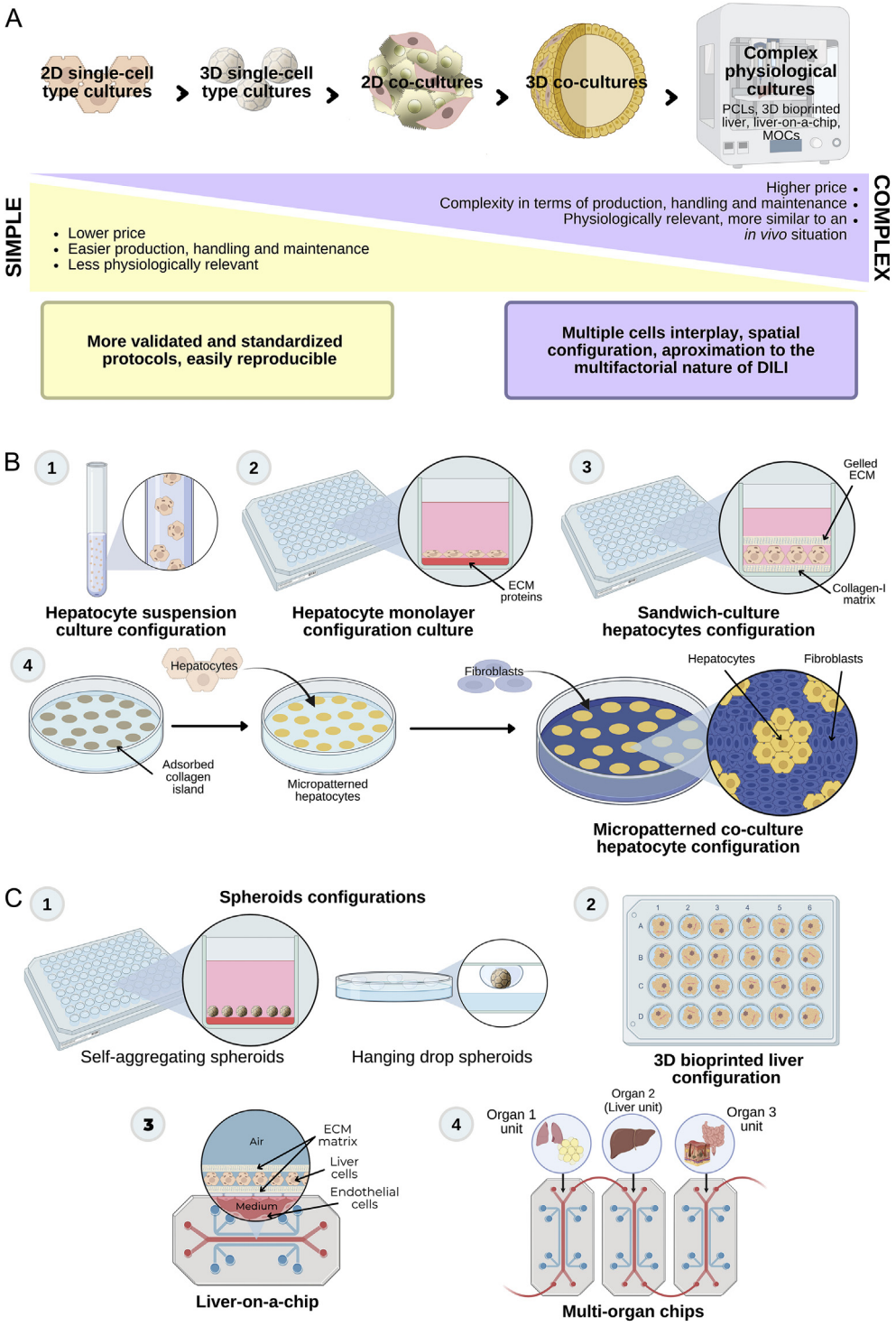

Figure 2 Overview of *in vitro* liver model configurations. (A) Flow diagram showing key features of *in vitro* hepatic cultures ranging from the simplest to the most complex. (B) Main 2D culture configurations used to study liver diseases include: 1) Suspension culture configuration, where hepatocytes 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[121], primary hepatocytes[122] or HLCs derived from hiPSC[123] 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[122]. 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[124].

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*[121]. These spheroids can be transferred to different multi-well plates for drug screening or toxicity testing[125].

Spheroids have been shown to promote higher functions in hepatocytes and increased sensitivity over liver toxicants compared to conventionally cultured hepatocytes[126], 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.[127] 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.[125] 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.[128] 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[129]. 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[130]. 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[131]. 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 acetaminophen (APAP)-associated damage to native liver tissues[132]. Other 3D bioprinted livers have also been used to model compound-induced fibrogenesis[133], and have recently been proposed for chronic hepatotoxicity studies *in vitro*[134].

- *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[135]. 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[51] and investigating drug-induced reactive oxygen species (ROS) formation and glutathione (GSH) depletion in HepG2/HepC3a cells[52].

Very recently, Rubiano et al.[53] 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 hepatotoxic 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.[136] designed a MOC

model with cell lines derived from lung, liver and fat to investigate drug biodistribution. Bricks et al.[55] 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.[54] designed a MOC with co-cultures of human artificial liver microtissues and skin biopsies, to study liver−skin interactions. Recently, Oleaga et al.[56] 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[137,138], 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[139].

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[140]. Some hypothesized pathophysiological mechanisms of iDILI are being studied *in vitro* using different cell models[141], 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[142,143]. 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[144]. 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[145], 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[146] 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[145,146].

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[147]. 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[148]. 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 (C_{max}) of the investigated drugs, since most hepatotoxic drugs show significant cytotoxicity within the 100-fold C_{max} range[149].

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[24,34]. On the other hand, Vorrink et al.[22] 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 whit 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[150].

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)[151].

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[152]. 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)[153].

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[154]. It can occur as a consequence of mitochondrial impairment or due to the formation of CRM[155] (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[156]. 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[25]. Using HepaRG cells, Anthérieu et al.[157] 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.[37] 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[158–163]. 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%[164].

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[165]. 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[166] are probably the most useful as predictor for iDILI involving mitochondrial dysfunction[167]. 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[168].

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.[169] 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[23,170–172]. 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[169].

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[30].

However, one important problem of mitochondrial damage assays is that the drug concentrations used are commonly much higher than the C_{max}, 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[173,174]. 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[175]. Autophagic flux dysfunction due to lysosome instability has also been described as a mechanism of diclofenac-iDILI[176], 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[177]. 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[178]. Moreover, it has been observed that proinflammatory cytokines contribute to cell damage *in vitro* in part through activation of ER stress[179].

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[180], as well as increased cytosolic free Ca^{2+} concentration and/or increased expression of calcium release-activated calcium channel protein 1 (CRAM1) and stromal interaction molecule 1 (STIM1), which are key components of the store-operated calcium entry[181].

In a similar way, Hep3B cells have been used to study the role of ER stress in EFV-iDILI. Apostolova et al.[31] 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 Ca^{2+} 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 the bile flow from the liver to the duodenum is referred 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[182]. 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[183]. Its inhibition and/or repression have been proposed as key mechanisms for drug-induced cholestasis[184]. 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[185], 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[186]. 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[187,188], 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 underestimate. 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 TCA 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 *ABCB11* 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[189].

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 (IC_{50}) value should be determined. However, there are huge discrepancies between the BSEP IC_{50} 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[190]. 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[191]. This system has been used to detect troglitazone and lopinavir repression of *ABCB11*[184]. 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[1]. 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.[192] 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.[193] 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.[194] 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[195] 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[196].

3.7. *Immune system activation*

Liver is a highly immunological organ[197] and dysregulation of its immune environment is thought to play a critical role in the initiation and progression of iDILI[198]. The common delayed onset of symptoms after taking the drug and the rapid onset after rechallenge[199], the presence of modified drug-peptide antigens (haptens) and the presence of autoantibodies or sensitized T cells in serum[200] 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 leucocyte antigen (HLA) alleles[201] 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 flucloxacillin[202–204]. Other HLA alleles with a strong iDILI association are *HLA DRB1*15:01-DQB1*06:02* for amoxicillin-clavulanic-associated hepatotoxicity[205–209] and *HLA-DRB1*07:01* for iDILI caused by ximelagatran-[210] and lapatinib-[211] 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 (idiosyncratic), the innate immune system previously activated by DAMPs, CRMs, etc. could be not idiosyncratic. 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[212,213]. 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[214].

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[215], and the lymphocyte transformation test (LTT), which measures the proliferation of T cells to a chemical compound *in vitro*[216]. However, this last technique exhibits limited sensitivity and specificity depending on the reaction and the responsible drug[217]. 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[218].

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.[219] 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 inflammasomes 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.[213] 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.[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[221].

To further investigate the role of inflammation in iDILI, Cosgrove et al.[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 (TNFα), interferon gamma (IFNγ), 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 TNFα to kill hepatocytes in culture[223–225], suggesting an essential role of cytokine signaling in iDILI caused by these compounds. Very recently, Oda et al.[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[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[60]. 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[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[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® have also been used, measuring different endpoints after hepatotoxic drugs exposure, such as nuclear size and intensity, cell permeability or mitochondrial integrity[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.

The main advantage of HLCs derived from hiPSCs is that they allow the comparison between different individuals' response in high-content imaging-based *in vitro* toxicity assays as well as drug toxicity potential screenings.

Whereas HCS is relatively simple for 2D conventional cell cultures, it is technically challenging in 3D systems due to inter-layer 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.[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[232]. Then, the images are processed automatically, and in this step, accurate cell segmentation is critical[233]. Finally, data analysis of all images by image algorithms is essential, and a complex and fine-tuned system is required[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 use 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.[240] 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.[241] 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 iDILI[242]. It has been observed that some miRNAs may be associated with iDILI development and progression[243]. miRNAs can be found in two different ways: free circulating or associated with extracellular vesicles (EV)[244]. Due to their ubiquity and stability, EV could be a promising source for studying transcriptomic profiles in iDILI and other liver diseases[245], as some groups have proven for example a significantly increased of circulating miRNA-192 and miRNA-30 in patients with alcoholic hepatitis[246] or alterations in the liver-specific RNA (albumin mRNA and *miR-122*) content of human primary hepatocytes-derived exosomes exposed at subtoxic doses of APAP[247].

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.[33] 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 C_{max} data. The authors showed that cytotoxicity measurements only predicted iDILI in the presence of exposure data. However, BSEP inhibition and mitochondrial disruption showed IC_{50} threshold effects around 30 μmol/L. The predictive value was improved when C_{max} 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 C_{max}) 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 vitro 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

Table 2 Principal endpoints, fluorescent probes, cell models, drugs tested and image analysis software used for HCS in iDILI modeling.

Parameter measured	Probe	Cell type	Example of drugs tested	Image analysis software	Ref.
Cell number Nuclear content Cell viability	Hoechst 33342 DRAQ5 Propidium iodide, WST-1 reagent, MTT colorimetric assay, Calcein AM, ATP-lite luminescence assay kit	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
Oxidative stress (ROS generation) Mitochondrial membrane potential Mitochondrial damage Intracellular GSH	CM-H2DCFDA, DHE, DCF, Carboxy-H2DCFD TMRM, MitoTracker, JC-10 TMRE mBCl, GSH-GLO Glutathione				
Intracellular lipid/fat deposits Cellular ATP content Caspase activity Endoplasmic reticulum status Membrane permeability Calcium levels Apoptosis	BODIPY 493/503, Steatosis Colorimetric Assay Kit, LipidTOX ATPlite CaspaseGLO 3/7 ER Tracker TOTO-3, YoYo-1 Fluo-4 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	25,27,75,235
Lysosomal activation Lipid peroxidation LDH leakage Albumin secretion Other parameters (*i.e.*, cholestasis, protein expression)	LysoTracker BODIPY 665/676 LDH cytotoxicity Assay Kit Albuwell kit 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[253].

Unfortunately, *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[254]. 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[255]. In fact, while animal toxicity studies can easily detect compounds with intrinsic toxicity, standard toxicology studies generally fail to identify drugs that produce iDILI[256]. Failure to predict adverse drug reactions using *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 *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[257].

Despite the *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 *in vivo* approaches in iDILI.

4.1. *Conventional animal toxicology 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[258]. APAP mechanisms of hepatotoxicity have been well documented[259,260] 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[261–268]. 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[269,270], rats are likely the most used animals when using CCl_4[261]. Interestingly, low doses of CCl_4 given repeatedly cause persistent liver injury with inflammation and fibrosis, thus CCl_4 can also be used to model chronic liver diseases, such as alcoholic liver disease, MAFLD and non-alcoholic steatohepatitis[271,272]. Co-administration of low doses of IHN and rifampicin (RIF) also resulted in liver injury[273].

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[274].

Another disadvantage of these models is that they are typically used in intrinsic DILI 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[275]. Metabolomic[276] and proteomic[277] 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[275–277].

Contrary to the situation of animal models of intrinsic DILI, the study of iDILI *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 *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 *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[1]. A number of studies have demonstrated the role of circulating gut-derived endotoxins and inflammatory processes in liver injury outcome and development[278]. 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[279].

Researchers have established several *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.[280], 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	Mostly intrinsic DILI, absence of delayed response	APAP, CCl_4, IHN, RIF, CsA, VPA, CBZ, TET, ADQ	↑↑ plasma/serum liver enzymes, histopathology alterations, liver necrosis, immune cell infiltration	258, 261–263, 265, 267
	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	Timing of LPS exposure, absence of delayed response, difficult to replicate, loss of sensitivity to LPS, mainly innate immune response	CPZ, TVX, AMD, DIC, SUL, RAN	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	Slight ↑ of ALT, modest liver injury, portal infiltration of lymphocytes	290–293
	Mitochondrial dysfunction	*Sod2*$^{+/-}$ 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, TVX, NIM	Discrete ↑ serum ALT activity, variable or delayed hepatocellular necrosis and/or apoptosis	294–296
	GSH knockdown	GSH-depleted rats through inhibition of γ-GCS or by	Possibility of linking GSH to iDILI drugs causing			Drug tolerance,	APAP, DIC, FTL,	Variable plasma ↑ ALT and AST,	297–299

(*continued on next page*)

Table 3 (*continued*)

Model type			Description	Advantage		Disadvantage		Example	Liver injury	Ref.
			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			Absence of iDILI drugs test, not widespread use	APAP	↑ serum ALT, extensive centrilobular coagulative necrosis	300
	Multifactorial models	Multiple determinant	Mice with selected specific risk factors (*e.g.*, eight weeks fed, female, BALB/c mice)	Consider more broadly the multifactorial origin and susceptibility factors of iDILI		High cost, difficulty of implementation, insufficient biological diversity to elucidate iDILI responses, poorly tested with iDILI drugs		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 models	Tissue humanization	Single tissue	Immunodeficient rodent hepatocytes are replaced with PHH or hiPSC-HLC using different strategies [*e.g.*, activation of plasminogen (uPA-SCID/NOG), accumulation of FAA (FRG), ganciclovir treatment (TK-NOG), treatment with FK506 (AFC8)].	Human liver functions, synthesis of human proteins, DMETs and transporters	Widespread use for studying idiosyncratic drug reactions (*e.g.*, PXB-mouse®)	Limited to special research facilities, humanization is often heterogeneous, cannot be spread to offspring	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	Mice co-transplanted with leukocyte progenitors ($CD34^+$ HSPC) and human hepatocytes (fetal or mature) (*e.g.*, AFC8-hu HSC/Hep, uPA-NOG HSC/Hep)		Inclusion of whole human HLA system provides the most complete		Lack of validation in iDILI studies, mostly used for the study of viral infections, occasionally low hepatocyte replacement, thymic	Not found	Immune cell infiltration (not DILI related)	308−310

					model to study human iDILI *in vivo* when high liver repopulation is achieved (*e.g.*, FRGN mice mixed model)		epithelium is often not humanized		
	Genetic humanization	HLA alleles	Introduction of human genes into the mouse genome	Mice carrying single or multiple human HLA linked to drug reactions (*e.g.*, *HLA-B*57:01, HLA-B*15:02*	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	ABC, CBZ, XMT	↑ of plasma ALT/GTP, histological hepatic lesions on *HLA-B*57:01* and *HLA-B*15:02*, no injury on *XMT- HLA-DR7* and *HLA-DQ2*	311–313
		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	DIC, RIF, IHN	Variable ↑ of serum markers, swelling and degeneration of hepatocytes	314–317

ABC, abacavir; ADQ, amodiaquine; AFC8, FK506 binding protein-caspase 8; AMD, amiodarone; APAP, acetaminophen; BOS, bosentan; CBZ, carbamazepine; CCl_4, 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, $Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}$; FRGN, FRG 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^{null}; 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 inflammagen models have been used to induce reproducible liver injury caused by different iDILI drugs at non-toxic doses[281–288] (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[286] (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[289].

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 1 protein (PD-1) KO female C57BL/6 mice co-treated with an antibody against cytotoxic 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 strategy has been used to develop a halothane-[291], IHN- and NVP-induced[292] liver injury models. The "Uetrecht-Pohl" model has shown to be capable of distinguishing between hepatotoxic and non-hepatoxic drugs of the same class and confirm an immune mechanism for troglitazone- and tolcapone-induced iDILI[293]. 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-D-tryptophan, an inhibitor of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO). Contrary to what was expected, co-treatment of amodiaquine with 1-methyl-D-tryptophan decreased liver injury. These results reaffirm the complexity of the immune response and highlight that drug-immune system interactions are still difficult to model and predict[318].

4.4. The mitochondrial dysfunction model (*Sod2*$^{+/-}$ mice)

Animal models with previous mitochondriopathies 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 humans[294]. 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 aconitase activity and complex I and III subunits expression[295]. Similar mitochondrial dysfunction was observed in *Sod2*$^{+/-}$ mice treated with trovafloxacin[296]. However, attempts to reproduce those results have had mixed success[319] and this model is not commonly used. Recent discovered associations between *Sod2* variants and risk of developing cholestatic/mixed iDILI[320] and hepatocellular[321] 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 strategy[322].

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 hepatotoxicity[298] and significant ALT elevations after treatment with diclofenac and flutamide[297]. 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 synthesis[323]. 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 hepatotoxicity[299,324]. The same strategy has been used to stablish 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 hepatotoxicity[297].

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 imaging[300]. 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 generated[301,302]. 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[301]. 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[303]. 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.[327], 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[328].

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[329] (Fig. 3).

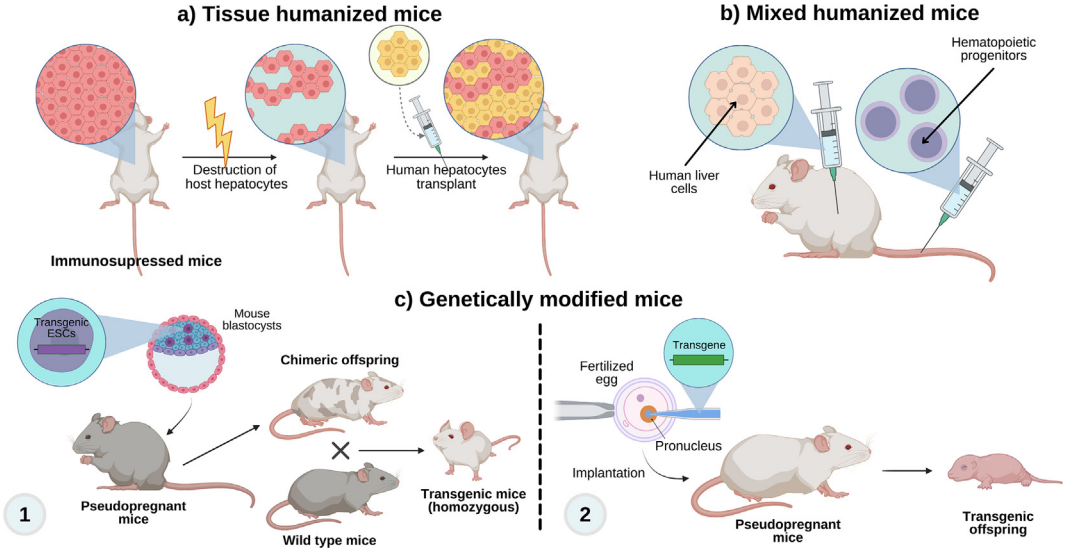

Figure 3 Main approaches for generating humanized mouse models. (a) Tissue humanized mice models can be generated in many different manners. These models have in common the use of immunosuppressed mice in which hepatocytes are eliminated and replaced with human hepatocytes. (b) Mixed humanized models 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[330]. These models can mimic human histologic and biochemical features of iDILI[331] 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[332]. The cells' sources for humanization are typically PHHs and HLCs, although there are also studies using immortalized cells such as HepaRG[333].

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[334].

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*)[335], inducing a severe immunodeficiency syndrome (SCID)[336] or knocking out interleukin 2 receptor common gamma chain (*Il2rg*)[337]. For example, NOG mouse is a highly immunodeficient non-obese diabetes (NOD) mouse strain, which harbors mutations inactivating both the innate ($Il2rg^{-/-}$) 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.[335] and Mercer et al.[336] 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-cyclohexedione (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[338]. 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)[339] and the diphtheria toxin receptor (DTR) under the control of the albumin promoter[340].

Humanized uPA/SCID mice[341] 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[304]. Tateno et al.[342] 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[343] and to characterize *in vivo* idiosyncratic hepatotoxicants such as troglitazone, flutamide[344] and more recently, to determine the metabolic fate of fenclozic acid, a potentially human iDILI toxin[305]. 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.[337] has also been used to study iDILI by thalidomide[306]. Moreover, a TK-NOG model has been used to introduce primary human hepatocytes[339] or HLCs derived from hiPSC[345], 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[346]. Moreover, human liver chimeric TK-NOG mice have also been used to identify drugs that cause animal-specific hepatotoxicity[347] and to model cholestatic liver toxicity induced by bosentan[307]. 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.[348] 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 metabolism 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 adaptative/innate immune system involvement is more difficult to adapt in animal models. Moreover, mouse immune system shows many differences in comparison to humans'[349].

- *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.[350] 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[308]. However, the immature fetal liver cells used by Washburn et al.[350] 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[309]. Similarly, Wilson et al.[310] 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 those double-chimeric FRGN mice thus limiting their utility for studying human T-cell responses. In a recent study, Kim et al.[351] 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.[352] 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.[353] 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[354]. 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[355] and induce inflammation in the skin[356]. However, the drug was tolerated *in vivo* and the skin reactions did not fully mimic that observed in humans.

Song et al.[311] 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[313], 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[202]. 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[357]. 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[358]. 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[359].

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[360]. Genetically humanized PXR-mice haven been useful in unraveling iDILI mechanism for diclofenac[314], or for the cotreatment with RIF and IHN[315]. Similarly, genetic humanization has also been performed for other receptors, such as the aryl hydrocarbon receptor[316] and many individual CYP450 genes, including the families CYP1, CYP2, CYP3 and CYP4; and have continuously been proposed for drug toxicity analyses[361]. 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.[317] 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 on 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[362]. 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[363]. 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[364].

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*)[365].

Statistical models are normally generated from a dataset of chemical structures, which defines the relationship between biological activity and chemical structure[366], together with associated toxicity data, which are used as training for an automatic algorithm[367]. QSARs models can reach accuracies of 53%−84% in correctly predicting iDILI within the retrospective studies conducted, as reviewed[368]. Frequently used algorithms in these type of models are Bayesian models[369], k-nearest neighbor quantitative structure (kNN-QSAR)[370], random forest[371] and artificial neural networks[372].

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[373]. 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[373]. 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)[373] and the new-R ratio[10].

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[374,375]. Recently, Gonzalez-Jimenez et al.[376] 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[377]. 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[378].

DILIsym® has been already used to model iDILI events associated with troglitazone[379], entacapone[380] and acetaminophen[381]. 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 field of *in silico* predictions of iDILI. For example, MITOsym® is an *in silico* tool developed in DILIsym® to understand the mechanism of clinical mitochondrial hepatotoxicity[382]. It has been successfully used to retrospectively determine the role of mitochondria in tolvaptan[383], macrolides[384] and TAK-875 hepatotoxicity[385].

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 market[386]. 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 method[373]. 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 development[373]. 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 LTKB[387], the LiverTox website (https://www.LiverTox.nih.gov), Pharmapendium, ACToR (Aggregated Computational Toxicology Resource)[388], PubChem (https://pubchem.ncbi.nlm.nih.gov) or Drug Induced Liver Injury Rank Dataset (DILIrank). 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 compounds[389]. 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 affected[390]. 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. Of these, the followings may be of particular interest: in Spain, the Spanish iDILI Registry founded in 1994, was the pioneer in the matter[391], 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 (SLatinDILI) network, founded in 2011[392]; and finally the incipient Indian Network for Drug-Induced Liver Injury (IN-iDILI), that has been collecting cases since 2013[393]. 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.[394], *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 potential[395].

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 to 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

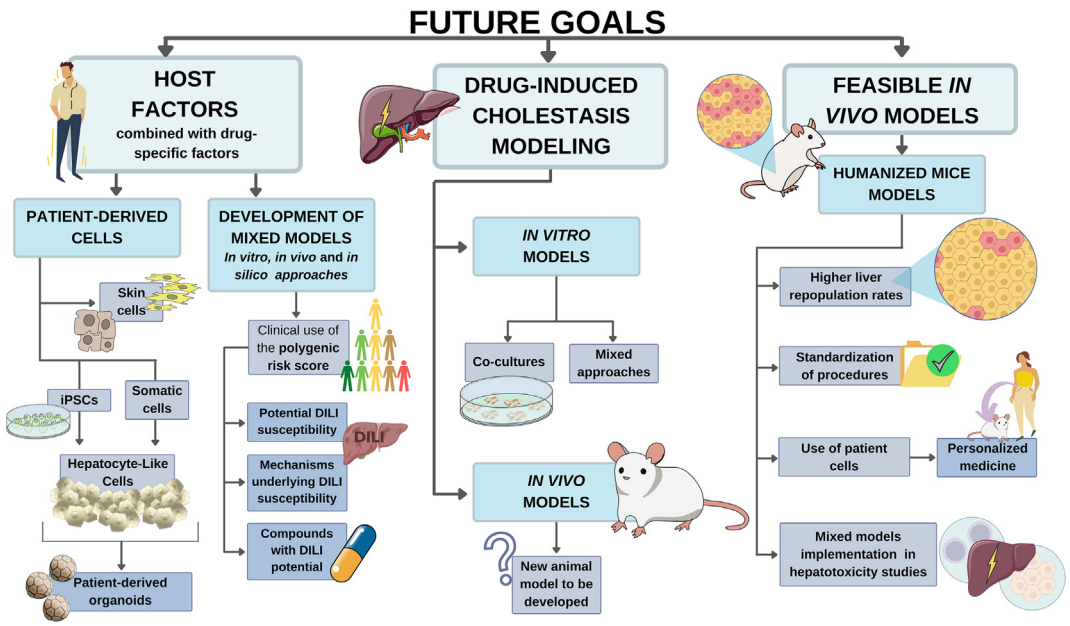

Figure 4 Overview of the future goals in iDILI modeling. Potential future next steps in iDILI preclinical modeling according to the authors' opinion are represented in the figure. The combination of host and drug-specific factors makes the use of patient-derived cells and the development of new cell cultures a pressing need. Drug-induced cholestasis modeling will have to be enhanced, by developing and improving *in vitro* and *in vivo* models. Regarding *in vivo* modeling, humanized mice models appear to be the most feasible ones, and many advantages might be implemented to improve iDILI's performance. iPSCs, induced-pluripotent stem cells. (This figure has been created using Biorender.com.)

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[396], could help to identify the drug responsible for iDILI episodes in cases of polypharmacy[397] and to identify potential individual susceptibility to iDILI due to specific drugs[398].

Some promising approaches include the use and characterization of patients-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[399], or expression of *Hnf4a* plus *FoxA1*, *FoxA2*, or *FoxA3*[400].

Since then, new tools and strategies have been found to obtain murine and human iHeps (hiHeps)[401–405] 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[406].

- *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%[391,407]. 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 allopurinol[409].

A relationship between dermatological manifestations and the severity and prognosis of the liver damage has been suggested[410–412]. 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 identified[412]. 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 liver[411]. 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 DRESS[413].

There are several HLA alleles considered as risk factors for the development of SCARs and iDILI produced by different drugs[414]. For example, the *HLA-A*31:01* allele has been identified as a risk factor for both iDILI and CBZ-induced SCARs[415].

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 skin[416], 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 hepatocytes[417] and primary human keratinocytes[418]. 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 iDILI[418].

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 system[416]. 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 process[419].

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 issues[420]. The composition of BAs 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 activity[421,422]. 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 an indispensable tool 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*$^{-/-}$ *IL-2R*γ*c*$^{-/-}$ 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 age[424], and may cause mitochondrial rejuvenation and an improvement in the cellular energy production capacity upon differentiation[425]. Therefore, exploring the use of patient-derived iHeps 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 consensual 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.[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 contributes 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,

UMA18-FEDERJA-194, PY18-3364, Spain) and grants of Consejería de Salud de Andalucía 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 Andalucía. C.L.G. holds a Juan de la Cierva Incorporación (IJCI-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.

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