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REVIEW

Mechanism-based drug safety testing using innovative *in vitro* liver models: from DILI prediction to idiosyncratic DILI liability assessment

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ABSTRACT

Introduction: Idiosyncratic drug-induced liver injury (iDILI) remains unpredictable. As adverse responses arise in a small fraction of patients, drugs often fail in later drug development stages or post-approval, thereby tremendously increasing costs and putting patients at risk, highlighting the need for accurate early identification of iDILI liabilities.

Covered areas: Using articles from the last 5 years (PubMed), iDILI risk factors are described, *in vitro* liver models and mechanism-based readout strategies are evaluated on their potential to enable iDILI liability assessment.

Expert opinion: Various *in vitro* liver models are established for disease modeling and DILI prediction. Drawbacks for each of these seem inevitable, making the evaluation of their application domain and iDILI liability assessment potential crucial. A tiered approach could be considered, whereby compounds are initially screened and flagged using simple fit-for-purpose models for DILI prediction, followed by multicellular liver models that integrate the current knowledge of iDILI onset in combination with mechanistic readouts. Multiplexing models within an integrated mechanism-based testing strategy could improve the safety assessment accuracy. Defined *in vitro* models should integrate critical hepatocyte intrinsic risk factors as well as adaptive immune system components to refine iDILI liability assessment.

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Advanced *in vitro* liver models; high-content imaging; idiosyncratic drug-induced liver injury; interindividual variability; mechanism-based drug safety testing; omics; readout strategies

1. Introduction

The liver is an important organ to consider in drug safety testing since it is prone to drug-induced liver injury (DILI) because of its blood-filtering and metabolizing function [1]. Even though DILI can be harmless and reversible at early stages, it could progress into irreversible and potentially fatal liver cirrhosis or cancer [2]. Moreover, many drugs fail relatively late in the development pipeline due to safety concerns related to the liver [3]. In the case of idiosyncratic DILI (iDILI), being highly unpredictable [4], this is often only observed when the drug is already on the market as it was not picked up during clinical trials with relatively small cohorts. Recent warnings and discontinuation of novel therapies obeticholic acid (Ocaliva®) and BGE-105 (Azelaprag) during clinical trials add to the list of drugs that failed due to liver safety issues [5]. Therefore, early (i)DILI liability assessment is crucial during drug development in combination with the characterization of patients who are at higher risk for iDILI development. The causes and mechanisms for iDILI are ambiguous, and therefore a generic one-fits-all strategy for the prediction and causality assessment unfortunately remains hardly possible.

iDILI cases are registered in databases such as LiverTox [6] and the modified Roussel Uclaf Causality Assessment Method (RUCAM) [6]. Even though these databases do not always contain fully validated data or lack database correlation [7], they currently provide the most appropriate iDILI causality and prediction framework. Especially, LiverTox has been subjected to criticism, as the data is of insufficient quality due to poor reporting of standard liver injury definition, incomplete or inaccurate description of alternative toxicity causes, or the causality was not validated using the RUCAM method as causality grading was defined based on published cases, a nonscientific approach [7]. RUCAM is currently used in almost 100 thousand iDILI cases worldwide [8] and is therefore believed to be the best and only robustly validated tool for causality assessment and risk prediction [6]. However, this data still lacks insight into the molecular mechanistic understanding of iDILI as it only focuses on the diagnosis. Moreover, the accurate prediction of the risk of the development of iDILI by novel drugs or chemicals is still rather limited without administering to large cohorts of humans and observing the effects. Systems biology and pre-clinical models are of use to predict intrinsic dose-dependent DILI, but the prediction of iDILI remains highly challenging due to the differences of responses between patients. There are currently no *in vitro* liver models that can predict iDILI available yet. Therefore, there is a high need to evaluate innovative models on their (potential) DILI predictive

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Article highlights

- Idiosyncratic drug-induced liver injury (iDILI) mainly occurs due to patient-dependent variations in metabolism and adaptive immune system responses, and therefore, a model should include these factors, ideally in a personalized manner, to enable iDILI liability assessment.
- Advanced multicellular *in vitro* liver models enable improved resemblance to human biology compared to traditional models but still require extensive validation of liver functionality, scalability, and applicability for iDILI liability assessment.
- Novel mechanism-based readout strategies, such as high content imaging or toxicogenomics, are increasingly being used for toxicological studies and could potentially serve as sensitive mechanistic measurements to capture iDILI liability in appropriate *in vitro* models.

capacity and the possibilities to extend this to iDILI liability assessment.

This review provides an overview of the current state of *in vitro* liver models and their readouts to assess iDILI liability. This has been done by evaluating recently published literature from the last 5 years using the PubMed database. First, an overview of the currently known crucial risk factors for developing iDILI is provided, which is used as a reference to evaluate the emerging liver *in vitro* models and their coverage of iDILI development biology. Lastly, novel readout strategies that could be combined with these *in vitro* models such as high content imaging and toxicogenomics approaches will be addressed. This overview of the current models and readout strategies could aid in carefully designing a testing strategy that potentially allows iDILI detection at an early stage of drug development and enables identification of critical points for improvement.

2. Body

2.1. Drug-induced liver injury

The liver is a primary target for toxic chemicals due to its main functions in blood filtration and metabolism. Most drugs and chemicals undergo metabolism and detoxification within the liver by drug-metabolizing enzymes (DMEs). These DMEs are located within the parenchymal cells of the liver, the hepatocytes, which account for approximately 60% of all liver cells [9]. However, metabolism and detoxification of these drugs or chemicals can also contribute to the development of DILI through the generation of reactive metabolites [1]. During DILI development, both the hepatocytes as well as the non-parenchymal cells (NPCs) may be affected, including liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs), immune cells such as Kupffer cells (KCs) and hepatic natural killer cells (hNK) [9]. Depending on affected cell types, DILI can lead to various liver disease phenotypes, such as hepatic necrosis, hepatitis, cholestasis, nonalcoholic fatty liver disease (NAFLD), fibrosis, cirrhosis or liver cancer. DILI can be classified as dose-dependent and predictable, referred to as intrinsic. The mechanisms of iDILI are unclear and believed to be dependent on multiple factors, including environmental, chemical, pharmacological and genetic factors [1,10,11]. Moreover, striking evidence suggests a critical role of the adaptive immune system in the development of iDILI [12,13]. It remains unclear as to what triggers the adaptive immune system, however it is likely the result of a protein modification by a reactive drug or metabolite species, resulting in the generation of a neoantigen. In most cases the injury appears to be mediated by CD8+ T cells, which are thought to be activated by damage-associated molecule patterns (DAMPs) presented on antigen-presenting cells [12,13]. The involvement of T cells has been evidenced by the work of Naisbitt [14,15] and Benesic [16], where they show immune cell responses after exposure to DILI-inducing drugs, specifically from CD8+ T cells. A 2D co-culture of primary hepatocytes and hypersensitive patient-derived reactive T cells showed increased proliferation and cytokine secretion in CD4+ and CD8+ T cells [15]. Even though striking insights can be obtained from these studies, some drawbacks exist regarding the use of primary patient-derived immune cells, including expansion and availability. To overcome these issues, protocols to derive immune cells (such as CD8+ T cells, monocytes and macrophages) from human induced pluripotent stem cells (hiPSCs) are also available, which could be applied in combination with *in vitro* liver models [17,18]. However, their use in iDILI research should still be explored. Further support for the involvement of the immune system is the genetic variation in human leukocyte antigens (HLA), which is shown to be responsible for some iDILI cases (further described in section 2.1.1) [19]. Since the profile of the immune system will differ between patients, it would be key to explore the possibilities of mimicking patient specific profiles of immune cells within a liver model. This will be very challenging and is one of the major factors why there is not a model yet that enables confident iDILI prediction. The extraction of the complete immune profile of a patient might solve this but this will be practically very challenging. Alternatively, immune profiling of patients that have developed iDILI in the past could allow for identifying markers for increased iDILI risk. These markers could then be included in a more generalized *in vitro* model. However, due to the expected large extent of variety it may be very challenging to include in an *in vitro* liver model. In this case, systems biology could be supportive by modeling the variation of immune responses. Even though immune system modeling is mostly performed on simulating the immune response to viruses or vaccines [20], this method may be extended to the immune response toward toxicants. While the role of the immune system in iDILI development is evident, the following section describes other important risk factors that should be considered.

2.2. Factors that impact the risk for iDILI development

Since iDILI development depends on several factors, it is important to enable the modeling of these factors in a test system to assess the iDILI risk. We further describe those factors in this section and will evaluate the *in vitro* models on their ability to implement those in section 2.3.

Environmental factors encompass different types of interactions, namely drug–drug interactions, dietary factors, host–drug interactions, or environmental chemical–drug interactions [21,22]. All these factors can accumulate during lifetime and

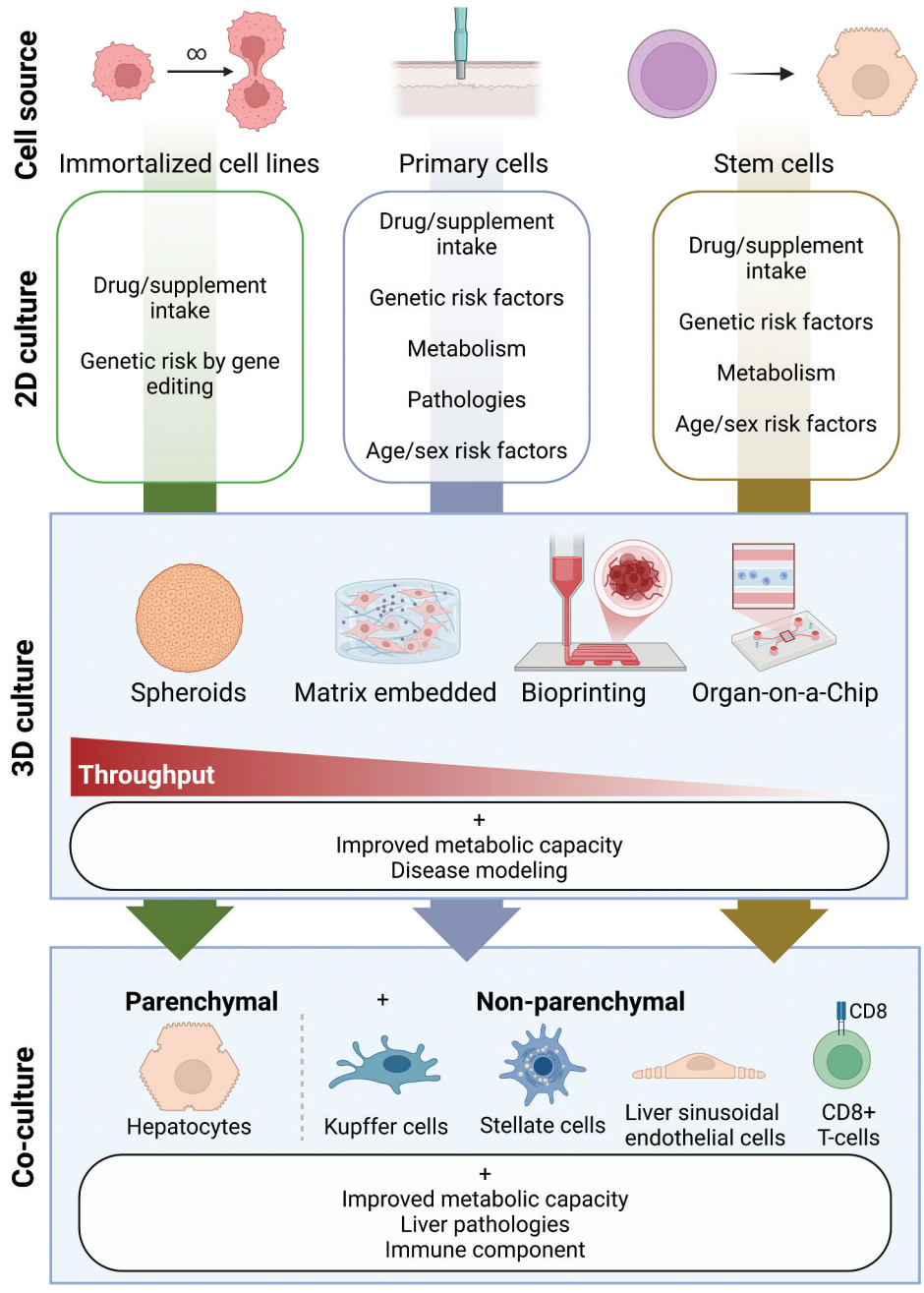

Figure 1. *In vitro* test system setups and their ability to model iDILI risk factors. a liver *in vitro* test system can be developed using several considerations: cell types, cell sources and the format. The liver cell types include hepatocytes, immune cells or Kupffer cells, fibroblasts or stellate cells, and (liver sinusoidal) endothelial cells. The cell source can be primary cells, stem cells or immortalized cell lines. The format can be either in a 2D monolayer of cells or in 3D within a matrix, as spheroids, bioprinted or within an organ-on-a-chip. The choice of cell types depends on a mono- or co-culture setup. Each test system has its limitations and possibilities. Crucial iDILI risk factors should be taken into consideration for the experiment design. Which of these factors are implemented in each test system is suggested in the rounded square blocks. These iDILI risk factors add up to those mentioned at the above test system level. Created in BioRender, Niemeijer, M. (2025) *https://BioRender.com/l99j166*.

are collectively called the exposome [23]. The exposome can also cause genetic variation as a result of direct interaction with the DNA, leading to mutations, and/or (long-lasting) epigenetic modulations. A complete description of the patient's exposome is impossible as there are too many factors to consider, however the implementation of key components of the exposome affecting iDILI risk in a model would be preferable [21]. Many drug–drug and supplement–drug interactions are known and affect metabolism enzymes, such as cytochrome P450 (CYP) enzymes and may impact on hapten formation [21]. Most iDILI cases are due to reactive metabolites, and therefore the drug metabolizing profile of a patient is an essential driver in most iDILI cases. However, supplement–drug interactions are usually not well reported due to insufficient reporting of patients, which calls for better registration of dietary supplement intake for patients [19]. Malnutrition or overnutrition can also increase the risk of iDILI [22,24]. An unhealthy diet often results in underlying liver pathologies, such as inflammation

and fatty liver, making the liver more prone to toxicity [22,25,26]. Diet also affects the gut microbiome, possibly leading to alterations in the metabolic system of the gut and thereby contributing to the distribution and uptake of (reactive) metabolites [22,27].

Genomic modifications in the germline can result in polymorphisms of transporters, metabolic enzymes, or other genes [28]. Alternatively, epigenetics can alter the DNA to affect gene expression or protein functionality. Certain modifications (i.e. GpC methylation and histone acetylation) regulate gene transcription and alter detoxification activity leading to iDILI [28,29]. Genetic polymorphisms that alter the expression of hepatic transporter proteins or their function affect drug clearance, and thereby the plasma drug concentration. The steady-state plasma drug concentration can consequently elevate above the breaking point for adversity development [21]. Although many drugs have been shown to interact with transporters and various transport protein polymorphisms have been identified, studies linking those polymorphisms directly to iDILI are still lacking [21]. Polymorphisms in metabolic genes, e.g. CYP enzymes, can result in lower compound detoxification or elevated toxic metabolite production and hapten generation. These include genetic polymorphisms causing autoinduction where drugs induce enzymes responsible for their metabolism increasing the risk of toxicity (e.g. carbamazepine) [21,30]. Substantial evidence is pointing toward reactive metabolites of drugs as the main contributor to the development of iDILI [31]. Hence, iDILI-causing drugs are often not toxic themselves, resulting in unexpected adverse reactions at lower dosages in patients with metabolism-related polymorphisms compared to the average population. Other genetic polymorphisms might result in deficient cytoprotective mechanisms lowering the breaking point for adversity development and thereby increasing the risk of toxicity during treatment. For instance, polymorphisms leading to decreased expression of antioxidant genes (e.g. nuclear factor erythroid 2-related factor 2 (NRF2) [32–34]) lower the detoxifying protection mechanisms toward reactive metabolites and increasing the changes of hapten formation. Moreover, decreased NRF2 functionality will make cells more susceptible toward DILI [21,35].

Certain genetic polymorphisms can cause iDILI specifically upon treatment with certain drugs or drug combinations, depending on their mode of action. For example, polymorphisms in the immune cell regulating gene protein tyrosine phosphatase non-receptor type 22 (PTPN22) are associated with iDILI to multiple drugs [19], while other polymorphisms are linked to iDILI only for specific drugs (e.g. HLA-B * 57:01 for flucloxacillin [36] and HLA-A *32:01 for vancomycin [37]) [7]. Human leukocyte antigen (HLA) subtypes were identified and showed a significant correlation with iDILI, showcasing the role of the immune system in iDILI [38,39]. These hints evidence even more the importance of the immune system in the development of iDILI.

Age and sex are other factors that can increase the risk of iDILI. Specific age groups can be more susceptible to iDILI. Younger age groups have a higher risk of valproate- and aspirin-related iDILI, whereas older age groups have a higher risk of isoniazid-related iDILI [22,29]. Also, sex has been shown to influence the risk of iDILI. Particularly females are more susceptible to iDILI due to variations in metabolic function and the influence of steroid hormones [22,29].

Altogether, these factors account for inter- and intra-individual variability in iDILI and can influence the variability that exists within the human population or a person, respectively. Therefore, studies can be performed on cells from individual donors to investigate the risk of iDILI development before administering drugs [40]. *In vitro* methods, such as exposing patient blood-derived immune cells (i.e. T-cells) to the drug before administering it to the patient, were developed to mechanistically predict the occurrence of (i)DILI by the group of Naisbitt [14]. Patient-derived cells provide information about the genetic background and possibly respond similarly to toxicants enabling (i)DILI prediction [16]. However, accurate iDILI prediction remains difficult and requires more advanced models that can take into account the factors that influence the probability of iDILI development in a patient. Therefore, a model that enables personalized drug screening and that includes genetic, environmental (such as underlying pathologies) and immune components is crucial. The next section evaluates the emerging *in vitro* models on their potential to predict iDILI.

2.3. Emerging novel in vitro models to screen for (i)DILI

Liver cells for *in vitro* models can originate from human tissue, immortalized cell lines or stem cells, and can be cultured as simple 2D monolayers, or in more complex 3D organizations (Figure 1). 3D models can consist of monocultures or co-cultures to improve the composition and functionality and increase *in vivo* physiological resemblance. Currently, primary human hepatocytes (PHHs) cultured in 2D are considered to be the 'gold standard' of drug safety testing for liver toxicity [41]. Other models that are currently used include 2D HepG2 or HepaRG cultures [42]. However, since iDILI development relies on multiple cell types (i.e. hepatocytes, KCs, and HSCs), monoculture models are probably too simplistic and unable to capture iDILI mechanisms potentially due to the lack of parenchymal cells and immune cells, the latter being essential in most iDILI responses. Therefore, more advanced models, such as multi-cellular liver spheroids, organoids or organ-on-a-chip models should be considered. In addition, a major issue in predicting iDILI is the big influence of interindividual variability. A liver model ideally should therefore be able to mimic these interindividual differences by using patient-derived materials, genetic modification, or mimicking liver disease. Given that DME-mediated hapten formation is an essential driver of iDILI, proper metabolic capacity of liver models that are reminiscent of human in vivo liver is essential. Alternatively, a defined average population model combined with accurate simulation results for the human population including interindividual variability could be used. This review will focus on*in vitro* liver models using primary tissue or cells, hepatic cell lines or hiPSC-derived liver cells.

2.3.1. Primary (multicellular) 3D models

PHHs can be cultured in 3D spheroids, either in suspension or embedded in a matrix, thereby mimicking the *in vivo* 3D

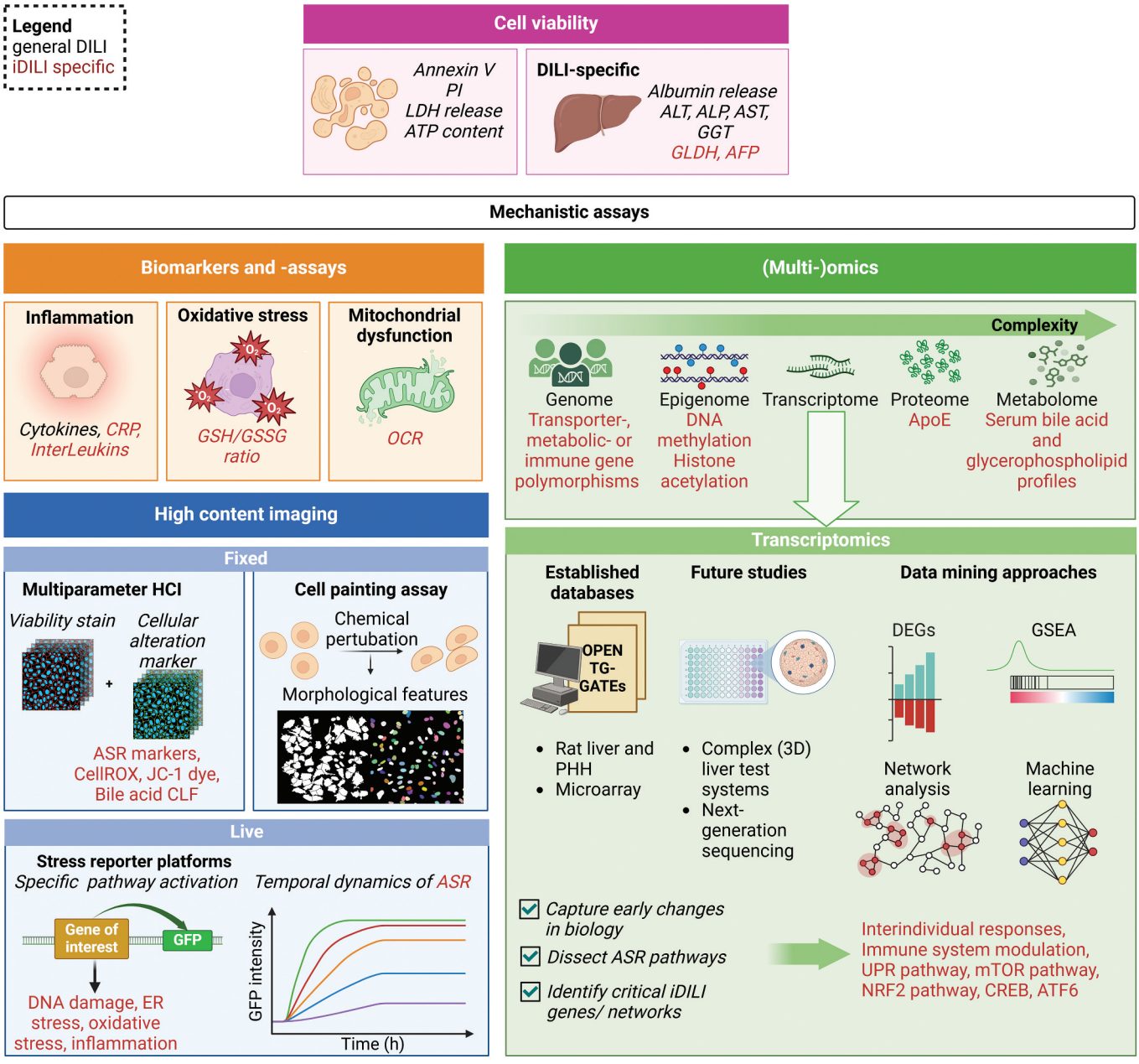

Figure 2. Strategies to study hepatotoxicity *in vitro*. Readout strategies that have been applied to identify drug-induced liver injury (DILI) include measurements for cell viability, DILI-specific or general biomarker detection, high-content imaging (HCI) for cell viability, cell painting or fluorescence reporter platforms, and omics approaches. Examples of general DILI markers are stated in black and idiosyncratic (i) DILI-specific markers are shown in red. Abbreviations: PI: propidium iodide, LDH: lactate dehydrogenase, ATP: adenosine triphosphate, ALT: alanine aminotransferase, ALP: alkaline phosphatase, AST: aspartate aminotransferase, GGT: gamma-glutamyl transferase, GLDH: glutamate dehydrogenase, AFP: alpha-fetoprotein, CRP: C-reactive protein, CCL5: chemokine C-C motif ligand 5, GSH/GSSG: glutathione/glutathione disulfide, OCR: oxygen consumption rate, ASR: adaptive stress response, JC-1 dye: mitochondrial membrane potential probe, CLF: cholyl-lysyl-fluorescein, ApoE: apolipoprotein E, DEG: differential expressed gene, GSEA: gene set enrichment analysis, UPR: unfolded protein response, NRF2: nuclear factor erythroid 2-related factor 2, CREB: cAMP-response element binding protein, ATF6: activating transcription factor 6. Created in BioRender. Niemeijer, M. (2025) *https://BioRender.com/q81o326*.

architecture. Culturing PHHs in 3D spheroids overcomes the drastic decrease in CYP activity upon 2D culture. 3D cultures remain functionally stable and viable for at least 21 days [43], compared to a maximum of 48–72 h in 2D [44]. 2D cultured PHHs can lose (metabolic) functionality already within hours after plating [44]. Even though the primary 3D cultures show potential, a relevant 3D model should incorporate NPCs to capture the multicellular processes related to iDILI. Besides, the addition of NPCs to a 3D culture of PHH further boosts hepatic functions of the system (i.e. increased CYP expression and albumin release) [45]. Commercially available models using spheroid co-cultures are, for example, human liver microtissue (hLiMT) (InSphero) [46,47]. This model contains both primary parenchymal cells and NPCs (i.e. LSECs, KCs, and with [46] or without HSC [47]) enabling the modeling of underlying liver diseases, such as steatosis and pre-fibrotic nonalcoholic steatohepatitis (NASH), toxicity assessment, or a combination of both [46,47]. Toxicity is being assessed in

this model using traditional readouts for cell viability multiplexed with novel readouts such as transcriptomics [48]. Current LiMT are typically based on larger donor pools to prevent donor variability; however, this would be contradictory to the highly personalized susceptibility of iDILI. Having access to primary human hepatocytes and NPCs from iDILI susceptible individuals is an extreme challenge. Additionally, introducing CRISPR-based genetic modification in the primary cells that would mimic susceptibility factors is very difficult. 2D PHH have been used for hapten formation in combination with immune system components, demonstrating the critical relevance of DME competent liver cells for iDILI assessment. Considering the capabilities of PHH-based models to include multiple important factors for iDILI prediction, we expect that this system could allow for iDILI liability assessment.

2.3.2. Liver tissue-derived organoids

Another 3D method to culture patient-derived liver tissue is by deriving intrahepatic cholangiocyte organoids from healthy liver biopsies and differentiating them toward hepatic organoids (HO) [49,50]. Alternatively, HOs were developed from isolated hepatocytes from fetal liver tissue showing increased hepatocyte-like phenotype and long-term stability [51,52]. HOs reflect the genetics of the donor showcasing its ability to include genetic and environmental factors (such as an underlying disease) [50,52]. Its applicability for *in vitro* toxicity testing was determined and compared to 2D PHH by Bouwmeester et al. [49]. In general, it was shown that the HO derived from adult liver tissue showed similar drug metabolism for CYP3A4, but CYP2B6 and CYP2D6 were expressed lower than in PHHs [49]. Moreover, similar toxicity results of four DILI compounds were shown in the HO compared to PHH [49]. Different RNA expression profiles were observed between HOs from different donors, thereby showing the capability to identify variability in responses between patients. Further characterization of the method should be performed to gain more insight into the composition of the HOs, the functional variability between donors and the predictive potential for iDILI. Utilizing HOs also enables gene editing [51]. Hendriks et al. showed that gene editing of single nucleotide polymorphisms (SNPs) in human fetal hepatocyte-derived HO lines can address the risk of steatosis [53]. However, drug screening performed on these HOs only focused on the hunt for drug targets to resolve steatosis. The HOs should first be assessed for overall DILI prediction and then further evaluated on their contribution to iDILI assessment. When using adult human hepatocytes as a source for HOs, limited long-term stability was observed and needs further optimization [52]. Therefore, this strategy might be suitable for (i)DILI prediction if the adult human hepatocyte-derived HOs' stability improves, or the low maturity state of the fetal-derived HOs is increased.

2.3.3. Enhanced immortalized and cancerous liver cell lines

Using patient-derived materials is a very costly approach, and the availability of material is limited. Therefore, other cell sources and approaches are evaluated. Researchers have developed methods to improve liver cell line models (i.e. immortalized hepatocytes HepaRG [54] or liver cancer cell lines HepG2 and Huh7 [55]). HepaRG cells differentiated in 3D showed increased levels of CYP enzymes reaching levels of freshly isolated PHHs stable up to 28 days [56]. HepG2 cells cultured within a Matrigel matrix [57] or amino acid-improved medium [58] improved their differentiation status into more mature hepatocytes with increased metabolism. The use of hepatic cell lines is not per se personalized and does not allow personalized screening, but it enables the genetic modification of genes to the mutant version of a patient. Another strategy for personalized screening is to 'educate' the HepG2 cells by cultivating in patient-derived depleted serum prepared from blood samples to phenotypically mimic the inter-individual variability [59]. Educated HepG2-monocyte-HSC co-cultured spheroids showed interindividual variability and it was stated that one patient with an increased troglitazone-related iDILI risk could be identified [60]. Of interest, only serum from 10 individuals was tested which would make chances very low to detect a very rare event such as iDILI (<0.005% of treated patients develop liver failure) [61]. This model uses cell viability as a classical readout for toxicity alongside the detection of IL-12 levels. It showed that IL-12 plays a protective role since adding additional IL-12 in the IL-12-low-expressed patient resulted in less cell death. Further mechanistic understanding and predictivity as a validation of the model are still lacking. Moreover, cell lines still exhibit nonpreferable cancerous characteristics. Therefore, it is of importance to evaluate the cell lines' accuracy to represent human liver cells compared to primary cells before using them. The fact that some studies have already identified a high-risk iDILI patient hint that using such systems could potentially be used for iDILI predictions, although further validation and definition of its applicability domain (e.g. application to other compounds) is needed. An important first critical assessment for these models would be the formation of the anticipated critical metabolites of iDILI drugs that trigger T cell activation [15].

2.3.4. hiPSC-derived liver organoids

A promising method to reduce and replace the use of primary cells is the use of stem cells such as the hiPSC-derived cell systems. hiPSCs have the advantages of being accessible, genetically modifiable and can be generated from patient-derived cells [62]. hiPSCs can develop directly into self-organizing 3D HOs (hiHO) [63]. The advantage is that all liver cell types could be captured within the organoid, however different protocols result in various cell type compositions [64]. Therefore, thorough analysis of the hiHO composition is required using FACS and single-cell sequencing. Moreover, the hepatocytes within the organoids remain functionally immature, therefore needing further optimization. The organoid's immaturity and differentiation method could result in altered composition and structure as shown in a study of Guan et al. [64].

Current hiHO models are mostly composed of hepatocytes and cholangiocytes. Generally, the strategy to produce these organoids is to first differentiate hiPSCs toward definitive endoderm using Activin A and WNT inhibitors [65]. Then, cells will be differentiated further into hepatoblasts or hepatic

endoderm under the influence of growth factors such as bone morphogenetic protein 4 (BMP4), hepatic growth factor (HGF), and fibroblast growth factor (FGF). Lastly, a maturation phase is implemented using, e.g. HGF, and dexamethasone [65–67]. Since this strategy mostly focuses on hepatocyte differentiation via the endodermal pathway, the NPCs originating from the mesoderm are often lacking. This makes the organoids more simplistic and physiologically less relevant.

In contrast, more recent hiHO models incorporated NPCs within their systems [66,67]. Kim et al. (2022) showed the integration of LSEC and plasma cells in the hiHO [67]. The hiHOs had enhanced metabolic expression compared to other hepatic models (hiPSC-derived hepatocyte-like cells (HLC) and HepG2 in 2D), making them suitable for toxicity testing of metabolism-dependent compounds. It was shown that the hiHOs had similar or even more sensitivity to well-known DILI compounds compared to 2D PHHs [67]. However, the current toxicity readouts used for these models are not relevant for iDILI. More relevant readouts, such as DAMPs-release or transcriptomics, should be done in combination with the hiHO models to validate their application domain in the field of iDILI. Moreover, even though the model does contain some NPC, the composition ratio of hepatocytes and NPCs is not representative of the *in vivo* situation. Harrison et al. (2023) were able to generate larger NPC fractions during organoid differentiation, which primarily consisted of endothelial cells and to a lesser extent HSCs and immune cells [66]. Due to the sheer force of an orbital shaker, the organoids even developed vasculature-like structures [66]. Further analysis by single-cell sequencing showed that the hepatocytes showed early zonation, a process that is not fully understood and only occurs very late in human development [66]. However, this model has only been validated for disease and transplantation purposes and not yet for toxicity screenings [66]. Moreover, although the models include some immune cells, their immune representation remains insufficient compared to the *in vivo* situation. Differentiation protocols should be further optimized to represent the *in vivo* human liver biology. Notably, it is possible to genetically modify hiPSCs for mutations that drive iDILI susceptibility. While hiPSC-derived liver organoids may hold promise for the future, major improvements regarding the maturation of the organoid models are still required to reach DME levels matching the adult human liver and their capacity to predict DILI should still be validated.

2.3.5. hiPSC-derived liver spheroids

Alternatively, methods to enable the control of the exact cell composition within hiPSC-derived liver spheroids have been developed. This approach includes differentiating hiPSCs toward liver-relevant cell types separately to pool them later in a spheroid. A recently developed hiPSC-derived model is based on independently differentiating cell types before aggregation by orbital shaking in U-bottom 96-well plates [68]. hiPSC differentiation is followed by previously established protocols for each cell type [58,69–71]. Cells were combined into spheroids on days 8, 10, 14 and 7 for hepatoblasts [58], HSCs [69], endothelial cells [70] and macrophages [71], respectively [68]. This strategy enables better control of the cell compositions and therefore requires less validation. The model was validated based on functionality and marker expression. Moreover, the possibility of screening for toxicity was established by exposing the spheroids to the DILI compound acetaminophen (APAP). The spheroids showed the expected increase in disease markers and a decrease in viability in a concentration-dependent manner. Further assessment of DILI should further substantiate the relevance of the model. At this stage, the potential of the model to also assess iDILI is not demonstrated yet, but the cell composition of the model contains all relevant liver cell types. Integration of the iPSC-derived adaptive immune system components will be an additional challenge. The use of hiPSCs as a source for 3D multicell type liver models shows potential for the application for iDILI prediction, as it can be personalized, genetically modified, and applied high throughput, but further validation studies still need to be performed to confirm this assumption.

2.3.6. (multi) Organ-on-a-chip models

Organ-on-a-Chip (OoaC) enables the inclusion of more complex morphologies and thereby improves tissue functionality. Microphysiological systems (MPSs) encompasses 'functional features of a specific tissue or organ of human or animal origin by exposing cells to a microenvironment that mimics the physiological aspects important for their function or pathophysiological condition' according to the U.S. Food and Drug Administration (FDA) [72]. For OoaC specifically, this implies a system that usually contains fluid flow, changing pressure and possible multi-organ aspects [72].

Important features that an OoaC model can provide are fluid flow in a vascular network, and stratified morphological features as found in the native liver. Mimicking the sinusoidal vessels has been attempted using different approaches. One approach separates two channels with a porous membrane (Emulate Chip). PHHs are placed in the top, and LSECs, HSCs, and KCs are in the bottom channel [73]. Both channels are perfused with medium to mimic blood flow. This method creates an environment reproducing hepatic lobules with sinusoid-like architecture [74]. The model has already been validated for toxicity testing and is currently even incorporated by the FDA pilot program [75]. Although its applicability for iDILI prediction has not yet been studied, an advantage of this system is its incorporation of primary cells, which makes it suitable for personalized screenings. As already mentioned for other primary cell models, the robustness of the model still requires validation for the use of different sources of the primary cells. Moreover, it would be interesting to evaluate the implementation of hiPSC-derived cells or organoids. The use of primary or hiPSC-derived cells from a patient allows the inclusion of the patient's unique genetic background. Alternatively, hiPSC-derived cell lines can be genetically modified to represent specific patient mutations. If the model demonstrates sufficient robustness, it holds great potential for iDILI prediction. However, it is not yet suitable for high-throughput screening applications needing further optimization.

It has been shown that in the right conditions, spontaneous formation of sinusoidal vessels occurs in 3D models. Researchers made use of the spontaneous vessel formation by co-culturing hepatocytes (i.e. HLCs) and NPCs (primary LSECs and HSCs) within a fibrin matrix and introducing flow

using gravity in an OoaC plate (the OrganoPlate of Mimetas) [76,77]. The hepatocytes structured themselves into plate-like islands and polarized around bile canaliculi [76]. The model has been demonstrated to enable disease modeling for steatosis and fibrosis by adding fatty acids or transforming growth factor beta (TGFβ), respectively [76]. An important feature that is missing in the model is the presence of immune cells. For iDILI prediction, immune cells seem to be crucial. Therefore, the possibility to include a variety of immune cells in the system is required. Moreover, the application for high-throughput toxicity testing is still lacking as the researchers focused on disease modeling. The model's ability to automate processes with robotics enables high-throughput screenings [76]. Validating the model for toxicity screenings using sequencing technologies is also required to evaluate the genetic and transcriptomic profiles. This has not been done yet, and therefore it is unclear whether the model resembles human liver biology accurately, or that it is only mimicking the morphology.

Other OoaC systems have been developed by Freag et al. [78], Kostrzewski et al. [79], and Ehrlich et al. [80]. These systems all contain multiple cell types including hepatocytes, HSCs, LSECs and immune cells, and enable the identification of hepatotoxicity and steatohepatitis. Therefore, these models might be suitable for iDILI prediction as well. Alternatively, multi-organ-on-a-chips are developed to study the interaction of other organs with the liver for the development of DILI. For example, the intestine-liver-on-a-chip (using the TissUse HUMIMIC Chip2) by Yu et al. studied the hepatotoxicity induced by APAP and the influence of intestinal cells [81]. However, a deeper mechanistic understanding and thorough comparison of the models and their response to toxic compounds is still lacking. Therefore, further evaluation of the models is required before implementation within toxicity screening strategies.

Even though many MPS models are still under development, researchers believe in the opportunities within these systems [82]. The MPS models offer a fit-for-serve cellular complexity, phenotype, dynamic mechanisms, and the possibility of multi-organ connection. The architecture and cellular composition are fully modifiable by altering the setup. Moreover, MPS enables different methods to test new drug modalities and therapies, such as cell therapies, (viral vector) gene therapies, and antibody therapies. However, using a complex system comes with limitations as well. Some technical considerations are the costs, robustness, scalability, and use of the system with robotics [82]. Mainly validation, scalability, and personalization would be a major drawback in previously described models. Most models are not fully validated for drug safety testing yet, or only use cytotoxicity as readout for DILI. This does not resemble the *in vivo* situation and does not provide information about the mechanisms driving the toxicity. Therefore, more advanced readout strategies should be used in combination with these models (further described in section 2.4). In addition, increasing the complexity in a model potentially decreases scalability and may complicate the possibility for personalizing the model. Due to the major variabilities between patients and their risk factors for iDILI (as described in section 2.2), it is of great interest to enable the modeling of these factors in a higher throughput and personalized way. Here, in particular, the metabolic profile and the immune cell profile are of interest due to their essential roles in iDILI development.

Whereas advanced 3D models may overcome the 2D culturing limitations, some endpoints could suffice with a simpler model. Therefore, careful consideration of 2D or 3D configuration and the required readouts is essential and may be integrated within an integrated testing strategy for iDILI [83]. Moreover, using *in vitro* systems in regulatory safety studies is still limited due to legal and regulatory limitations. The FDA states that animal testing of compounds is essential to test their pharmacological activity and toxicity (21CFR Part 314) [84]. This is partially due to limited confidence in the predictive value of the *in vitro* models [82]. To improve this confidence, the models could be validated using sophisticated and sensitive readout strategies, such as transcriptomics, after exposure to a set of reference compounds.

2.4. Basic and complex readouts to investigate iDILI in vitro

2.4.1. Cell viability

To allow for appropriate iDILI investigation and prediction *in vitro*, readout strategies should be able to capture the complex and unpredictable nature of the disease. The most straightforward approach to predict intrinsic DILI risk *in vitro* is determining the degree of cytotoxicity following drug exposure. Methods for measuring the cytotoxicity include well-described assays such as the lactate dehydrogenase (LDH), ATP-based luminescence and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assays, or quantification of apoptotic markers, such as Annexin-V and propidium iodide (PI). Cytotoxicity tests have effectively been used to assess the ability of novel *in vitro* models to outperform conventional models in capturing DILI risk [85–88]. Clinical approaches more specific to hepatotoxicity could also be applied to preclinical *in vitro* liver models [89–91]. These monitor liver injury by measuring the release of hepatocellular proteins, such as alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT), but also elevated bilirubin [92,93]. However, elevated levels of these biomarkers are not specific to (i)DILI as other liver injury etiologies could show similar profiles. Therefore, there is a need to identify more specific and sensitive biomarkers to detect (i)DILI. Novel biomarkers for (i)DILI, recently systematically reviewed by Atallah et al., include Alpha-Fetoprotein (AFP), glutamate dehydrogenase (GLDH), miR-122, apolipoprotein E (ApoE), and c-glutamyl dipeptides potentially being more specific and sensitive [92].

Multiple attempts have been made to use conventional cell viability assays to identify idiosyncratic drugs *in vitro* employing different types of immunocompetent co-culture systems [60,91,94–96]. However, models used in most of these studies merely include antigen presenting cells, thereby neglecting the adaptive immune responses which are predominant mediators iDILI. Other drawbacks of cell viability measurements are that cytotoxicity relies on the dose-dependency of the

compound within the selected model and neglects molecular signatures associated with iDILI. Additionally, considering the short lifespan of most *in vitro* models, cytotoxicity assessment in these short-term (24–48 h) studies predominantly emulates acute toxicity responses, whereas the development of iDILI can be a timely process taking weeks or months [41]. Another critical point is that the concentration used to provoke a cytotoxic response *in vitro* hardly translates to human exposure levels [97]. Even though cytotoxicity measurements are useful for flagging important DILI drugs, other analyses are required to allow the prediction and reveal underlying mechanisms of iDILI (Figure 2).

2.4.2. Mechanistic biomarkers and assays

Considering the evident role of immune activation in the onset of iDILI, the analysis of inflammatory markers, such as C-reactive protein (CRP) and various cytokines, is of great relevance for iDILI research [91,98]. For that reason, few studies have evaluated the mechanisms behind iDILI using immunocompetent co-culture models. Roser et al. investigated the effect of the iDILI compound aldesleukin by exposing different immune cells in co-culture with HepaRG cells or PHH. Here, CD8+ T cell co-culture was found to most prominently model aldesleukin-mediated toxicity, as shown by increased pro-inflammatory mediator release and cytotoxicity [98]. Roux et al. studied the role of immune responses in iDILI onset caused by the antidiabetic drug troglitazone. Healthy-donor-blood-educated HepG2-HSC cell line co-culture spheroids were used [60]. Cytotoxicity below Cmax was observed in one out of 10 individuals after troglitazone exposure, indicating iDILI risk. After exposing macrophages derived from the different individuals, a panel of cytokines was found to be differentially induced. From these, only IL-12a transcript was downregulated for the individual who showed iDILI risk, suggesting a role of IL-12 in the occurrence of troglitazone-mediated iDILI [60].

Other injury-specific markers to study iDILI *in vitro* could be related to metabolization by generation of reactive oxygen species (ROS) leading to mitochondrial stress. A previous study therefore investigated the added value of measuring oxygen consumption rate (OCR) next to caspase-3/7 activity in PHH, which are endpoints of mitochondrial function and apoptosis, respectively [99]. Herein, it was found that idiosyncratic troglitazone caused mitochondrial dysfunction, while there was no effect on cell viability. This finding demonstrates that solely cytotoxicity testing is not sufficient to identify an iDILI drug, while additional mechanistic assays capture cellular perturbation, supporting the use of combined assays for *in vitro* iDILI risk determination [99]. However, the determined IC50 values were higher than the therapeutic Cmax of the drugs indicating that this assay was not sensitive enough to capture the iDILI risk at therapeutic relevant concentration levels. Therefore, it is important to consider the translatability of the IC50 derived from specific assays to human doses, which proves a challenging task.

The aforementioned efforts for modeling and analyzing iDILI using various biomarkers and assays *in vitro* validate the added value of mechanistic studies on top of cytotoxicity tests for iDILI research. However, the number of *in vitro* iDILI studies remains limited and most studies have been conducted using cell line- or primary cell-based systems. In contrast, Mun et al. tested the capacity of hiHOs to exhibit toxicological responses to iDILI compounds compared to HLCs in 2D culture [85]. Interestingly, the organoids showed higher sensitivity to idiosyncratic antidiabetic drug troglitazone and structurally similar antibiotic trovafloxacin at clinically relevant concentrations compared to the 2D HLCs based on cytotoxicity experiments [85]. Even though the organoids lack presence of resident immune cells, an inflammatory response could be induced by high-dose APAP treatment as shown by increased ROS generation, reduced ATP content and glutathione/glutathione disulfide (GSH/GSSG) ratio, and increased expression of inflammatory mediators [85]. In this study, however, the capability of iDILI drugs to induce such a response has not been tested yet, therefore no conclusions can be drawn on translatability of this experimental setup for iDILI risk identification. The use of organoids for both DILI and iDILI research, however, is currently still at a very early stage and requires extensive validation of their exact predictive capacity.

As described before, for iDILI it is of importance that an *in vitro* model captures the complex mechanisms of the disease, which can be validated using mechanistic markers and assays. Notably, due to the various possible underlying mechanisms for iDILI, analyzing multiple disease mechanisms in parallel in a high-throughput fashion would be favorable. Using conventional assays and biomarkers, this can be a challenging task. Therefore, novel high-content imaging (HCI) studies using a multiparametric approach could allow for simultaneous analysis of possible iDILI mechanisms.

2.4.3. High-content imaging studies increasingly provide mechanistic insights on (i)DILI

Conventional imaging-based iDILI prediction strategies solely rely on the presence of apoptotic markers, such as Annexin V and PI, as endpoint measurements. However, these approaches neglect early molecular signatures potentially leading to injury and lack mechanistic information on iDILI development. Therefore, HCI studies increasingly focus on detecting molecular and morphological markers associated with specific cellular alterations. Even though the application of these technologies for the investigation of iDILI remains limited, the following section will discuss their current use for (i)DILI research and outline the possibilities for future iDILI studies.

2.4.3.1. Probe-based multiparametric testing using HCI.

HCI allows for simultaneous multiparametric testing of cellular perturbation markers within a selected model. These cellular perturbations (e.g. oxidative stress, mitochondrial dysfunction, DNA damage, endoplasmic reticulum (ER) stress or inflammation) are induced upon occurrence of a molecular initiating event (MIE) by a drug. The design of HCI setups using compatible fluorescent probes for investigation of DILI has been extensively described in previous studies using HepG2 cells [100–102]. These include probes for cellular stress responses such as mitochondrial damage, lipid accumulation and oxidative stress. When combined with appropriate models, analysis

of these probes is a promising approach for studying iDILI mechanisms *in vitro*.

A first study applying a multiparametric HCI experimental design to iDILI research, used HepG2 cells engineered to express differential CYP enzyme levels, thereby mimicking population variability in drug metabolism [103]. The researchers used a set of eight fluorescent probes including BODIPY493/503 for lipid accumulation, CellROX for oxidative stress detection, and MitoSOX Red as mitochondrial superoxide indicator. This study could identify the specific type of injury after drug exposure while also relating these injuries to specific CYP isoforms [103]. However, image data quantification can be rather challenging in 3D models, being a critical point for multiparametric HCI-based experiments. Nevertheless, a more recent study successfully evaluated hepatotoxicity of idiosyncratic serotonin and norepinephrine reuptake inhibitor duloxetine in a 3D HepaRG spheroid model [104]. Here, a set of dyes was used to evaluate various types of toxicity, including Nile Red for lipid accumulation, CellROX, and JC-1 dye for mitochondrial membrane potential (MMP). Initiation of oxidative stress by duloxetine ultimately led to mitochondrial dysfunction and induced hepatic steatosis and cholestasis [104]. Another study showed the development of an HCI assay using hiHOs to analyze the interplay between cholestasis and mitochondrial stress. To measure cholestatic function, a fluorescent bile acid cholyl-lysyl-fluorescein (CLF) was used. The hiHOs were exposed to 238 test compounds, of which known cholestatic drugs tended to reduce CLF intensity. The predictability of the method was validated with sensitivity and specificity ratios of 88.7% and 88.9%, respectively. By multiplexing cholestatic markers with mitochondrial health as measured by MMP, this study found cholestatic stress as dominating factor for liver injury compared to mitochondrial stress [88].

2.4.3.2. Cell painting for phenotypic perturbation analysis. Even though the biochemical probes described above provide mechanistic insight into DILI onset, a common limitation of fluorescence-based imaging studies is that these are limited to measuring a relatively small number of parameters. To enrich the data obtained from HCI experiments, Carpenter et al. developed the Cell Painting assay [105]. The Cell Painting assay employs morphological profiling to assess cellular perturbations. This assay uses a set of six dyes marking different compartments of the cell (e.g. nucleus, cytoplasm, cell membrane), which translate to morphological features (e.g. shape, intensity, texture). Large sets of morphological features, typically thousands, can be quantified and used to investigate subsequent perturbations in an unbiased approach [105]. For iDILI research, such an unbiased endpoint analysis could be of interest, as iDILI can occur via various (unknown) mechanisms depending on the drug and morphological perturbations might therefore be a sensitive indicator for a wide range of injuries. So far, the Cell Painting assay has been shown to be compatible with, among other cell lines, 2D HepG2 cells [106]. To evaluate the utility of Cell Painting for DILI research, it was investigated whether similar morphological perturbations induced by DILI compounds also shared similar modes of action based on transcriptomics analysis. Indeed, it appeared that chemicals inducing similar morphological patterns also hold similar modes of action [107]. The major limitation of this assay, however, remains that true mechanistic insights can currently not be provided based on cell painting data alone.

The previously described studies illustrate the potential of biochemical and morphological multiparametric HCI for investigating iDILI *in vitro*. The drawback of the above-mentioned biochemical markers, however, is that they represent conventional late markers of cellular injury and provide limited insights into molecular adaptive stress response (ASR) pathway activation [108]. These pathways function to restore homeostasis upon drug-induced cellular perturbations. Key ASR pathways are the NRF2-mediated oxidative stress response, unfolded protein response (UPR), p53-mediated DNA damage response, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-mediated inflammatory signaling [109]. The use of ASR markers related to the mode of action of drug-related adversities could further deepen the understanding of (i)DILI and enhance the predictive capacity by increasing the sensitivity.

2.4.3.3. Fluorescent reporter cell lines for DILI liability. Detecting well-defined proteins associated with specific stress response pathways has recently been used as an alternative imaging-based strategy to enhance DILI risk predictivity and provide a mechanistic understanding of DILI onset [57,108–112]. Reporter cell lines are useful for mechanistic toxicology studies, as the expression of known stress response activators is coupled to the expression of a reporter gene, such as enhanced green fluorescent protein (eGFP). This allows for real-time quantification of stress pathway activation upon exposure. In 2017, Wink et al. generated a panel of Bacterial Artificial Chromosome (BAC)-GFP HepG2 reporter cell lines for the evaluation of oxidative stress, endoplasmic reticulum (ER) stress and DNA damage response. Using these reporter HepG2 cell lines, the temporal order of activation of adaptive stress reporters could be defined after exposure to drugs [110]. Effective incorporation of GFP-reporter cells in a 3D system resulted in improved identification of DILI liability compared to 2D monolayer HepG2 [57,113]. Especially, immunoglobulin-binding protein (BiP)-GFP and sulfiredoxin-1 (SRXN1)-GFP reporter cell lines seem most predictive for DILI assessment, allowing for evaluation of UPR and oxidative stress pathway activity, respectively [57]. In a screen of >1500 FDA-approved drugs, Vlasveld et al. employed several HepG2 BAC-GFP reporter cell lines to assess ASR activation. Of these, >150 drugs were identified as critical activators of C/EBP homologous protein (CHOP), a key protein within the UPR upon ER stress. Further transcriptomic analysis and OCR assays found that mitochondrial perturbation occurs in conjunction with CHOP-activation, indicating the utility of the CHOP-GFP cell line to serve as a molecular indicator for mitochondrial perturbation [111].

Even though the previously described studies using the BAC-GFP HepG2 reporter cell lines have provided promising results on DILI risk prediction, there are obvious limitations related to the use of tumor-derived cell line monocultures for iDILI research. Since there is increasing interest in the use of hiPSC-derived models for drug safety predictions, Snijders

et al. applied the reporter technology for the development of a hiPSC-based system. Here, CRISPR/Cas9 technology was utilized to generate a heme oxygenase 1 (HMOX1)-GFP reporter hiPSC line for image-based oxidative stress detection in, among other cell types, HLCs [112].

So far, fluorescent reporter technology has been proven to be suitable for 3D models as well as hiPSC-derived systems [57,112,113]. Moreover, interesting ASR pathway activations for iDILI, such as oxidative stress and mitochondrial injury, can be effectively analyzed [57,109–112]. However, for each specific stress response marker the exact predictivity for iDILI risk should be further explored. For future iDILI research, it could additionally be of interest to expand the hiPSC-reporter platform to cover a broader spectrum of ASR pathways and identify the most promising ASR markers for iDILI specifically.

2.4.4. Transcriptomic analysis as mechanistic endpoint in toxicology studies

Transcriptomic analysis has the potential to serve as a sensitive endpoint measurement for toxicological studies as it captures early changes in the biology of a system in response to exposure. With the onset of transcriptomics technology, large toxicogenomics databases have been established and exploited to predict hepatotoxicity of several compounds as well as provide mechanistic insights into underlying stress responses [114–118].

2.4.4.1. (i)DILI predictivity of toxicogenomics data. Using transcriptomic data of liver models exposed to large compound sets, such as the Open Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System (TG-GATEs) datasets, several studies have demonstrated the ability to distinguish DILI from non-DILI compounds [114–118]. As a result of the massive amount of toxicogenomics data available for drugs and other compounds, there is a growing need for the development of novel biological interpretation approaches regarding big data. Standard analyses of transcriptomics data focus on quantification of differentially expressed genes (DEGs) and enrichment analysis of annotated pathways through gene set enrichment analysis (GSEA). These approaches have been useful but are biased toward well-annotated genes and might fail to capture meaningful mechanisms related to DILI and iDILI specifically [119]. Moreover, transcriptomics data suffer from high data dimensionality and variability issues among target models as well as between species [120]. Therefore, efforts have been made to reduce dimensionality of the data by employing systems biology approaches.

Sutherland et al. aimed to circumvent these issues by applying weighted gene co-expression analysis (WGCNA) to toxicogenomics data to analyze groups of co-expressed genes and gain mechanistic information on toxicogenomic pathways [121]. This approach has later been used to investigate DILI using TG-GATEs PHH data and for safety testing of cosmetic chemicals using targeted templated oligo-sequencing (TempO-Seq) in PHH and HepaRG cells [119,122]. Other methods that could reduce transcriptomic data dimensionality include machine-learning (ML) based approaches. For instance, Kohonen et al. used probabilistic modeling to uncover associations between compound treatments and DEG sets. They generated a predictive toxicogenomics space (PTGS), which represented the minimally sized component gene space that captured dose-dependent cytotoxicity based on probabilistic modeling of transcriptomics and cytotoxicity data [120]. Their prediction model was verified using the PHH and rat liver TG-GATEs data, which showed that the model could predict DILI with 71% sensitivity and 100% specificity. Interestingly, they also showed an activated dose-dependent DILI score for idiosyncratic compounds (nimesulide and benzbromarone) [120]. Due to the increasing efforts made to identify gene networks and toxicity pathways to predict DILI, there is also a growing need for methods to measure the importance of different molecular pathways [123]. Therefore, a recent study described entropy weight method (EMW) combined with ML model XGBoot-SHAP to evaluate the contribution of specific pathways to perturbation under DILI circumstances and rank toxicity pathways' importance based on ALT elevation, respectively [123]. This study established a panel of 11 toxicity pathways (e.g. UPR, mTOR and NRF2 signaling pathways) that should capture DILI and iDILI mechanisms. Additionally, the prediction capacity of their model was validated using 89 drugs, which yielded in a precision of 86% and specificity of 85% at the ratio cutoff that achieved the highest accuracy of 78% [123].

2.4.4.2. Mechanistic insights from toxicogenomics studies. Expression profiling of *in vitro* models after compound exposures provides the opportunity to elucidate molecular mechanisms underlying hepatotoxicity. Upon GSEA of TG-GATEs PHH data, a previous study aimed to identify transcription factors (TFs) whose activation was most associated with high DILI-concern drugs. After identification of NRF2, ETS TF ELK1 (ELK1), cAMP-response element binding protein 1 (CREB1), and E2F TF (E2F) as hits, these TFs were experimentally validated in HepG2 using luciferase reporter vectors [124]. Metabolism in HepG2 was first induced by adding metabolizing enzyme cocktails. A strong polyserial correlation coefficient of CREB between reporter assay and idiosyncratic toxicity risk was found, demonstrating a key role of the TF as a key downstream hub exerting important hepatoprotective effects [124].

Using the same PHH TG-GATEs data, Callegaro et al. employed WGCNA modules to rapidly identify compound-induced stress responses [119]. Specifically, they investigated the transcriptional response of cyclosporine A (CSA), which showed high similarity to tunicamycin response, a prototypical ER inducer. Modules that were most strongly correlated between treatments included well-known ER-stress inducers, showing the lowest p-value for gene ontology – cellular component (GO-CC) term "endoplasmic reticulum." Moreover, these modules were enriched for activating TF 6 (ATF6) target genes [119]. Another recent study described the development of a prediction model constructed using logistic regression with elastic net regularization to form a framework for transcriptomics-based hepatotoxicity prediction [125]. Their analysis showed distinct DILI mechanisms induced by

different compounds and coupled morphological changes in the ER and mitochondrion to DILI risk through integration of cell image data.

Considering that the onset of iDILI is often unexpected due to interindividual variations, it is important to note that by using transcriptomics, differences in sensitivity and activation of stress pathways can be identified. An approach to quantify the sensitivity is through identification of benchmark concentrations (BMCs) of genes or co-regulating pathways through dose–response modeling. The BMC determines the concentration at which a gene or network is significantly differentially expressed reaching a certain level of response, which is often defined as the point of departure (PoD) [126,127]. Using targeted TempO-Seq technology, Niemeijer et al. found large differences in BMCs of specific stress response inducers in a PHH panel derived from 50 different donors. Specifically, the toxicological responses after exposure to tunicamycin and tumor necrosis factor-alpha (TNFα), inducing UPR and NF-κB-related genes, respectively, showed the highest variability [40]. This variation in sensitivity could be particularly significant for further iDILI research, as immune system modulation and UPR have both been linked to iDILI [31].

Most of the studies described above have made use of the large open TG-GATEs toxicogenomics database. Even though the use of this database has led to significant insights into DILI development, there are some drawbacks to this data. Notably, TG-GATEs consist of microarray data, which is generally considered less reliable than RNA-sequencing for gene expression measurement. For DILI research specifically, RNA-sequencing more accurately predicts toxicity as it captures additional DEGs and canonical pathways relevant to liver injury [128]. However, considering the large quantity of compounds needed to be tested for drug safety assessments, and the high cost of performing whole transcriptome RNA sequencing, other avenues should be considered. As a result, advanced targeted RNA sequencing technologies, such as TempO-Seq technology and L1000, have been applied for toxicity testing to increase cost-efficiency [40,116,129,130]. Moreover, other studies exploiting transcriptomics to investigate DILI have primarily used monocultured PHH or cell lines as model, which have their limitations regarding iDILI study. For future transcriptomic iDILI research, it would be of high value to gather transcriptomic data on advanced liver *in vitro* models mimicking idiosyncratic responses.

2.4.4.3. Toxicogenomics in advanced hepatic in vitro models.

The combination of toxicogenomics with one of the innovative DILI models as described above could provide even more promising results with improved *in vitro*-to-*in vivo* translation of the obtained data. Only a few studies, however, have already exploited the use of transcriptomics to study DILI in advanced MPS models [89,131–133]. In response to a clinical study showing unexpected severe DILI in patients receiving combined therapy of inarigivir/tenofovir, Zhang et al. performed an investigation for this specific case using hiHOs. Herein, a proof-of-concept for single-cell transcriptomic prediction combined with biochemical and phenotypic analysis was demonstrated [89]. Genes related to fatty acid, triglyceride, and lipid storage were differentially expressed in hepatocytes based on the single-cell transcriptomic data, which was in concordance with an increase in lipid content as shown by LipidTox staining. Even though previous research efforts showed promising results for the integration of transcriptomics analysis and advanced *in vitro* models for (i)DILI prediction of specific compounds, large-scale toxicogenomics screens in these models are still lacking.

2.4.5. Multi-omics approaches

Besides transcriptomics, proteomics and metabolomics could be of great interest to study biochemical changes induced by iDILI-associated compounds. Until now, proteomics [134] and metabolomics [135,136] profiling have been primarily applied to DILI studies using liquid biopsies, leading to the discovery of several candidate proteins and metabolite markers. Proteomic analysis revealed a set of 92 serum proteins which were significantly differentially expressed between DILI and control groups. Of these, many were related to inflammation and immune responses, as well as hepatotoxicity-specific pathways such as steatosis and cirrhosis. ApoE levels, in particular, had the greatest power to distinguish DILI patients from controls [134]. Cytoplasmic aconitate hydratase, argininosuccinate synthase, carbamoylphosphate synthase, fumarylacetoacetase, fructose-1,6-bisphosphatase were discovered as potent metabolite markers to distinguish DILI patients from healthy controls with high accuracy [136]. Moreover, metabolomic analysis identified serum bile acid and glycerophospholipid profiles that could accurately discriminate between cholestatic and hepatocellular DILI phenotypes [135]. Metabolomic analysis has additionally been shown to not only distinguish specific DILI phenotypes but also capture variable DILI responses in different individuals after treatment with the same drug, which could be of great interest for iDILI research [137]. *In vitro*, metabolomics has yet to be translated to (i)DILI-specific research. However, studies focusing on other types of toxicity show promising results for metabolomics-based toxicity assessment [138,139]. Moreover, novel technology enables simultaneous analysis of transcriptomic and proteomic profiles in single cells, providing interesting opportunities for investigating (i)DILI *in vitro* [140]. For future research, complementing transcriptomics studies with other omics analyses *in vitro* would be a valuable approach to explore. This may identify discrepancies between translation of gene expression into proteins and assess the functionality of expressed proteins and metabolic pathways among the *in vitro* models.

3. Conclusion

The current field of DILI testing is rapidly evolving, and increasingly complex *in vitro* models and endpoint measurements are being developed and validated. Regarding the development of models, there is an increasing focus on mimicking human liver physiology as closely as possible while maintaining robustness and suitability for high-throughput screens. For iDILI, it is of great importance that the developed models exhibit metabolic capacity, considering the role of reactive metabolites, contain an immune component (in particular T cells) and enable the capturing of inter-

individual variability within the model or characterized and combined with simulations to extrapolate the human population. Until now, most of these complex *in vitro* models have merely been validated using only relatively simple readout methods or have not been applied for toxicity studies at all. Novel readout strategies, such as HCI or transcriptomics, seem to be promising for accurate prediction of iDILI and can additionally provide useful mechanistic information on drug toxicity. Future studies should focus on the integration of these innovative HCI and omics approaches within models that enable personalized risk assessment to increase the understanding of iDILI occurrence and may even enable the prediction of iDILI.

4. Expert opinion

This review described potential novel *in vitro* liver models and the readouts that might enable the prediction of iDILI. The advances in the current field of *in vitro* liver model development and toxicology are already impacting toxicity screening approaches. Indeed, *in vitro* drug screening approaches are increasingly used to predict toxicity. However, advanced *in vitro* liver models still need further characterization and validation to allow for integration within a safety testing strategy to improve the prediction of liver toxicity by new drugs or chemicals and gradually move away from animal testing. The FDA made the first step by accepting the Emulate liver-on-a-chip as a model to predict toxicity. Even though this implementation is still in the first phase, it shows willingness of regulators to take strides in replacing animal experimentation for *in vitro* testing. After many studies that establish the value of current *in vitro* models in terms of disease and toxicity testing, we can no longer undermine the potency of using *in vitro* models during drug development and chemical screening intending to replace animal studies.

However, each *in vitro* model has its advantages and limitations, as described before in this review, therefore it is essential to identify its applicability domain. The most important limitations are the (metabolic) functionality and maturity of cells, lack of an immune profile component, costs of cells but also materials and labor intensity. Capturing the personalized metabolic and immune cell variety into a model is essential to assess the iDILI liability due to its crucial roles in iDILI development. However, the diversity of HLA haplotypes and T cell receptors that underlie various idiosyncratic drug responses poses a major hurdle for this approach. First, efforts should be made to study immune profiles of patients who suffer idiosyncratic responses in these *in vitro* models. Hopefully, generic signatures might be identified from these models and aid in future predictions of idiosyncrasy. Nevertheless, the limited availability of the patient material required for these studies remains a serious challenge.

Given the diverse potential applications of each model, it is important to adopt systematic approaches to model selection. Careful consideration should be made on which systems to include in the study design, and the model that has no more complexity than required should be chosen. Also, a tiered integrated approach to testing and assessment (IATA) for iDILI that combines multiple models could be a possibility. Multiplexing readouts is already common practice, and novel readouts should be combined with traditional readouts to gain more and earlier mechanistic insights. Until now, the lack of systematic validation of several advanced *in vitro* models in combination with these mechanism-based readouts prevents them from being used in regulatory safety assessments. Implementing omics systematically will improve the understanding of the models and identify gaps in biology necessary to capture iDILI mechanisms as it shows the complete transcriptional profile of the system and benchmark to human liver in healthy and diseased state.

Even though *in vitro* liver models are rapidly being developed, the importance of validating appropriate readout strategies for the assessment of iDILI liabilities should not be underestimated. Considering the limitations related to standard cytotoxicity studies as mentioned earlier in this review, the use of early molecular signature detection through HCI and omics approaches for drug safety testing would be promising avenues to further explore. Large quantities of data are already being collected, and several ways of data interpretations are being applied. However, a consensus on how the data obtained from *in vitro* models translates to human biology is still lacking. It remains unclear which transcriptomic perturbed profiles are strong indicators for the development of adversity in humans. Questions that still arise are, for example, 'Which perturbed pathways or networks are most predictive for the development of adversity?,' and 'Which level of perturbation results in adversity, and what level and duration of ASR activation results in adaptation or allow for regeneration?.' Defining the crucial pathways and ASR levels that are contributing and are predictive for (i)DILI is crucial for improved interpretation of transcriptomics data.

There are various characteristics that would define the ideal *in vitro* model for (i)DILI investigation. First, the system should be functionally mature, stable in culture for periods that allow repeated-dose experiments, and amendable to high-throughput and personalized setups. Moreover, to capture the full biology of iDILI development, all relevant liver cell types should be included (especially the immune component) which should be either donor-dependent or genetically modifiable to enable the study of interindividual variability. These characteristics within a single model might enable the full prediction of iDILI. Having these characteristics within a single model would remove the need to multiplex models, thereby simplifying the analysis. Further advancements of such a model include the incorporation of disease modeling approaches to mimic underlying pathologies, such as inflammation and fatty liver. Realistically, having a single model fit-for-all in the context of iDILI prediction will be a big challenge to accomplish, since advanced models still exhibit their limitations. Performing large high-throughput screenings on an advanced system will most certainly be a costly task. Therefore, a tiered approach might be more suitable in which simple models are used for initial screening purposes followed by safety evaluations using advanced liver models at smaller scales.

In our opinion but also seen in recent studies, hiPSC-derived culture systems provide tremendous possibilities using defined differentiation strategies that result in functional

and mature liver tissue. hiPSCs are easily available and genetically modifiable, which enables high-throughput screenings with novel readouts, such as transcriptomics and reporter panel screening. However, a lot of advancements are still required to achieve a fully mature hiPSC-derived liver model. Novel readout strategies such as single-cell sequencing and spatial omics approaches enable a full understanding of the functionality and cellular composition of the model. It is often seen that hiHOs have a low maturity state or lack the NPCs of the liver. The presence of NPCs may improve the maturation of the system by increasing the fidelity of the liver environment. Moreover, several events during iDILI occur in the NPCs, such as KC activation, LSEC capillarization and HSC activation. Therefore, protocols should focus on optimizing the inclusion of NPCs within the current models. For inclusion of the adaptive immune component often critical for iDILI development, these liver models could be combined with CD8+ T cells, either derived from patients or being hiPSC-based, which may allow for personalized iDILI screening approaches.

Currently, many research groups are focusing on developing mature hiPSC-derived liver models. This field is evolving quickly, and we believe that developing a validated IATA for accurate *in vitro* DILI detection will set the ground for applications for recognition of iDILI liabilities upon inclusion of inter-individual variability and critical immune components. Together, this will enhance the potential of accurate (i)DILI predictions at an early stage during drug development and contribute to the replacement of animal experiments.

Abbreviations

AFP	Alpha-fetoprotein
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APAP	Acetaminophen
ApoE	Apolipoprotein E
ASR	Adaptive stress response
AST	Aspartate aminotransferase
ATF6	Activating transcription factor 6
BAC	Bacterial artificial chromosome
BiP	Immunoglobulin-binding protein
BMCs	Benchmark concentrations
BMP4	Bone morphogenetic protein 4
CHOP	C/EBP homologous protein
CLF	Cholyl-lysyl-fluorescein
CREB1	CAMP-response element binding protein 1
CRP	C-reactive protein
CSA	Cyclosporine A
CYP	Cytochromes P450
DAMP	Damage-associated molecular patterns
DEG	Differentially expressed gene
DILI	Drug induced liver injury
DME	drug-metabolizing enzyme
E2F	E2F transcription factor
eGFP	Enhanced green fluorescent protein
ELK1	ETS transcription factor ELK1
EMW	Entropy weight method
ER	Endoplasmic reticulum
FDA	The U.S. Food and Drug Administration
FGF	Fibroblast growth factor
GGT	Gamma-glutamyl transferase
GLDH	Glutamate dehydrogenase
GO-CC	Gene ontology – cellular component
GSEA	Gene set enrichment analysis
GSH/GSSG	Glutathione/glutathione disulfide
HCI	High-content imaging
HGF	Hepatocyte growth factor
hiHO	HiPSC-derived hepatic organoids
HiPSC	Human induced pluripotent stem cell
HLA	Human leukocyte antigen
HLC	HiPSC derived hepatic like cells
hLiMT	Human liver microtissue
HMOX1	Heme oxygenase 1
hNK	Hepatic natural killer cell
HO	Hepatic organoids
HSC	Hepatic stellate cell
IATA	Integrated approaches to testing and assessment
iDILI	Idiosyncratic drug induced liver injury
KC	Kupffer cell
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cell
MDR-1	Multi-drug resistance-1
MIE	Molecular initiating event
ML	Machine learning
MMP	Mitochondrial membrane potential
MPS	Microphysiological systems
MTT	ATP-based luminescence and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NPC	Non-parenchymal cell
NRF2	Nuclear factor erythroid 2-related factor 2
OCR	Oxygen consumption rate
OoaC	Organ-on-a-chip
PHH	Primary human hepatocytes
PI	Propidium iodide
PoD	Point of departure
PTGS	Predictive toxicogenomics space
PTPN22	Protein tyrosine phosphatase non-receptor type 22
RECAM	Revised electronic causality assessment method
ROS	Reactive oxygen species
RUCAM	Rousell Uclaf causality assessment method
SNP	Single nucleotide polymorphisms
Srxn1	Sulfiredoxin-1
TempO-Seq	Templated oligo-sequencing
TF	Transcription factor
TGFβ	Transforming growth factor beta
TG-GATEs	Open toxicogenomics project-genomics assisted toxicity evaluation system
TNFα	Tumor necrosis factor-alpha
UPR	Unfolded protein response
WCGNA	Weighted gene co-expression analysis

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