Biotechnology Journal
www.biotechnology-journal.com

# 3D Primary Hepatocyte Culture Systems for Analyses of Liver Diseases, Drug Metabolism, and Toxicity: Emerging Culture Paradigms and Applications

*Volker M. Lauschke, Reza Z. Shafagh, Delilah F. G. Hendriks, and Magnus Ingelman-Sundberg**

**Recent research has shown that the maintenance of relevant liver functions ex vivo requires models in which the cells exhibit an in vivo-like phenotype, often achieved by reconstitution of appropriate cellular interactions. Multiple different models have been presented that differ in the cells utilized, media, and culture conditions. Furthermore, several technologically different approaches have been presented including bioreactors, chips, and plate-based systems in fluidic or static media constituting of chemically diverse materials. Using such models, the ability to predict drug metabolism, drug toxicity, and liver functionality have increased tremendously as compared to conventional in vitro models in which cells are cultured as 2D monolayers. Here, the authors highlight important considerations for microphysiological systems for primary hepatocyte culture, review current culture paradigms, and discuss their opportunities for studies of drug metabolism, hepatotoxicity, liver biology, and disease.**

## 1. Introduction

Drug discovery and development relies on model systems that can faithfully predict human drug response and toxicity before entering clinical stages. However, pronounced species differences, particularly in hepatic enzyme expression and specificity, drastically reduce their predictive accuracy and constitute a major obstacle for the development of new medicines.[1] To overcome these problems, isolated primary human hepatocytes (PHH) cultured in 2D configuration have been used for studies of liver biology and function, as well as drug metabolism and toxicity. The phenotypes of such systems are however drastically different from the liver in vivo. Specifically, seeding of hepatocytes on stiff, collagen-coated plastic culture plates entails a rapid loss of hepatic functions, mediated by a burst of different microRNAs that inhibit hepatic gene expression already 30 min after exposure to the substratum.[2,3] Accordingly, in the last decade, it has become evident that 2D cultures of hepatocytes have important limitations and do not faithfully inform about liver biology and drug response in vivo.

## 2. 3D Culture Paradigms for PHH

In recent years, an arsenal of different 3D model systems for studies of liver function has been developed, many of which are in a commercial frame (**Table 1**). Their applications are in the areas of i) prediction of acute and chronic drug toxicity, ii) pharmacokinetic analyses, iii) drug–drug interactions (DDIs), iv) analyses of metabolite formation, v) studies of liver function and regulation, and vi) modeling of different liver diseases (**Figure 1**). Their properties and advantages differ and here we provide an update of the most common systems employed.

### 2.1. Liver Spheroid Cultures

PHH can be cultured as 3D aggregates, termed spheroids, with diameters between 200 µm and 300 µm. Spheroid culture supports the maintenance of mature hepatic phenotypes, resulting in long-term stable hepatic functionality. Multiple methods for the generation of spheroids have been presented, including stirring

Prof. V. M. Lauschke, Dr. R. Z. Shafagh, Dr. D. F. G. Hendriks, Prof. M. Ingelman-Sundberg
Department of Physiology and Pharmacology
Section of Pharmacogenetics, Biomedicum 5B
Karolinska Institutet
SE-171 77, Stockholm, Sweden
E-mail: magnus.ingelman-sundberg@ki.se

Dr. R. Z. Shafagh
Department of Micro and Nanosystems
School of Electrical Engineering
Royal Institute of Technology
SE-100 44, Stockholm, Sweden

Dr. D. F. G. Hendriks
Present address: Hubrecht Institute
Royal Netherlands Academy of Arts and Sciences (KNAW) and University Medical Centre (UMC) Utrecht
3584 CT, Utrecht, The Netherlands



**DOI: 10.1002/biot.201800347**

bioreactors, aggregation in hanging drops, or culture on ultralow attachment (ULA) surfaces with the latter seemingly becoming the predominant spheroid-formation technique in recent years.

Spheroid formation in stirring bioreactors allows production of PHH spheroids at large scales. Spheroids hereby generated form functional bile canaliculi and relatively stable rates of albumin secretion and urea synthesis over the course of at least two weeks in culture.[4] In addition, cells in this study retained their RNA expression levels of various phase I (CYP1A2, CYP2C9, and CYP3A4) and phase II enzymes (GSTA1 and UGT2B7). Coculturing PHH in stirred bioreactors with an outer layer of bone marrow mesenchymal stem cells have been shown to further increase the expression of these cytochrome P450s (CYPs), whereas functional parameters (urea and albumin) were not affected.[5] While these results are promising regarding the molecular phenotypes and activities of the cultured cells, bioreactors require large numbers of cells and form spheroids of heterogeneous sizes, which complicates analyses and is incompatible with high-throughput testing of different conditions.

Formation of spheroids of defined sizes is possible in hanging-drop cultures without the use of specialized equipment by pipetting small drops of culture media with defined cell concentrations onto the bottom of a normal cell culture dish lid. Subsequently, the lid is inverted, placed onto the liquid-filled bottom of the plate, and cultured in a standard cell culture incubator.[6] Once cells have aggregated by gravity, the spheroids can be transferred into a culture plate for long-term maintenance. Furthermore, commercial plates and media are available for PHH hanging-drop culture, in which spheroids remain viable and functional for five weeks in culture.[7] However, protein levels of multiple drug-metabolizing enzymes and transporters, including CYP2C8, CYP2C9, CYP2D6, OCT1, and OATP1B1, decrease fivefold to tenfold compared to isolated cells[8] and the use of nondisclosed media components in this system complicates the interpretation of results.

In addition, spheroids can be formed in plates with surface chemistry preventing cell attachment, often referred to as ULA plates. PHH formed in ULA plates, using chemically defined and fully disclosed media compositions, maintained viability and hepatic functions, such as albumin secretion and CYP enzyme activity, for at least five weeks in the culture (**Figure 2**).[9]

**Volker M. Lauschke** obtained his Ph.D. from the EMBL and the University of Heidelberg. In 2014, V.M.L. moved to Karolinska Institutet as a MarieCurie fellow to develop physiological hepatic model systems. Since 2017 he is the group leader and since 2018 associate professor of Personalized Medicine and Drug Development at K.I. His research group uses 3D cell culture systems of primary human cells, microfluidics, and integrative bioinformatics approaches to develop better model systems for prediction of drug toxicity and to identify novel therapeutic strategies, for example, type 2 diabetes. Since 2018 V.M.L. heads the Karolinska Micro- and Nanoengineering facility.

**Magnus Ingelman-Sundberg**, Ph.D., BSc.Med., is senior professor of Molecular Toxicology and research group leader in Pharmacogenetics at the Department of Physiology and Pharmacology, Karolinska Institutet, Biomedicum since 2006.

Furthermore, comprehensive proteomic and metabolomic profiling revealed that PHH retained their molecular signatures and metabolic configuration in spheroid cultures, whereas cells from the same donors cultured as conventional 2D monolayers rapidly deteriorated.[9,10] PHH in this culture paradigm outperformed other emerging cell models, such as 2D cultures of HepaRG cells and induced pluripotent stem cell (PSC)-derived hepatocyte-like cells (HLCs) concerning transcriptomic profiles and sensitivity to a range of hepatotoxic model compounds.[3] Moreover, cross-sectorial multicenter studies using

**Table 1.** Current overview of advanced hepatic in vitro systems utilizing PHH.

| System | Properties | Established applications | Examples of commercial suppliers |
|---|---|---|---|
| Spheroid systems | Static monoculture system; allows coculture with NPCs; phenotypically stable for >5 weeks; HTS-compatible | Hepatotoxicity, disease models for NASH, fibrosis and cholestasis, and toxicogenomics | InSphero, HepaPredict, and Cyprotex |
| MPCC | Static coculture of hepatic cells with murine fibroblasts; hepatocyte function stable for weeks; HTS-compatible | Hepatotoxicity, pharmacokinetic studies, and toxicogenomics | BioIVT HepatoPac |
| Liver-on-a-chip | Microfluidic devices with different layouts and designs; setup allows modeling of hepatic zonation; stable for weeks | Hepatotoxicity, pharmacokinetic studies, NASH, and viral hepatitis models | Mimetas, CN Bio Innovations, and HemoShear |
| Bioprinting | Scaffold-free assembly of PHH and NPCs | Proof-of-concept hepatotoxicity and fibrosis disease models | Organovo |
| Microfluidic multiorgan devices | Microfluidic, organoid cultures, and transwell system possible | As of yet, only proof-of-concept studies | Draper, CN Bio Innovations, and TissUse |

HTS, high-throughput screening.

 

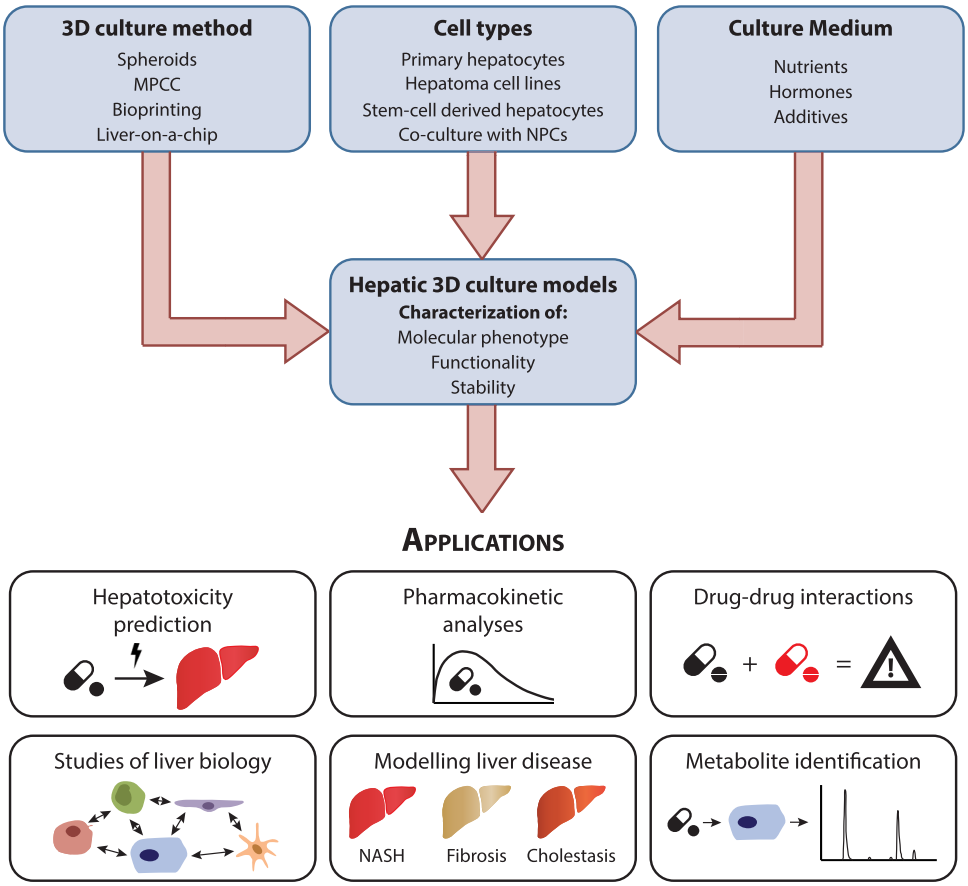


**Figure 1.** The diversity and versatility of hepatic 3D culture models. In recent years, various hepatic 3D models have been presented that differ in their culture methods, as well as cell types and materials used. Furthermore, there are stark differences regarding their characterization and benchmarking status and their utility for downstream applications.

standardized culture protocols found that PHH in the spheroid culture were metabolically more active and exhibited increased sensitivity to hepatotoxins when compared to cells from the same donors cultured as a 2D sandwich culture.[11]

Importantly, the spheroid culture paradigm supports the coculture of hepatocytes with other nonparenchymal liver cells, such as Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), and hepatic stellate cells (HSCs). Coculture of hepatocytes with nonparenchymal cells (NPCs) in the spheroids has been shown to be stable for many weeks[9] and has been suggested to further support hepatic functionality of PHH in spheroid cultures.[12] Hepatic cocultures open up possibilities to study aspects of liver biology that require the interplay between the different hepatic cell types. Combined, the presented studies demonstrate that the spheroid method constitutes a versatile experimental paradigm that supports the long-term culture of PHH and other hepatic cell types with physiologically relevant molecular phenotypes.

### 2.2. Micropatterned Coculture (MPCCs)

In MPCCs, PHH are seeded on micropatterned islands of extracellular matrix and cocultured with surrounding stromal mouse 3T3-J2 fibroblasts, resulting in improved maintenance of hepatic phenotypes and functionality.[13] The fibroblasts support hepatic functionality by supplying PHH with high levels of growth factors, cytokines, and adhesion molecules.[14,15] In this configuration, MPCCs permit the culture of PHH for at least three weeks with stable levels of albumin secretion, urea synthesis, and metabolic functions.[13]

Notably, in recent years, MPCC was demonstrated to support coculture with KCs, allowing to mimic hepatic responses to inflammatory cues.[16] Moreover, PHH in MPCC can be cocultivated with LSECs.[17] However, these authors found that LSECs cannot replace murine fibroblasts to support PHH functionality in this culture configuration. Combined, these studies paved the way towards establishing MPCC as a model, in which the reciprocal interactions between PHH and NPC types can be recapitulated in physiology and disease.

### 2.3. Liver-on-a-Chip Platforms

In the intact liver, hepatocytes are exposed to various biophysical factors, including hemodynamics and shear stress. In an attempt to mimic these conditions, devices have been developed that permit perfusion of 2D hepatocyte cultures and

 

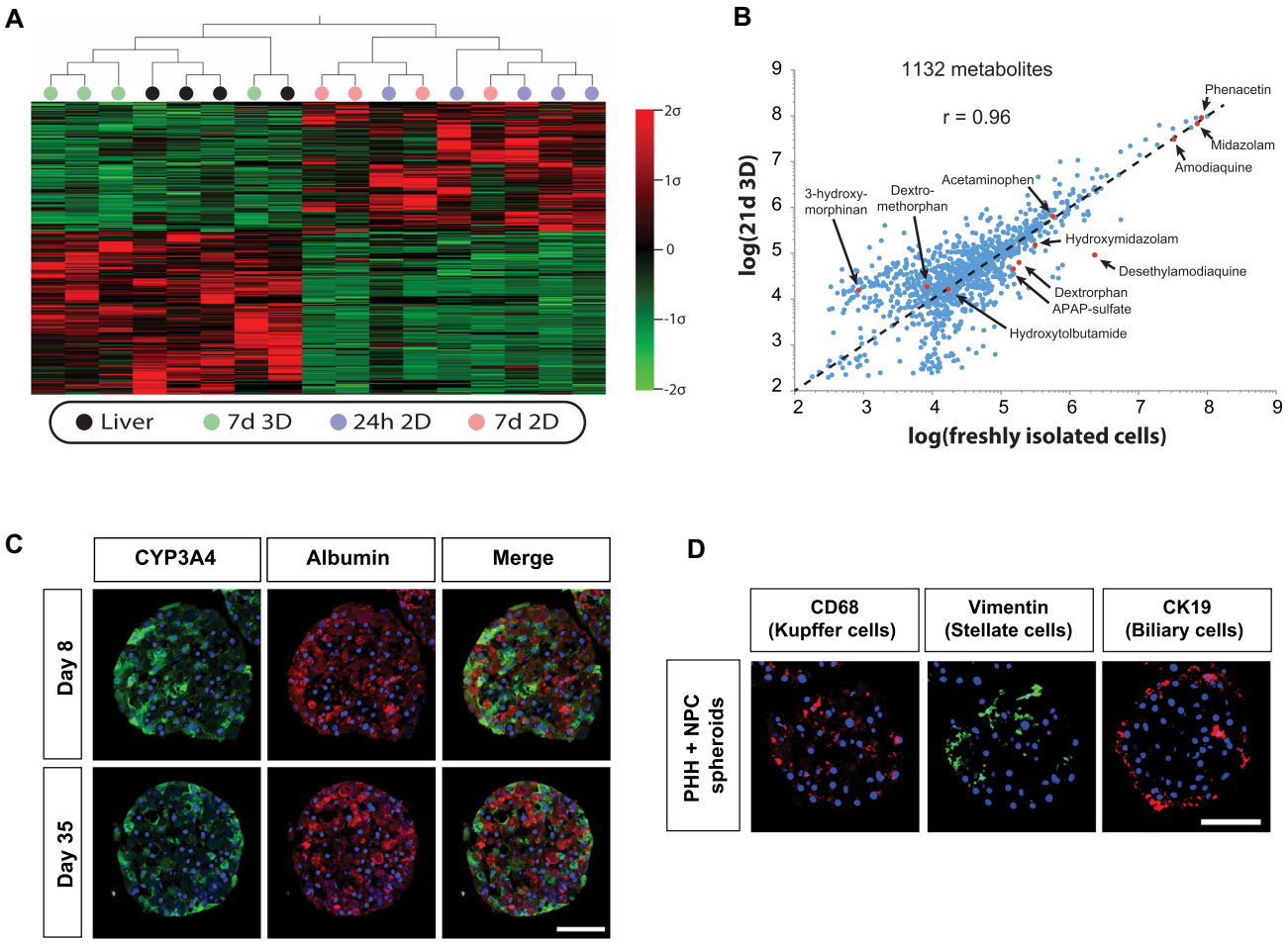


**Figure 2.** Phenotypic characterization of 3D hepatic spheroids in chemically defined media. A) Whole-proteome analyses reveal that the proteomic phenotype of 3D spheroids closely resemble their in vivo counterpart, whereas the proteomes of 2D-cultured hepatocytes isolated from the same liver cells are distinctly different. B) The metabolomic signature analyzed by a combination of targeted and untargeted mass spectrometry of PHH in 3D culture remains stable over 21 days in the culture ($r = 0.96$). The scatterplot shows extracellular metabolite abundances averaged across six biological replicates. Red dots indicate probe substrate metabolites for CYP enzymes, unambiguously identified with internal standards. C) Immunohistochemistry for perivenous (CYP3A4) and periportal (albumin) markers of spheroids after 8 days and 35 days reveals maintenance of their zonal identity. D) Primary hepatocytes can be stably cocultured with human NPCs, such as KCs, stellate cells, and biliary cells. Figure A, C, and D reproduced with permission.[9] Copyright 2016, Nature Publishing Group and Figure B reproduced with permission.[10] Copyright 2017, Federation of American Societies for Experimental Biology.

such experimental setups have been reported to improve hepatic functions, such as urea and albumin secretion.[18] Furthermore, perfusion allows the establishment of oxygen gradients and hepatic zonation, resulting in graded CYP expression and metabolism, as well as the recapitulation of zone-specific patterns of drug-induced liver injury (DILI).[19–21]

Microfluidic chips provide appealing models to incorporate hemodynamics, and various models have been developed in both academic and commercial frames. Specifically, these technologies have been successfully used to culture microphysiological biomimetics of liver sinusoids (**Figure 3**). Taylor and colleagues developed a microfluidic chip, in which PHH and NPCs are cocultured at physiological ratios in a layered 3D organization, consisting of a vascular channel separated from the hepatic chamber by a porous membrane that resembles the liver acinus.[22,23] The system can mimic hepatic oxygen gradient-drive zonation and allows to study leukocytic infiltration of the hepatic chamber. A similar microphysiological coculture chip with fenestrated architecture was presented by Du et al.[24]

An array of perfused bioreactors, each containing up to 600 000 cells, originally developed by Griffith and colleagues[25,26] is provided commercially by CN Bio Innovations. Cells in this model remain viable and functional for at least seven days, as judged by calcein acetoxymethyl (AM) and albumin staining, respectively. The model has been used for a variety of applications, including pharmacokinetic studies,[27,28] evaluation of hepatotoxicity,[29] modeling of nonalcoholic fatty liver disease (NAFLD),[30] and hepatitis B viral infections.[31] In addition, a multitude of small companies and start-ups provide liver-on-a-chip platforms for which at present very limited peer-reviewed benchmarking data are available.

Liver-on-a-chip systems (as well as spheroid and MPCC platforms) can be utilized as stand-alone models or can be integrated with other organs-on-chips in microfluidic perfusion systems. In this context, a plethora of multiorgan systems have

 

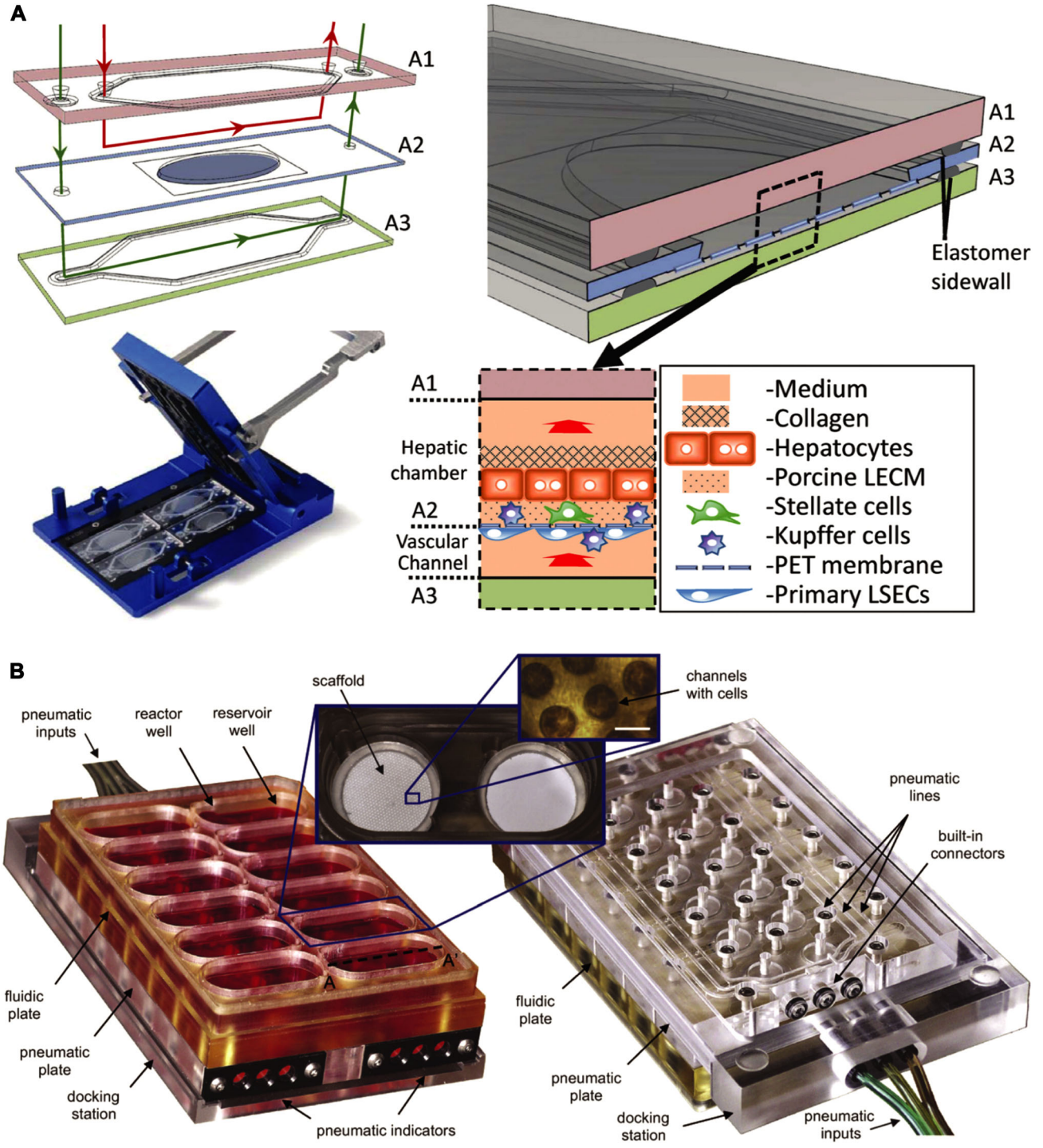


**Figure 3.** Exemplary designs of liver-on-a-chip models. A) Overview of the vascularized liver acinus microphysiology system (vLAMPS). The model is built from three glass layers (A1–A3) of which A2 contains an elliptical opening with a PET membrane onto which PHH and NPCs are seeded. The vascular channel between A2 and A3 contains LSECs and KCs, and the upper hepatic chamber between A1 and A2 harbors the PHH and stellate cells. Red arrows in the schematic indicate the direction of flow. B) Top view (left) and bottom view (right) of perfused multiwell plates. Insets show close-ups of a bioreactor chamber with scaffold and the channels loaded with cells. Individual valves can be addressed using air pressure selectively distributed by pneumatic lines. Scale bar = 300 mm. Figure A-D reproduced with permission.[23] Copyright 2018, Royal Society of Chemistry. Figure E reproduced with permission.[26] Copyright 2010, Royal Society of Chemistry. LECM, lung extracellular matrix; PET, polyethylene terephthalate.

 

been presented, in which a organotypic hepatic model is integrated with models of other tissues in perfused devices. However, as these applications are outside of the scope of this review, we refer the interested reader to excellent recent reviews on this matter.[32–34]

### 2.4. Bioprinting of Hepatic Structures

Bioprinting includes a number of technically diverse methods that aim to recapitulate the physiological architecture of the tissue of interest by allowing highly precise control of the placement of cells, as well as bioactive cofactors, such as extracellular matrix proteins, growth factors, cytokines, or drug molecules. Bioprinting approaches can be subdivided into three main methods: i) droplet-based bioprinting, ii) extrusion-based bioprinting, and iii) light-assisted bioprinting. For technical details, we refer the interested reader to excellent recent reviews on this topic.[35–38]

As of now, the field of bioprinting uses primarily hepatoma cell lines. Skardal and colleagues used a hybrid bioink of methacrylated gelatin (GEMA) and methacrylated hyaluronic acid (HAMA) that was previously shown to allow printing of HepG2 cells with high cell viability[39,40] to incorporate tissue-derived decellularized extracellular matrix-based solutions to more closely mimic the native microenvironment.[41] PHH spheroids were then bioprinted using extrusion-based setup, and cells cultured in this scaffold exhibited stable albumin production and urea synthesis for up to two weeks in culture. Furthermore, Organovo developed a bioprinting platform, in which, PHH, stellate cells, and KCs are bioprinted using nondisclosed bioink, where the cells showed five to ten times increased viability and albumin secretion compared to 2D culture after two weeks.[42] Furthermore, the authors recently demonstrated that the model can recapitulate aspects of methotrexate-induced fibrogenesis.[43]

## 3. Special Considerations

### 3.1. Cells

The in vitro system used should to a great extent comply with the phenotype present in vivo. In 3D liver spheroids, the perivenous and periportal phenotypes of hepatocytes are retained for many weeks, although these two types of hepatocytes are randomly distributed within the spheroids.[9] For every new system developed, a comparison with the cellular phenotype of the donor tissue is of high importance. Importantly, hepatic drug response and toxicity are influenced by a variety of genetic, physiological, pathophysiological, and environmental factors.[44] In our experience, physiological characteristics of the donor tissue, such as insulin resistance and intracellular lipid accumulation, are retained in the spheroid culture.[45] Interdonor variation in sensitivity to external hormones or dietary factors is extensive, whereas intradonor variability between different experiments using material from the same donor is minimal. Thus, the in vitro system of choice should ideally allow mimicking this inter-individual variation using material from several different donors.

Hepatocytes from about 40% of the liver donors are not plateable in a 2D format in Petri dishes and only plateable hepatocytes are able to form spheroids, with an approximate success rate of 80%.[12] However, as the capacity to form spheroids in cultures appears to be a donor-specific property, commercial suppliers can screen their hepatocyte batches for plateability or spheroid-formation capacity beforehand.

PHH are considered as the most physiologically relevant cell model and in this review, we focus specifically on 3D culture applications for PHH. However, as a variety of other cell models has been extensively utilized to study hepatic drug response and toxicity, we here concisely discuss their strengths and weaknesses. These include hepatoma cell lines, such as HepG2, Huh7, and HepaRG, as well as stem cell-derived HLCs. Hepatoma cell lines are readily proliferating, which makes them a cheaper and more easily scalable alternative to PHH, useful for early stages of the drug development pipeline. Furthermore, as they have been used for many years there is already substantial body of available information, whereas for PHH, interindividual differences require thorough characterizations for every new donor. However, the molecular phenotype and functionality of these cell lines are very poor, resembling extensively dedifferentiated PHH after multiple days in the monolayer culture.[46–48] As a result, hepatoma cells exhibit low metabolic activity and are substantially less sensitive to hepatotoxic drugs.[49]

HLCs can be derived from induced PSCs (iPSCs), which, in turn, can be generated from any somatic tissue, thereby allowing to study drug metabolism and response in hepatic cells from patients who experienced idiosyncratic drug reactions without the need for invasive procedures, such as liver biopsies. However, iPSC lines commonly contain contaminations and karyotype abnormalities, thus mandating extensive characterization.[50] Moreover, stem cell-derived HLCs generated using current protocols only acquire immature hepatic differentiation states, retain expression of fetal markers, and express low levels of metabolic enzymes,[51,52] resulting in low sensitivity to hepatotoxic compounds.[53,54] However, advanced hepatic culture methods improve the molecular phenotypes of HLCs, to some extent, thereby increasing their functionality and predictive power.[55–57]

### 3.2. Media

The liver function is to a great extent dependent on the hormonal and dietary context, as well as damaging environmental cues. Liver diseases have a multitude of underlying etiologies, including exposure to high levels of dietary fats and carbohydrates, excessive alcohol consumption, and hepatitis virus infections. It is a clear advantage to culture hepatocytes in chemically defined media for studying liver functions and pathophysiology. Notably, however, many commercially available media, such as standard Williams' E formulations, contain >1000-fold higher insulin and >3-fold higher glucose concentrations than what is physiological. It is thus important to carefully check the specific content of the media employed for these applications.

Serum generally contains many growth factors and mediators, which differ extensively between different batches of sera, and a more controlled liver in vitro system is obtained in the absence of serum. Such a defined medium as a basis allows pathophysiological modifications, which can be used to provoke

 

pathophysiologically relevant perturbations as seen in metabolic disorders. Albumin constitutes a component that should be considered particularly for pharmacological applications due to drug-specific absorption. However, when exposure concentrations are appropriately adjusted, albumin-free media might be used and, e.g., the 3D spheroid system works well under such conditions. It is important to ensure that the media is oxygenated and accessible to the cells. Spheroids <300 μm do not show evident signs of hypoxia and no central necrosis is observed even after several weeks in standard nonperfused cell culture incubators with periodic medium changes. Static culture can thus apparently support sufficient nutrient supply.

### 3.3. Choice of Substratum Materials

The choice of material can affect cellular phenotypes of 3D human liver systems and impact the interpretation of biological and pharmacological results, particularly for liver-on-a-chip models. Different materials can directly impact cellular functions. Glass, silicon, and Teflon are amenable choices for cell applications when inertness of the material is being considered. Some materials, however, are prone to absorption of soluble molecules. For instance, polydimethylsiloxane can absorb estrogen and other hydrophobic molecules, resulting in biologically significant signaling perturbations in cultured cells and confounding of drug exposure studies.[58–60] In addition, uncross-linked oligomers can leach out and contaminate the culture medium, potentially eliciting unphysiological cellular responses.[61]

Furthermore, substrate stiffness constitutes a key parameter that modulates biological function and responses. Stiffness optima are highly cell type-dependent with Young's moduli varying between <1 kPa for soft tissue, such as brain and fat, and 1000 kPa for bone.[62] In the context of hepatocytes, culturing on soft substrates (2 kPa) results in increased albumin and urea synthesis as well as activity of drug-metabolizing enzymes compared to culturing on the relatively stiff substratum (50 kPa).[63] Thermoplastic and thermoset polymers often render stiff materials of medium to high Young modulus depending on the crosslink density in the polymer network. Elastomers, in contrast, have rubbery mechanical property and can be exposed to reversible elongation. Recently, a thermosetting material toolbox, coined as off-stoichiometry thiol-ene (OSTE), with tunable mechanical property for lab-on-a-chip applications has emerged.[64,65]

## 4. Applications of Advanced Primary Human Hepatocyte Culture Models

### 4.1. Prediction of DILI

Importantly, the risk of safety failures in clinical stages of drug development decreases with the increasing quality of preclinical safety assessments. For instance, a longitudinal review of AstraZeneca's small-molecule drug projects from 2005 to 2010 revealed that overall 29% ($n = 11$ out of 38 projects) of projects with low confidence in the preclinical safety profile were closed due to safety issues, whereas none of the compounds with high-quality safety data was terminated due to safety concerns in the clinics ($n = 0$ out of 13).[66] In light of these findings, it is thus not surprising that much research has focused on the development of preclinical model systems that improve the prediction of hepatotoxic liabilities.[33,67–69]

DILI encompasses a wide range of mechanistically heterogenous insults that can differ widely with regard to clinical pattern, frequency, severity, and prognosis.[70] Thus, categorization of compounds into DILI-positive and -negative is not unequivocal and classification schemata for certain drugs, such as paroxetine, fenofibrate, and phenelzine, can differ.[71–74] As the evaluation of systems for the preclinical testing of hepatotoxicity requires faithful training sets of DILI-positive and -negative compounds, it is thus essential to comprehensively evaluate the DILI classification of each drug and include only those in the test set that can be unambiguously classified.

In clinics, DILI occurs most often only after multiple weeks or several months of drug treatment. In order to faithfully mimic such delayed hepatotoxicity events, the methodological focus shifted away from using short-lived hepatic in vitro systems, such as 2D monolayer cultures, liver slices, or suspension cultures, towards the use of 3D culture methods. For hepatotoxicity screens, spheroids and MPCC cultures are particularly suitable due to their intrinsic high-throughput compatibility and multiple studies have systematically assessed their predictive power using large panels of drugs with and without direct evidence of causing DILI. Notably, these assays commonly account for human exposure levels by testing toxicity at a peak serum concentration (and multiples thereof) measured in patients and might thus be less suitable for hepatotoxicity assessments in early stages of drug development when exposure levels have not yet been determined.

Khetani et al.[75] used PHH cultured as MPCC to evaluate the hepatotoxicity of 35 DILI-positive and 10 DILI-negative compounds. Using repeated exposures over the course of nine days, they correctly predicted the toxicity of 66% (23/35) with only one out of ten false-positives (specificity = 90%). Furthermore, PHH-MPCCs have been utilized for the identification of biomarkers for idiosyncratic DILI induced by tolvaptan.[76]

Two systematic benchmarking studies for hepatotoxicity tests have been published in PHH spheroids to date. Proctor et al.[77] used a commercial proprietary model to test the toxicity of 110 drugs (69 DILI-positive and 41 DILI-negative) during 14 days of repeated exposures using the InSphero model and reported overall sensitivity and specificity of 59% and 80%, respectively. In contrast, we used chemically defined media and ULA 3D PHH spheroids to expose a panel of 123 drugs (70 DILI-positive and 53 DILI-negative), correctly predicting the hepatotoxicity of 48 out of 70 compounds (sensitivity = 69%) without a single false-positive result (specificity = 100%; **Figure 4**A).[78] Classification of compounds into DILI-positive and -negative for this study was based on the current expert consensus and regulatory classifications.

Notably, exposure of PHH spheroids to physiological subtoxic concentrations of hepatotoxic compounds with different toxicity mechanisms faithfully mimicked the transcriptional perturbations observed in patients. Exposure to the genotoxic agent aflatoxin B1 resulted in induction of genes active in nucleotide excision repair and DNA replication.[3] Genes involved in the bile acid synthesis was strongly downregulated in spheroids exposed to the cholestatic drug chlorpromazine,

 

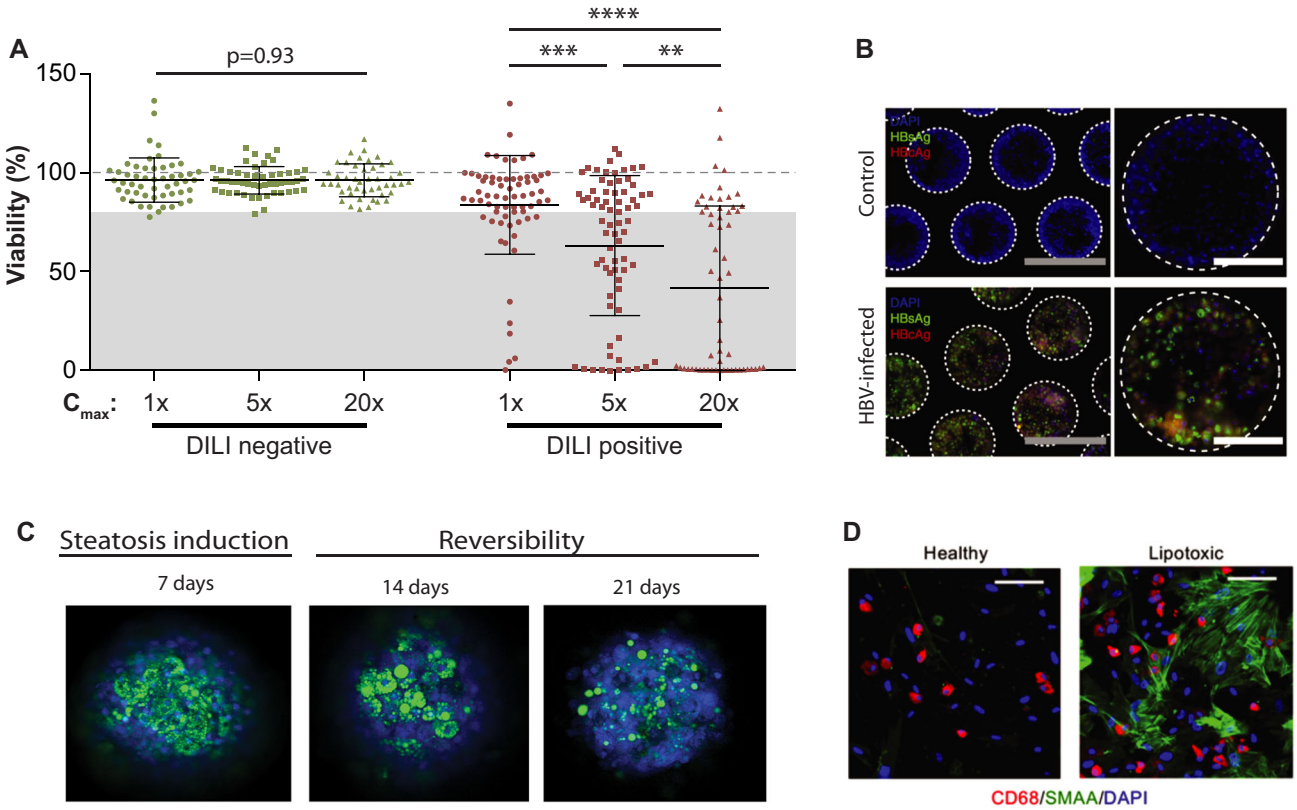


**Figure 4.** Data demonstrating the utility of hepatic 3D spheroids for hepatotoxicity predictions and disease modeling. A) A total of 123 drugs with or without direct implication in clinical DILI were screened for hepatotoxicity in 3D spheroids using two-week exposures at ×1, ×5, and ×20 of the therapeutic serum concentration ($c_{max}$). Note that viability decreases dose-dependently when hepatocytes are exposed to DILI-positive but not to DILI-negative compounds. Error bars indicate SD $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Reproduced with permission.[78] Copyright 2018, Society of Toxicology. B) PHH cultured as liver-on-chip are susceptible to hepatitis B virus (HBV) infections as judged by immunofluorescence of HBV viral antigens (HBsAg and HBcAg) ten days after infection. Reproduced with permission.[31] Copyright 2018, Nature Publishing Group. C) Hepatic steatosis can be induced in human spheroids by exposure to elevated FFA levels in the culture medium. Notably, these effects are reversible upon FFA withdrawal. Reproduced with permission.[45] Copyright 2018, Nature Publishing Group. D) Representative photomicrographs of NPCs in the human HemoShear NAFLD model under lipotoxic stress. Note the increase in HSC activation (green SMAA-positive cells) in lipotoxic but not healthy conditions. All scale bars = 100 μm. Reproduced with permission.[101] Copyright 2016, American Society for Clinical Investigation.

mirroring changes in primarily the expression of the *CYP7A1* gene observed in patients with cholestasis.[3] Similar toxicogenomic approaches in MPCC revealed transcriptomic perturbations upon treatment with troglitazone, nefazodone, ibufenac, and tolcapone. Interestingly, the authors found that the number of differentially expressed genes was higher in MPCCs treated with these hepatotoxins compared to those cultures dosed with the corresponding nontoxic analogues (rosiglitazone, buspirone, ibuprofen, and entacapone).[80]

While advanced hepatic 3D models have clearly improved hepatotoxicity predictions, they generally do not address patient-specific susceptibility factors, such as environmental exposures, hepatic morbidities, or genetic predisposition. Notably, 3D hepatic models are not likely to successfully identify the idiosyncratic hepatotoxicity of compounds, that is related to specific, often rare HLA alleles and mediated by the immune system, such as flucloxacillin, amoxicillin-clavulanate, and ticlopidine.[81] However, important recent in vitro studies showed that CD4+ and CD8+ T-cells isolated from risk allele carriers can be activated when the respective drug antigen being presented,[82–84] opening possibilities for testing of idiosyncratic DILI risk due to candidate HLA alleles during drug development.

### 4.2. Metabolite Identification and Pharmacokinetic Studies

Predicting the biotransformation and main metabolic routes of new drugs is critically important for drug development projects. However, metabolic profiles differ drastically between humans and preclinical model species. Additionally, conventional systems for metabolite profiling predicted only less than half of circulating metabolites, primarily because they were too short-lived to reveal the full metabolic fingerprint of the parent.[85] To improve success rates, 3D culture systems that maintain physiological levels of drug-metabolizing enzymes for extended periods of time provide appealing tools to obtain realistic metabolite profiles.

By incubating cells for 1 week without changing the medium with 27 drugs with available quantitative human metabolite profile data, MPCC allowed one to detect 82% and 75% of their

 

abundant excretory and circulating metabolites.[86] In contrast, human liver microsomes or PHH suspension cultures identified only 39% and 55% of metabolites, respectively.[87] Furthermore, PHH in MPCC have been successfully used to predict human clearance of low-turnover compounds. Notably, clearance of 19 out of 26 (73%) and 10 out of 17 (59%) slowly metabolized compounds was quantified within twofold of the observed human clearance in vivo.[88,89] In addition, the system recapitulated the effects of CYP3A4 induction (rifampicin) and CYP3A4 and CYP2D6 inhibition (ritonavir and midazolam, respectively) on clearance of the respective substrates.[89]

Similarly, phase I and phase II metabolites of acetaminophen, diclofenac, midazolam, propranolol, salbutamol, and lamotrigine observed in vivo could be identified in PHH spheroids.[90] Moreover, spheroids were successfully used to evaluate the impact of genetic polymorphisms in drug-metabolizing enzymes on the metabolic fate of their substrates. Dextromethorphan is primarily metabolized by CYP2D6 to dextrorphan; however, when cells from a donor were used who was phenotypically and genotypically categorized as a poor CYP2D6 metabolizer (CYP2D6*4/*10), the metabolic flux was shunted towards CYP3A4-dependent metabolism, resulting in the predominant formation of 3-methoxymorphinan.[10] These findings incentivize the use of such systems to evaluate the effects of genetic polymorphisms, many of which are common in the general population,[91] on the metabolism and metabolic fingerprints of drugs of interest. Moreover, PHH spheroids have been suggested as promising tools for the identification of DDIs by systematically evaluating the effects of co-exposures on metabolic profiles.[92]

### 4.3. Infectious Liver Disease

Recreation of the 3D microenvironment is crucial to model the initiation and progression of host–pathogen interactions in vitro.[93] PHH in the MPCC model is permissive to infection by *Plasmodium falciparum*, the most virulent parasite responsible for most malaria-related deaths. Although infections remained low compared to in vivo, the full liver cycle of this pathogen was supported, including the release of infected merozoites and infection of overlaid human erythrocytes.[94] Furthermore, 3D liver models are permissive to viral infections, opening avenues for the ex vivo study of hepatitis viruses (Figure 4B). HBV infection has been difficult to model in human liver cell lines as most of these lack the necessary factors for HBV entry. Ortega-Prieto et al.[31] recently established a promising 3D microfluidic system where PHH were permissive to patient-derived HBV at low titers. Cocultivation with KCs allowed the identification of cell-specific immune responses and opened the possibility to compare host factors for HBV susceptibility. However, the viral spread was not detected in this model and it will be interesting to determine whether cocultivation of additional liver cell types and inclusion of other immune cells could further improve HBV modeling.

### 4.4. Nonalcoholic Fatty Liver Disease

NAFLD is a rising health problem in many parts of the world and the most rapidly growing etiology for liver failure and liver transplantation in the United States. The disease encompasses a wide spectrum of clinical and histological phenotypes, ranging from benign hepatic steatosis to nonalcoholic steatohepatitis (NASH), which can further progress to fibrosis, cirrhosis, and hepatocellular carcinoma. Currently, the global prevalence of NAFLD is approximately 25%[95] with associated annual medical costs of ≈103 billion USD in the United States alone.[96] Despite the global economic and clinical burden, no FDA-approved drug therapies for NASH exist and the cornerstone of therapy includes lifestyle intervention and weight control.[97]

Modeling of NAFLD in vitro has been challenging in conventional 2D liver models due to the chronic nature of the disease, which requires long-term stable cultures; the need for adequate in vivo-like metabolic responses to hormones and nutrients, as well as the disease complexity that requires the intricate interplay between both parenchymal and nonparenchymal liver cells to mediate disease progression and inflammatory responses.[98] PHH in the MPCC model are responsive to alterations in glucose levels in the culture media. Under hyperglycemic conditions, hepatocytes developed insulin resistance and subsequent steatosis.[99] Furthermore, in the same model, coculture with activated stellate cells promoted steatogenesis that could be ameliorated by treatment with obeticholic acid, a bile acid analogue in clinical trials for the treatment of NASH.[100]

In spheroids, exposure to a mixture of free fatty acids (FFAs), insulin, and carbohydrates induced reversible accumulation of lipid droplets and was accompanied by features of insulin resistance (Figure 4C). A reduction in the hepatocellular lipid content was observed by treatment with various antisteatotic agents, such as metformin and the antioxidant vitamin E, indicating a reversal of hepatic steatosis. PHH spheroids were also recently used to interrogate the protective role of nitrite in reducing chemically and metabolically induced steatosis.[101] Moreover, spheroid cultures were used to reveal the critical role of insulin-like growth factor binding protein 7, a factor secreted by liver macrophages that binds to the hepatocyte insulin receptor and induces lipogenesis and gluconeogenesis, thus revealing how macrophages contribute to insulin resistance independent of inflammation.[102]

Microfluidic devices have also shown promise for modeling NAFLD. Feaver et al.[79] engineered a multicellular system composed of PHH, HSCs, and macrophages that incorporates sinusoidal hemodynamics. Exposure to a lipotoxic milieu induced a lipidomic signature similar to clinical NASH biopsies with increased transforming growth factor-β secretion, indicative of a fibrotic response (Figure 4D). Importantly, phenotypic responses from individual cell types can be examined in this model. Kostrzewski et al.[30] employed the LiverChip platform to study transcriptomic and proteomic profiles of PHH upon fat loading. Fat-exposed PHH displayed numerous transcriptional changes in genes associated with insulin resistance and lipid metabolism, including increased *CYP2E1* expression. In addition, fat loading increased the secretion of fibrinogen and tissue inhibitors of metalloproteinases-1 and factors associated with fibrosis and wound healing. No secretion of interleukin-1β (IL-1β) and IL-6 were observed, likely due to the lack of nonparenchymal liver cell types that constitute the major source for these important proinflammatory cytokines.

 

### 4.5. Cholestatic Liver Disease

Retention of bile acids (cholestasis) can injure hepatocytes and biliary epithelial cells and constitute a major mechanism underlying the liver insult caused by various drugs. Modeling cholestasis in vitro requires an intact bile canalicular network with an adequate expression of bile acid transporters. Hepatocyte polarity is crucial for the establishment of bile canaliculi,[103] which is lost upon 2D-cultivation of hepatocytes. PHH re-establish bile canalicular networks when cultured in sandwich culture[104] or spheroid culture,[4] and PHH spheroids were able to discriminate cholestatic hepatotoxins from hepatotoxins with other mechanisms of injury.[105] Importantly, identification of the cholestatic liability of drugs improved upon prolonged exposure.[106] Precision-cut human liver slices have also been shown responsive to typical cholestatic compounds with appropriate transcriptomic signatures.[107] A major drawback of the latter model is however the short viability of cells and the inability to replicate findings using material from the same donor.

## 5. Conclusions

In recent years, there has been a rapid expansion in the 3D models for mimicking drug pharmacokinetics and toxicity, as well as for the formation of pathophysiological liver systems. Increased predictability is now achieved in primary human systems for chronic drug toxicity and metabolism of low clearance drugs. In addition, the novel models allow for a new era in the field of drug screening for the treatment of NAFLD, hepatitis, and hepatocellular cancer. For such purposes, many of the models are high-throughput and high-content compatible. Currently, several novel hepatic in vitro systems are produced for commercial purposes. However, information about their hepatic phenotype and properties as well as comprehensive benchmarking data are mostly lacking. Overall, we anticipate that the rapid development of the hepatic 3D systems will be of high importance for future drug development efforts and facilitate the discovery of novel intercellular mediators as well as mechanisms for the development of liver disease and their treatments.

## Acknowledgements

V.M.L. and M.I.-S. are founders and owners of HepaPredict AB. The work in the authors' laboratories is supported by the Swedish Research Council (grant agreement numbers: 2015-02760, 2016-01153, and 2016-01154), by the Strategic Research Programme in Diabetes at Karolinska Institutet, by the Lennart Philipson Foundation, by the Harald and Greta Jeansson Foundation, and by the ERC Advanced Grant HepaSphere (grant agreement number 742020). No writing assistance was utilized in the production of this manuscript. The copyright information of figure 2, 3, and 4 are updated on July 2, 2019.

## Conflict of Interests

The authors declare no conflict of interest.

## Keywords

cytochrome P450, drug metabolism, hepatotoxicity, liver fibrosis, liver-on-a-chip, microfluidic systems, spheroids, steatosis

Received: January 29, 2019
Revised: March 26, 2019
Published online: May 20, 2019

[1] M. Martignoni, G. M. M. Groothuis, R. de Kanter, *Expert. Opin. Drug Metab. Toxicol.* **2006**, *2*, 875.
[2] V. M. Lauschke, S. U. Vorrink, S. M. L. Moro, F. Rezayee, Å. Nordling, D. F. G. Hendriks, C. C. Bell, R. Sison-Young, B. K. Park, C. E. Goldring, E. Ellis, I. Johansson, S. Mkrtchian, T. B. Andersson, M. Ingelman-Sundberg, *Hepatology* **2016**, *64*, 1743.
[3] C. C. Bell, V. M. Lauschke, S. U. Vorrink, H. Palmgren, R. Duffin, T. B. Andersson, M. Ingelman-Sundberg, *Drug Metab. Dispos.* **2017**, *45*, 419.
[4] R. M. Tostões, S. B. Leite, M. Serra, J. Jensen, P. Björquist, M. J. T. Carrondo, C. Brito, P. M. Alves, *Hepatology* **2012**, *55*, 1227.
[5] S. P. Rebelo, R. Costa, M. M. Silva, P. Marcelino, C. Brito, P. M. Alves, *J. Tissue. Eng. Regen. Med.* **2017**, *11*, 2034.
[6] R. Foty, *J. Visualized Exp.* **2011**, 1.
[7] S. Messner, I. Agarkova, W. Moritz, J. M. Kelm, *Arch. Toxicol.* **2013**, *87*, 209.
[8] S. Messner, L. Fredriksson, V. M. Lauschke, K. Roessger, C. Escher, M. Bober, J. M. Kelm, M. Ingelman-Sundberg, W. Moritz, *Appl. In Vitro Toxicol.* **2018**, *4*, 1.
[9] C. C. Bell, D. F. G. Hendriks, S. M. L. Moro, E. Ellis, J. Walsh, A. Renblom, L. Fredriksson Puigvert, A. C. A. Dankers, F. Jacobs, J. Snoeys, R. L. Sison-Young, R. E. Jenkins, Å. Nordling, S. Mkrtchian, B. K. Park, N. R. Kitteringham, C. E. P. Goldring, V. M. Lauschke, M. Ingelman-Sundberg, *Sci. Rep.* **2016**, *6*, 25187.
[10] S. U. Vorrink, S. Ullah, S. Schmidt, J. Nandania, V. Velagapudi, O. Beck, M. Ingelman-Sundberg, V. M. Lauschke, *FASEB J.* **2017**, *31*, 2696.
[11] C. C. Bell, A. C. A. Dankers, V. M. Lauschke, R. Sison-Young, R. Jenkins, C. Rowe, C. E. Goldring, K. Park, S. L. Regan, T. Walker, C. Schofield, A. Baze, A. J. Foster, D. P. Williams, A. W. M. van de Ven, F. Jacobs, J. Houdt, T. Lähteenmäki, J. Snoeys, S. Juhila, L. Richert, M. Ingelman-Sundberg, *Toxicol. Sci.* **2018**, *162*, 655.
[12] A. Baze, C. Parmentier, D. F. G. Hendriks, T. Hurrell, B. Heyd, P. Bachellier, C. Schuster, M. Ingelman-Sundberg, L. Richert, *Tissue Eng., Part C* **2018**, *24*, 534.
[13] S. R. Khetani, S. N. Bhatia, *Nat. Biotechnol.* **2008**, *26*, 120.
[14] S. R. Khetani, G. Szulgit, J. A. Del Rio, C. Barlow, S. N. Bhatia, *Hepatology* **2004**, *40*, 545.
[15] S. R. Khetani, A. A. Chen, B. Ranscht, S. N. Bhatia, *FASEB J.* **2008**, *22*, 3768.
[16] T. V. Nguyen, O. Ukairo, S. R. Khetani, M. McVay, C. Kanchagar, W. Seghezzi, G. Ayanoglu, O. Irrechukwu, R. Evers, *Drug Metab. Dispos.* **2015**, *43*, 774.
[17] B. R. Ware, M. J. Durham, C. P. Monckton, S. R. Khetani, *Cell Mol. Gastroenterol. Hepatol.* **2018**, *5*, 187.
[18] M. Hegde, R. Jindal, A. Bhushan, S. S. Bale, W. J. McCarty, I. Golberg, O. B. Usta, M. L. Yarmush, *Lab Chip* **2014**, *14*, 2033.
[19] J. W. Allen, *Toxicol. Sci.* **2005**, *84*, 110.
[20] F. T. Lee-Montiel, S. M. George, A. H. Gough, A. D. Sharma, J. Wu, R. DeBiasio, L. A. Vernetti, D. L. Taylor, *Exp. Biol. Med.* **2017**, *242*, 1617.
[21] L. Tomlinson, L. Hyndman, J. W. Firman, R. Bentley, J. A. Kyffin, S. D. Webb, S. McGinty, P. Sharma, *Front. Bioeng. Biotechnol.* **2019**, *7*, 253.

 

[22] X. Li, S. M. George, L. Vernetti, A. H. Gough, D. L. Taylor, *Lab Chip* **2018**, *18*, 2614.
[23] L. A. Vernetti, N. Senutovitch, R. Boltz, R. DeBiasio, T. Ying shun, A. Gough, D. L. Taylor, *Exp. Biol. Med.* **2016**, *241*, 101.
[24] Y. Du, N. Li, H. Yang, C. Luo, Y. Gong, C. Tong, Y. Gao, S. Lü, M. Long, *Lab Chip* **2017**, *17*, 782.
[25] K. Domansky, W. Inman, J. Serdy, A. Dash, M. H. M. Lim, L. G. Griffith, *Lab Chip* **2010**, *10*, 51.
[26] M. J. Powers, K. Domansky, M. R. Kaazempur-Mofrad, A. Kalezi, A. Capitano, A. Upadhyaya, P. Kurzawski, K. E. Wack, D. B. Stolz, R. Kamm, L. G. Griffith, *Biotechnol. Bioeng.* **2002**, *78*, 257.
[27] U. Sarkar, K. C. Ravindra, E. Large, C. L. Young, D. Rivera-Burgos, J. Yu, M. Cirit, D. J. Hughes, J. S. Wishnok, D. A. Lauffenburger, L. G. Griffith, S. R. Tannenbaum, *Drug Metab. Dispos.* **2017**, *45*, 855.
[28] N. Tsamandouras, T. Kostrzewski, C. L. Stokes, L. G. Griffith, D. J. Hughes, M. Cirit, *J. Pharmacol. Exp. Ther.* **2017**, *360*, 95.
[29] C. Rowe, M. Shaeri, E. Large, T. Cornforth, A. Robinson, T. Kostrzewski, R. Sison-Young, C. Goldring, K. Park, D. Hughes, *Toxicol. In Vitro* **2018**, *46*, 29.
[30] T. Kostrzewski, T. Cornforth, S. A. Snow, L. Ouro-Gnao, C. Rowe, E. M. Large, D. J. Hughes, *World J. Gastroenterol.* **2017**, *23*, 204.
[31] A. M. Ortega-Prieto, J. K. Skelton, S. N. Wai, E. Large, M. Lussignol, G. Vizcay-Barrena, D. Hughes, R. A. Fleck, M. Thursz, M. T. Catanese, M. Dorner, *Nat. Commun.* **2018**, *9*, 682.
[32] R. Prantil-Baun, R. Novak, D. Das, M. R. Somayaji, A. Przekwas, D. E. Ingber, *Annu. Rev. Pharmacol. Toxicol.* **2018**, *58*, 37.
[33] L. Ewart, E.-M. Dehne, K. Fabre, S. Gibbs, J. Hickman, E. Hornberg, M. Ingelman-Sundberg, K.-J. Jang, D. R. Jones, V. M. Lauschke, U. Marx, J. T. Mettetal, A. Pointon, D. Williams, W.-H. Zimmermann, P. Newham, *Annu. Rev. Pharmacol. Toxicol.* **2018**, *58*, 65.
[34] K. Ronaldson-Bouchard, G. Vunjak-Novakovic, *Cell Stem Cell* **2018**, *22*, 310.
[35] X. Ma, J. Liu, W. Zhu, M. Tang, N. Lawrence, C. Yu, M. Gou, S. Chen, *Adv. Drug Deliv. Rev.* **2018**, *132*, 235.
[36] J. Jang, H.-G. Yi, D.-W. Cho, *ACS Biomater. Sci. Eng.* **2016**, *2*, 1722.
[37] S. Derakhshanfar, R. Mbeleck, K. Xu, X. Zhang, W. Zhong, M. Xing, *Bioact. Mater.* **2018**, *3*, 144.
[38] W. Peng, D. Unutmaz, I. T. Ozbolat, *Trends Biotechnol.* **2016**, *34*, 722.
[39] A. Skardal, J. Zhang, L. McCoard, X. Xu, S. Oottamasathien, G. D. Prestwich, *Tissue Eng.,Part A.* **2010**, *16*, 2675.
[40] T. Billiet, E. Gevaert, T. De Schryver, M. Cornelissen, P. Dubruel, *Biomaterials* **2014**, *35*, 49.
[41] A. Skardal, M. Devarasetty, H.-W. Kang, I. Mead, C. Bishop, T. Shupe, S. J. Lee, J. Jackson, J. Yoo, S. Soker, A. Atala, *Acta Biomater.* **2015**, *25*, 24.
[42] D. G. Nguyen, J. Funk, J. B. Robbins, C. Crogan-Grundy, S. C. Presnell, T. Singer, A. B. Roth, *PLoS One* **2016**, *11*, e0158674.
[43] L. M. Norona, D. G. Nguyen, D. A. Gerber, S. C. Presnell, M. Mosedale, P. B. Watkins, *PLoS One* **2019**, *14*, e0208958.
[44] V. M. Lauschke, M. Ingelman-Sundberg, *Int. J. Mol. Sci.* **2016**, *17*, 1714.
[45] M. Kozyra, I. Johansson, Å. Nordling, S. Ullah, V. M. Lauschke, M. Ingelman-Sundberg, *Sci. Rep.* **2018**, *8*, 14297.
[46] S. N. Hart, Y. Li, K. Nakamoto, E. Subileau, D. Steen, X. Zhong, *Drug Metab. Dispos.* **2010**, *38*, 988.
[47] M. Lübberstedt, U. Müller-Vieira, M. Mayer, K. M. Biemel, F. Knöspel, D. Knobeloch, A. K. Nüssler, J. C. Gerlach, K. Zeilinger, *J. Pharmacol. Toxicol. Methods* **2011**, *63*, 59.
[48] R. L. C. Sison-Young, D. Mitsa, R. E. Jenkins, D. Mottram, E. Alexandre, L. Richert, H. Aerts, R. J. Weaver, R. P. Jones, E. Johann, P. G. Hewitt, M. Ingelman-Sundberg, C. E. P. Goldring, N. R. Kitteringham, B. K. Park, *Toxicol. Sci.* **2015**, *147*, 412.
[49] R. L. Sison-Young, V. M. Lauschke, E. Johann, E. Alexandre, S. Antherieu, H. Aerts, H. H. J. Gerets, G. Labbe, D. Hoët, M. Dorau, C. A. Schofield, C. A. Lovatt, J. C. Holder, S. H. Stahl, L. Richert, N. R. Kitteringham, R. P. Jones, M. Elmasry, R. J. Weaver, P. G. Hewitt, M. Ingelman-Sundberg, C. E. Goldring, B. K. Park, *Arch. Toxicol.* **2017**, *91*, 1385.
[50] N. Salomonis, P. J. Dexheimer, L. Omberg, R. Schroll, S. Bush, J. Huo, L. Schriml, S. Ho Sui, M. Keddache, C. Mayhew, S. K. Shanmukhappa, J. Wells, K. Daily, S. Hubler, Y. Wang, E. Zambidis, A. Margolin, W. Hide, A. K. Hatzopoulos, P. Malik, J. A. Cancelas, B. J. Aronow, C. Lutzko, *Stem Cell Rep.* **2016**, *7*, 110.
[51] R. E. Schwartz, H. E. Fleming, S. R. Khetani, S. N. Bhatia, *Biotechnol. Adv.* **2014**, *32*, 504.
[52] M. Baxter, S. Withey, S. Harrison, C.-P. Segeritz, F. Zhang, R. Atkinson-Dell, C. Rowe, D. T. Gerrard, R. Sison-Young, R. Jenkins, J. Henry, A. A. Berry, L. Mohamet, M. Best, S. W. Fenwick, H. Malik, N. R. Kitteringham, C. E. Goldring, K. Piper Hanley, L. Vallier, N. A. Hanley, *J. Hepatol.* **2015**, *62*, 581.
[53] K. Takayama, K. Kawabata, Y. Nagamoto, K. Kishimoto, K. Tashiro, F. Sakurai, M. Tachibana, K. Kanda, T. Hayakawa, M. K. Furue, H. Mizuguchi, *Biomaterials* **2013**, *34*, 1781.
[54] C. N. Medine, B. Lucendo-Villarin, C. Storck, F. Wang, D. Szkolnicka, F. Khan, S. Pernagallo, J. R. Black, H. M. Marriage, J. A. Ross, M. Bradley, J. P. Iredale, O. Flint, D. C. Hay, *Stem Cells Transl. Med.* **2013**, *2*, 505.
[55] R. L. Gieseck III, N. R. F. Hannan, R. Bort, N. A. Hanley, R. A. L. Drake, G. W. W. Cameron, T. A. Wynn, L. Vallier, *PLoS One* **2014**, *9*, e86372.
[56] J. H. Kim, Y. J. Jang, S. Y. An, J. Son, J. Lee, G. Lee, J. Y. Park, H.-J. Park, D.-Y. Hwang, J.-H. Kim, J. Han, *Toxicol. Sci.* **2015**, *147*, 190.
[57] B. R. Ware, D. R. Berger, S. R. Khetani, *Toxicol. Sci.* **2015**, *145*, 252.
[58] M. W. Toepke, D. J. Beebe, *Lab Chip* **2006**, *6*, 1484.
[59] K. J. Regehr, M. Domenech, J. T. Koepsel, K. C. Carver, S. J. Ellison-Zelski, W. L. Murphy, L. A. Schuler, E. T. Alarid, D. J. Beebe, *Lab Chip* **2009**, *9*, 2132.
[60] B. J. van Meer, H. de Vries, K. S. A. Firth, J. van Weerd, L. G. J. Tertoolen, H. B. J. Karperien, P. Jonkheijm, C. Denning, A. P. IJzerman, C. L. Mummery, *Biochem. Biophys. Res. Commun.* **2017**, *482*, 323.
[61] G. R. McDonald, A. L. Hudson, S. M. J. Dunn, H. You, G. B. Baker, R. M. Whittal, J. W. Martin, A. Jha, D. E. Edmondson, A. Holt, *Science* **2008**, *322*, 917.
[62] Y. Yang, K. Wang, X. Gu, K. W. Leong, *Engineering* **2017**, *3*, 36.
[63] V. Natarajan, E. J. Berglund, D. X. Chen, S. Kidambi, *RSC Adv.* **2015**, *5*, 80956.
[64] C. F. Carlborg, A. Vastesson, Y. Liu, W. van der Wijngaart, M. Johansson, T. Haraldsson, *J. Polym. Sci., Part A* **2014**, *52*, 2604.
[65] N. Sandström, R. Z. Shafagh, A. Vastesson, C. F. Carlborg, W. van der Wijngaart, T. Haraldsson, *J. Micromech. Microeng.* **2015**, *25*, 075002.
[66] D. Cook, D. Brown, R. Alexander, R. March, P. Morgan, G. Satterthwaite, M. N. Pangalos, *Nat. Rev. Drug. Discov.* **2014**, *13*, 419.
[67] V. Y. Soldatow, E. L. LeCluyse, L. G. Griffith, I. Rusyn, *Toxicol. Res.* **2013**, *2*, 23.
[68] S. S. Bale, L. Vernetti, N. Senutovitch, R. Jindal, M. Hegde, A. Gough, W. J. McCarty, A. Bakan, A. Bhushan, T. Y. Shun, I. Golberg, R. DeBiasio, O. B. Usta, D. L. Taylor, M. L. Yarmush, *Exp. Biol. Med.* **2014**, *239*, 1180.
[69] V. M. Lauschke, D. F. G. Hendriks, C. C. Bell, T. B. Andersson, M. Ingelman-Sundberg, *Chem. Res. Toxicol.* **2016**, *29*, 1936.
[70] M. D. Leise, J. J. Poterucha, J. A. Talwalkar, *Mayo Clin. Proc.* **2014**, *89*, 95.

 

[71] P. J. O'Brien, W. Irwin, D. Diaz, E. Howard-Cofield, C. M. Krejsa, M. R. Slaughter, B. Gao, N. Kaludercic, A. Angeline, P. Bernardi, P. Brain, C. Hougham, *Arch. Toxicol.* **2006**, *80*, 580.
[72] J. J. Xu, P. V. Henstock, M. C. Dunn, A. R. Smith, J. R. Chabot, D. de Graaf, *Toxicol. Sci.* **2008**, *105*, 97.
[73] M. Z. Sakatis, M. J. Reese, A. W. Harrell, M. A. Taylor, I. A. Baines, L. Chen, J. C. Bloomer, E. Y. Yang, H. M. Ellens, J. L. Ambroso, C. A. Lovatt, A. D. Ayrton, S. E. Clarke, *Chem. Res. Toxicol.* **2012**, *25*, 2067.
[74] M. Chen, A. Suzuki, S. Thakkar, K. Yu, C. Hu, W. Tong, *Drug Discovery Today* **2016**, *21*, 648.
[75] S. R. Khetani, C. Kanchagar, O. Ukairo, S. Krzyzewski, A. Moore, J. Shi, S. Aoyama, M. Aleo, Y. Will, *Toxicol. Sci.* **2013**, *132*, 107.
[76] M. Mosedale, J. S. Eaddy, O. J. Trask, N. S. Holman, K. K. Wolf, E. LeCluyse, B. R. Ware, S. R. Khetani, J. Lu, W. J. Brock, S. E. Roth, P. B. Watkins, *Toxicol. Sci.* **2018**, *161*, 149.
[77] W. R. Proctor, A. J. Foster, J. Vogt, C. Summers, B. Middleton, M. A. Pilling, D. Shienson, M. Kijanska, S. Ströbel, J. M. Kelm, P. Morgan, S. Messner, D. Williams, *Arch. Toxicol.* **2017**, *91*, 2849.
[78] S. U. Vorrink, Y. Zhou, M. Ingelman-Sundberg, V. M. Lauschke, *Toxicol. Sci.* **2018**, *163*, 655.
[79] R. E. Feaver, B. K. Cole, M. J. Lawson, S. A. Hoang, S. Marukian, B. R. Blackman, R. A. Figler, A. J. Sanyal, B. R. Wamhoff, A. Dash, *JCI Insight* **2016**, *1*, e90954.
[80] B. R. Ware, M. McVay, W. Y. Sunada, S. R. Khetani, *Toxicol. Sci.* **2017**, *157*, 387.
[81] V. M. Lauschke, Y. Zhou, M. Ingelman-Sundberg, *Pharmacol. Ther.* **2019**, [Epub ahead of print]
[82] M. M. Monshi, L. Faulkner, A. Gibson, R. E. Jenkins, J. Farrell, C. J. Earnshaw, A. Alfirevic, K. Cederbrant, A. K. Daly, N. French, M. Pirmohamed, B. K. Park, D. J. Naisbitt, *Hepatology* **2013**, *57*, 727.
[83] S.-H. Kim, K. Saide, J. Farrell, L. Faulkner, A. Tailor, M. Ogese, A. K. Daly, M. Pirmohamed, B. K. Park, D. J. Naisbitt, *Hepatology* **2015**, *62*, 887.
[84] T. Usui, A. Tailor, L. Faulkner, X. Meng, J. Farrell, A. K. Daly, G. J. Dear, B. K. Park, D. J. Naisbitt, *Chem. Res. Toxicol.* **2018**, *31*, 1022.
[85] S. Anderson, D. Luffer-Atlas, M. P. Knadler, *Chem. Res. Toxicol.* **2009**, *22*, 243.
[86] W. W. Wang, S. R. Khetani, S. Krzyzewski, D. B. Duignan, R. S. Obach, *Drug Metab. Dispos.* **2010**, *38*, 1900.
[87] D. Dalvie, R. S. Obach, P. Kang, C. Prakash, C.-M. Loi, S. Hurst, A. Nedderman, L. Goulet, E. Smith, H.-Z. Bu, D. A. Smith, *Chem. Res. Toxicol.* **2009**, *22*, 357.
[88] T. S. Chan, H. Yu, A. Moore, S. R. Khetani, D. Tweedie, *Drug Metab. Dispos.* **2013**, *41*, 2024.
[89] C. Lin, J. Shi, A. Moore, S. R. Khetani, *Drug Metab. Dispos.* **2016**, *44*, 127.
[90] T. Ohkura, K. Ohta, T. Nagao, K. Kusumoto, A. Koeda, T. Ueda, T. Jomura, T. Ikeya, E. Ozeki, K. Wada, K. Naitoh, Y. Inoue, N. Takahashi, H. Iwai, H. Arakawa, T. Ogihara, *Drug. Metab. Pharmacokinet.* **2014**, *29*, 373.
[91] Y. Zhou, M. Ingelman-Sundberg, V. Lauschke, *Clin. Pharmacol. Ther.* **2017**, *102*, 688.
[92] M. Ingelman-Sundberg, V. M. Lauschke, *Pharmacogenomics* **2018**, *19*, 1133.
[93] J. Barrila, A. Crabbé, J. Yang, K. Franco, S. D. Nydam, R. J. Forsyth, R. R. Davis, S. Gangaraju, C. M. Ott, C. B. Coyne, M. J. Bissell, C. A. Nickerson, *Infect. Immun.* **2018**, *86*, e00282.
[94] S. March, S. Ng, S. Velmurugan, A. Galstian, J. Shan, D. J. Logan, A. E. Carpenter, D. Thomas, B. K. L. Sim, M. M. Mota, S. L. Hoffman, S. N. Bhatia, *Cell Host Microbe* **2013**, *14*, 104.
[95] Z. M. Younossi, A. B. Koenig, D. Abdelatif, Y. Fazel, L. Henry, M. Wymer, *Hepatology* **2016**, *64*, 73.
[96] Z. M. Younossi, D. Blissett, R. Blissett, L. Henry, M. Stepanova, Y. Younossi, A. Racila, S. Hunt, R. Beckerman, *Hepatology* **2016**, *64*, 1577.
[97] A. M. Oseini, B. K. Cole, D. Issa, R. E. Feaver, A. J. Sanyal, *Hepatol. Int.* **2018**, *12*, 6.
[98] J. Boeckmans, A. Natale, K. Buyl, V. Rogiers, J. De Kock, T. Vanhaecke, R. M. Rodrigues, *Pharmacol. Res.* **2018**, *134*, 257.
[99] M. D. Davidson, K. R. Ballinger, S. R. Khetani, *Sci. Rep.* **2016**, *6*, 28178.
[100] M. D. Davidson, D. A. Kukla, S. R. Khetani, *Integr. Biol.* **2017**, *9*, 662.
[101] I. Cordero-Herrera, M. Kozyra, Z. Zhuge, S. McCann Haworth, C. Moretti, M. Peleli, M. Caldeira-Dias, A. Jahandideh, H. Huirong, J. C. Cruz, A. L. Kleschyov, M. F. Montenegro, M. Ingelman-Sundberg, E. Weitzberg, J. O. Lundberg, M. Carlstrom, *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 217.
[102] C. Morgantini, J. Jager, X. Li, L. Levi, V. Azzimato, A. Sulen, E. Barreby, C. Xu, M. Tencerova, E. Näslund, C. Kumar, F. Verdeguer, S. Straniero, K. Hultenby, N. K. Björkström, E. Ellis, M. Rydèn, C. Kutter, T. Hurrell, V. M. Lauschke, J. Boucher, A. Tomcala, G. Krejcova, A. Bajgar, M. Aouadi, *Nature Metabolism* **2019**, In press
[103] A. Treyer, A. Müsch, *Compr. Physiol.* **2013**, *3*, 243.
[104] B. Swift, N. D. Pfeifer, K. L. R. Brouwer, *Drug Metab. Rev.* **2010**, *42*, 446.
[105] D. F. G. Hendriks, L. Fredriksson Puigvert, S. Messner, W. Mortiz, M. Ingelman-Sundberg, *Sci. Rep.* **2016**, *6*, 35434.
[106] C. Parmentier, D. F. G. Hendriks, B. Heyd, P. Bachellier, M. Ingelman-Sundberg, L. Richert, *Toxicol. Lett.* **2018**, *295*, 187.
[107] S. Vatakuti, P. Olinga, J. L. A. Pennings, G. M. M. Groothuis, *Arch. Toxicol.* **2017**, *91*, 1401.

 