MINIREVIEW

3D human liver spheroids for translational pharmacology and toxicology

Magnus Ingelman-Sundberg | Volker M. Lauschke

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

Correspondence
Volker M. Lauschke, Department of Physiology and Pharmacology, Karolinska Institutet, SE-171 77 Stockholm, Sweden. Email: volker.lauschke@ki.se

Abstract
Drug development is a failure-prone endeavour, and more than 85% of drugs fail during clinical development, showcasing that current preclinical systems for compound selection are clearly inadequate. Liver toxicity remains a major reason for safety failures. Furthermore, all efforts to develop pharmacological therapies for a variety of chronic liver diseases, such as non-alcoholic steatohepatitis (NASH) and fibrosis, remain unsuccessful. Considering the time and expense of clinical trials, as well as the substantial burden on patients, new strategies are thus of paramount importance to increase clinical success rates. To this end, human liver spheroids are becoming increasingly utilized as they allow to preserve patient-specific phenotypes and functions for multiple weeks in culture. We here review the recent application of such systems for i) predictive and mechanistic analyses of drug hepatotoxicity, ii) the evaluation of hepatic disposition and metabolite formation of low clearance drugs and iii) the development of drugs for metabolic and infectious liver diseases, including NASH, fibrosis, malaria and viral hepatitis. We envision that with increasing dissemination, liver spheroids might become the new gold standard for such applications in translational pharmacology and toxicology.

KEYWORDS
drug development, drug discovery, hepatotoxicity, NASH, organotypic culture, pharmacokinetics

Abbreviations: ACE2, Angiotensin-Converting Enzyme 2; AFP, Alpha-Fetoprotein; AhR, Aryl Hydrocarbon Receptor; AKT, AKT Serine/Threonine Kinase 1; ALB, Albumin; COVID, Coronavirus Disease; CYP, Cytochrome P450; DILI, Drug-Induced Liver Injury; EGFR, Epidermal Growth Factor Receptor; ERK, Extracellular Signal-Regulated Kinase; EUA, Emergency Use Authorization; FDA, US Food and Drug Administration; FFA, Free Fatty Acids; HBV, Hepatitis B Virus; HCV, Hepatitis C Virus; HLC, Hepatocyte-Like Cell; HNF4A, Hepatocyte Nuclear Factor 4 Alpha; IGFBP7, Insulin-Like Growth Factor-Binding Protein 7; iPSC, Induced Pluripotent Stem Cell; JAK, Janus Kinase; MAPK, Mitogen-Activated Protein Kinase; NAFLD, Non-Alcoholic Fatty Liver Disease; NASH, Non-Alcoholic Steatohepatitis; NPC, Non-Parenchymal Cell; NRF2, Nuclear Factor Erythroid 2-Related Factor 2; NTCP, Na+/Taurocholate Transport Protein; PHH, Primary Human Hepatocytes; PIM, Proto-Oncogene Serine/Threonine-Protein Kinase; PNPLA3, Patatin-Like Phospholipase Domain Containing 3; ROS, Reactive Oxygen Species; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; STAT, Signal Transducer and Activator of Transcription; TM6SF2, Transmembrane 6 Superfamily Member 2; ULA, Ultra-Low Attachment.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2021 The Authors, Basic & Clinical Pharmacology & Toxicology published by John Wiley & Sons Ltd on behalf of Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society).
Despite record spending on pharmaceutical research and development, success rates for new molecular entities remain low with likelihoods of approval from phase I to launch stagnating below 10% across therapeutic areas. This low success rate is at least in part attributed to the divide between basic research in a controlled laboratory setting and subsequent clinical results. Safety issues are the most common reason for drug failures in early-stage trials and the liver constitutes one of the tissues most commonly involved in toxicity and safety warnings. Importantly however, the risk of failing due to safety exhibited a strong inverse correlation with the confidence in the preclinical safety assessment, indicating that the careful evaluation of toxicity in cell and animals provides a useful measure to reduce adverse drug events in the clinics. While toxicity is the main reason for project closures in phase I, lack of efficacy constitutes accounts for the majority of phase II and phase III failures.

Translational pharmacology and toxicology aim at obtaining an in-depth understanding of the molecular mechanisms and underpinnings of drug effects to facilitate translation of this fundamental knowledge from cell culture into living organisms, that is, animal models or humans. Two of the major hurdles for such endeavours are the phenotypic differences at the organizational, cellular and molecular level, between preclinical models, and the organs they are supposed to emulate, as well as the drastic increase in complexity that impairs extrapolation from cell culture to tissues and organisms. There has been an emerging trend in recent years to introduce cell culture models that more accurately mimic in vivo tissue phenotypes. These models include spheroids, micropatterned co-cultures bioreactor cultures and microfluidic devices. For comparisons and benchmarking to other 3D liver

**FIGURE 1** Applications of human 3D liver spheroids in translational pharmacology and toxicology. APAP, acetaminophen; COL1A1, collagen 1A1; FASN, fatty acid synthase; G6PC, glucose-6-phosphatase; HBV, hepatitis B virus; HCV, hepatitis C virus; IS, insulin-sensitive; IR, insulin-resistant; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PCK1, phosphoenolpyruvate carboxykinase 1; PHH, primary human hepatocytes; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2. Individual panels were modified with permission from 61,81,82.
models, we refer the interested reader to recent comprehensive reviews.9-16 Spheroids are of value in drug discovery and development where they provide more relevant information regarding drug pharmacokinetics and toxicity (Figure 1). Furthermore, they provide promising tools for the identification of novel targets and drug screening for different liver diseases, including viral hepatitis and other infectious diseases, non-alcoholic fatty liver disease (NAFLD) and hepatic fibrosis. Here, we review the latest development in the field of primary human liver spheroids.

2 | SHORTCOMINGS OF CONVENTIONAL HEPATIC CELL CULTURE SYSTEMS

Conventional cell systems are based on culturing hepatic cells in 2D monolayers, most commonly on rat tail collagen-coated dishes. It has, however, become increasingly clear that such culture conditions do not provide physiological biochemical and biomechanical cues and, as a consequence, human liver cells rapidly lose their phenotypes and liver-specific functions.17 In recent years, substantial research efforts have attempted to elucidate the molecular mechanisms underlying hepatocyte dedifferentiation. High-resolution longitudinal transcriptomic analysis of primary human hepatocytes (PHH) during dedifferentiation showed that first phenotypic alterations were apparent as early as 30 minutes after plating in 2D culture and more than 5000 genes being differentially expressed after 4 hours.18 Dedifferentiation particularly affects genes involved in the complement system, fatty acid turnover and in xenobiotic metabolism. Specifically, expression of important phase I and phase II enzymes, as well as activity of CYP1A2, CYP2C8, CYP2C9, CYP2D6 and CYP3A4, was downregulated between 90% and 99% after 24 hours of monolayer culture.19 These transcriptomic observations align with proteomic data, demonstrating extensive phenotypic remodelling that is most pronounced in drug-metabolizing enzymes and mitochondrial proteins.20,21 Furthermore, dedifferentiation is associated with altered lipid signatures from polyunsaturated fatty acids to saturated fatty acids and mono-unsaturated fatty acids.22

In an attempt to overcome these limitations, monolayer cultures were extended to so-called sandwich cultures, in which hepatocyte monolayer cultures were overlaid with an additional layer of collagen or matrigel.23 In this configuration, hepatocytes retain their cellular morphology and polarity, and feature increased expression of drug transporters for multiple days, which renders them useful for hepatobiliary transport studies. However, dedifferentiation in sandwich culture is only delayed but not prevented and ECM overlays can complicate experiments due to mass transfer barriers and batch-to-batch variability. In light of this background, there remained a need for in vitro liver systems, particularly for long-term repeated dose applications.

3 | LIVER SPHEROIDS—HISTORY, METHODS, ADVANTAGES AND LIMITATIONS

From a historical perspective, it is surprising that a decade passed between the first isolation of hepatocytes from perfused liver in the mid-1970s24 until the first liver spheroids were presented in 1985.25 Even more surprising is that it took an additional two decades for the use of spheroid cultures to become more widespread despite the promising seminal findings of striking improvements in cytoarchitecture, albumin secretion and phenotypic stability25 (Figure 2). The delay in adoption might be at least in part explained by the dogmatic view that 2D hepatocyte cultures were the gold standard for in vitro work using liver cells. Such preparations have been used a long time for investigations of drug metabolism and drug toxicity particularly in industry. Today, we know, as described above, that 2D liver culture systems can be used for short-term investigations of acute toxicity and drug metabolism, but not for repeated dose studies or analyses of chronic drug toxicity, long-term drug effects or ex vivo treatment of hepatic pathologies.

3D liver spheroid systems were initially developed using hepatic cell lines. The first paper describing human liver cell spheroids was published in 1993 using the hepatocellular carcinoma cell line HepG226; however, characterization and further development into a very versatile model for determination of drug hepatotoxicity occurred only almost a decade later.27,28 In 2013, we began using 3D liver spheroids in collaboration with the Heinzle group using the HepaRG cell line, which is a human bipotent progenitor cell line established in an attempt to overcome these limitations, monolayer cultures were extended to so-called sandwich cultures, in which hepatocyte monolayer cultures were overlaid with an additional layer of collagen or matrigel.23 In this configuration, hepatocytes retain their cellular morphology and polarity, and feature increased expression of drug transporters for multiple days, which renders them useful for hepatobiliary transport studies. However, dedifferentiation in sandwich culture is only delayed but not prevented and ECM overlays can complicate experiments due to mass transfer barriers and batch-to-batch variability. In light of this background, there remained a need for in vitro liver systems, particularly for long-term repeated dose applications.

FIGURE 2 Number of PubMed-indexed publications in the area of liver spheroids since 1985. Data represent the result of a systematic database search using the key words “liver spheroids” OR “hepatocyte spheroids” OR “spheroids from liver”
from a liver tumour associated with chronic hepatitis C that can be induced to differentiate into both biliary-like and hepatocyte-like cells. Using this cell model, we found better functionality and performance in spheroids compared to 2D cultures.

We then continued our spheroid work by using PHH from the Hepatology unit at Karolinska University Hospital Huddinge. PHH have the advantage that they have the closest resemblance to human hepatocytes in situ. However, hepatocyte phenotypes can differ extensively between donors, which might increase the number of replicate experiments required. Furthermore, PHH cannot be expanded and the number of available cells from a given donor is finite, which can complicate the confirmation of results, particularly across study sites. Liver spheroids can be generated in hanging drops, in ultra-low attachment (ULA) plates, or, more recently, using bioprinting. In hanging drop culture, cell suspensions aggregate into spheroids in a drop of medium commonly placed on the inside of the lid of a culture dish, and, once formed, the spheroids are transferred into the culture plate format of choice. For spheroid formation in ULA plates, cell suspensions are directly seeded into U-shaped wells with specially treated surfaces that prevent cell attachment and allow for experiments in the same culture vessel. In contrast to the aforementioned methods in which spheroid formation takes 3-7 days depending on the cell model, bioprinted spheroids are fabricated by forced extrusion and layered deposition of hepatic cells in bioink solutions (eg, alginate or gelatin), resulting in the rapid formation of 3D aggregates in <24 hours.

We initially used the hanging drop systems, but due to higher reproducibility and reduced handling time we shifted to ULA plates, and this method has by now virtually replaced hanging drops for spheroid formations. Using proteomic analyses, we found that ULA-cultured PHH spheroids exhibited a striking resemblance in proteomic signatures compared to mature freshly isolated liver cells, whereas conventional 2D monolayer cultures from the same donors rapidly deteriorated. Furthermore, we showed that spheroids retain their overall metabolomic configuration even after multiple weeks in culture. The physiologically relevant molecular phenotypes combined with long-term functional stability opened up possibilities for a multitude of applications in translational pharmacology and toxicology.

4 LIVER SPHEROID APPLICATIONS IN DRUG DISCOVERY AND DEVELOPMENT

4.1 Hepatotoxicity studies

Evaluation of the hepatotoxic liability of drugs or drug candidates constitutes arguably the most prevalent application of liver spheroids. We here provide an update of the use of liver spheroids for drug-induced liver injury (DILI) predictions and mechanistic analyses.

A variety of different liver cell models have been used to generate liver spheroids for prediction of drug hepatotoxicity (Table 1). Early work focused primarily on spheroids generated from hepatic cell lines. Small-scale studies in HepG2 and HepaRG spheroids show elevated metabolic activity and increased sensitivity to diverse sets of hepatotoxins in spheroid formation compared to conventional 2D culture. Furthermore, automated imaging of HepG2 transgenic reporter lines for DNA damage, unfolded protein response or NRF2 activation showed increase reporter stress response in 3D spheroids compared to 2D monolayer culture.

HepaRG cells are generally considered to more closely resemble human hepatocytes than HepG2 cells on both transcriptomic and functional level particularly related to expression of drug-metabolizing enzymes and drug transporters. Phenotypes of HepaRG spheroids could be further

| Table 1 | Overview of the predictive accuracy of different spheroid models using human hepatic cells for the prediction of drug-induced liver injury risk |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Cell model** | **N** (pos/neg) | **Time** | **Endpoint** | **Sens** | **Spec** | **PPV** | **NPV** | **Ref** |
| HepG2 | 149 (97/52) | 4 days | ATP | 58% | 83% | 86% | 51% | 45 |
| HepG2 | 34 (27/7) | 6 days | Imaging-based | 70%c | 83%c | 95%c | 38%c | 39 |
| HepaRG | 149 (97/52) | 4 days | ATP | 47% | 86% | 86% | 45% | 45 |
| HLC | 48 (42/6) | 1-3 days | Imaging-based | Not analysed; lacks classification criteria | | | | |
| PHH | 100 (62/38) | 5 days | ATP | 61% | 79% | 80% | 56% | 63 |
| PHH | 110 (69/41) | 14 days | ATP | 59% | 80% | 84% | 54% | 56 |
| PHH | 123 (70/53) | 14 days | ATP | 69% | 100% | 100% | 71% | 58 |

Abbreviations: HLC, stem cell-derived hepatocyte-like cells; NPV, negative predictive value; PHH, primary human hepatocytes, PPV, positive predictive value; Sens, sensitivity; Spec, specificity.

*Total number of compounds tested. Numbers in brackets indicate the number of DILI positive and DILI negative compounds.

*Refers to time between first exposure and endpoint measurement.

*Statistical measures are based on clustering in contrast to cut-off classifications for all other studies.
improved by culture in liver biomatrix scaffolds generated from decellularized native rat livers. Recent studies used both HepG2 and HepaRG spheroids for detection of genotoxicity using the Comet assay, an electrophoretic measurement for the detection of DNA strand breaks in single cells. While both studies concluded that the respective models are suitable for the identification of carcinogenic action of various genotoxins and, in the case of HepaRG spheroids, also pro-genotoxins, the low number of compounds tested and, importantly, the lack of a sufficient number of non-genotoxic negative controls does not allow to draw firm conclusions. Interestingly, despite the improved functionality of HepaRG cells, a recent large scale benchmarking study using 150 compounds concluded that HepG2 spheroids were more sensitive to hepatotoxic drugs, albeit both systems having overall low accuracy (58% sensitivity and 83% specificity in HepG2 compared to 47% sensitivity and 86% specificity for HepaRG spheroids). In recent years, trends seem to have shifted to the use of induced pluripotent stem cell (iPSC)-derived hepatocyte-like cells (HLCs) and primary mature liver cells for most DILI-related applications. iPSCs can be easily acquired using minimally invasive methods from individuals with specific genetic signatures of interest and self-renew in culture while maintaining genomic stability. One impressive example of this utility is the investigation of toxicity mechanisms of the tyrosine kinase inhibitor pazopanib. In this study, HLCs were established from patients that tolerated pazopanib or experienced pazopanib-induced hepatotoxicity, and pazopanib sensitivity could be carried over into the in vitro context, which resulted in the identification of specific alterations of genes related to iron homeostasis and iron metabolism in susceptible HLCs.

On the flip side, the current differentiation protocols only achieve partial differentiation. Specifically, HLCs display strongly (up to 1000-fold) increased AFP expression (a marker of foetal, immature hepatocytes), whereas HNF4A and ALB levels (markers of fully differentiated hepatocytes) are 10- to 100-fold lower than in mature human hepatocytes, thus suggesting “only” foetal gene expression patterns that resemble dedifferentiated hepatocytes in 2D culture. Multiple studies showed that spheroid culture could improve HLC phenotypes, resulting in increased sensitivity to hepatotoxins. However, even in spheroids, detection of toxicity required compound concentrations in substantial excess of therapeutically relevant concentrations, particularly for those compounds whose toxicity mechanisms involve the metabolic activation into reactive metabolites. Furthermore, no HLC spheroid study published to date provided predictive measures, therefore not allowing to directly compare sensitivity and specificity to other 3D culture models.

Spheroids generated from primary human liver cells constitute in our view the most accurate model for the prediction of hepatotoxic liabilities. In a study using 110 drugs, PHH spheroids cultured in media with undisclosed formulation achieved sensitivity and specificity of 59% and 80%, respectively, which was not higher than in 2D sandwich culture, possibly due to the supplementation with unphysiological levels of serum or growth factors. By contrast, PHH spheroids cultured in chemically defined conditions achieved 69% sensitivity and 100% specificity at therapeutically relevant exposure levels in a largely overlapping set of compounds, considerably outperforming 2D sandwich cultures.

Besides the prediction of hepatotoxicity, human liver spheroids have been used to investigate toxicity mechanisms. Due to their increased metabolic activity, PHH spheroids are particularly suitable to evaluate toxicity caused by drug-drug interactions. For instance, the proton-pump inhibitor omeprazole, a known activator of the aryl hydrocarbon receptor (AhR), induced CYP1A2 activity in PHH spheroids, which in turn resulted in increased metabolic activation and elevated hepatotoxicity of the CYP1A2 substrate dacarbazine.

Using exposure at sub-toxic concentrations and transcriptomic analyses, gene alterations could be identified that were specific for different classes of toxicity mechanisms, including amiodarone for mitochondrial toxicity, chlorpromazine for cholestasis and aflatoxin B1 for genotoxicity. In addition, selective silencing of specific mitochondrial genes led to the elucidation of the mechanisms underlying fuluirdine hepatotoxicity, a compound that resulted in multiple cases of acute liver failure during clinical trials, whose toxic liability had been missed during preclinical development.

Current developments in the use of PHH spheroids for hepatotoxicity studies include the addition of primary human non-parenchymal cells (NPCs). Complementation with Kupffer cells allows to recapitulate inflammatory responses and sensitizes spheroids to specific hepatotoxins, such as trovafoxacin, paroxetine and indomethacin, whereas the majority of compounds show no difference. Furthermore, NPCs have been shown to modulate CYP expression in co-cultured PHH and exert protective effects against acetaminophen toxicity. We anticipate that liver spheroids will be increasingly used for drug toxicity studies in disease models, including viral hepatitis, alcoholic fatty liver disease, NAFLD and other inflammatory conditions, to evaluate potentially sensitizing parameters that can be difficult to assess using current clinical trial designs. For further toxicogenomic analyses of DILI mechanisms, we refer the interested reader to recent comprehensive reviews on this subject.

### 4.2 Clearance predictions

In the past, microsomes, hepatocyte suspension cultures and 2D monolayers of hepatocytes have been used for prediction of drug hepatic clearance and metabolite identification,
whereas only 2D cultures were used for studies of drug-mediated enzyme induction. Importantly, however, the functional lifespan of these cultures is very short (<24 hours for 2D cultures and cell suspensions), which limits the utility of these models for slowly metabolized compounds. 3D models, such as spheroids, have the advantage that they can be used for long-term studies, thus allowing the evaluation of metabolite formation and pharmacokinetic parameters from low clearance drugs. Intrinsic clearance (CL\textsubscript{int}) values of the low clearance compounds tested successfully predicted in vivo CL\textsubscript{int} within 3-fold. Due to improved phenotypes and the possibility for long-term measurements, the 3D system requires much less cells than compared to conventional 2D assays. As a result, the turnover of low clearance compounds can be reproducibly measured using only a single spheroid, composed of 1500-2000 cells, and both primary and secondary major metabolites observed in vivo are identified in spheroid cultures. Notably, however, as in other culture systems, in vivo CL\textsubscript{int} of high clearance compounds was systematically underpredicted, whereas clearance of very low clearance compounds, such as warfarin, remained difficult to measure despite longer measurement horizons. To overcome these issues, attempts were made to pool spheroids. While these remained unsuccessful in conventional ULA plates due to spheroid fusion followed by rapid dedifferentiation, multi-well plates in which each well harbours multiple (up to hundreds) of individual indentations to physically separate spheroids appear promising. Furthermore, the use of mathematical models that simulate spheroid drug disposition constitutes an interesting avenue to optimize exposure regimens.

### 4.3 CYP induction studies

In addition to clearance predictions, the improved hepatic phenotype with intact nuclear hormone receptor signalling allows to study mechanisms of drug-dependent enzyme induction that cannot be adequately evaluated in 2D PHH models. 3D spheroids showed excellent sensitivity and specificity correctly identifying 11 inducers and 13 non-inducers at therapeutic concentrations without false positives or false negatives, outperforming conventional 2D cultures on the same sets of test compounds. Spheroids were moreover able to recapitulate induction mechanisms due to mechanisms that can result in receptor phosphorylation and target gene activation independent of direct ligand binding. One such example is AZD1208, a PIM kinase inhibitor that failed in clinical development due to unexpected CYP3A4 autoinduction. AZD1208 induction was not detected in conventional 2D cultures, whereas 3D spheroids exhibited strong induction and involving the EGFR-MAPK signalling axis. Based on these data, 3D spheroids appear to be the most competent in vitro liver system for prediction of drug-inducible enzyme expression.

### 4.4 Non-alcoholic steatohepatitis (NASH) and fibrosis

NAFLD is a chronic liver disease characterized by excessive intracellular triglyceride accumulation in the absence of excessive alcohol intake. NAFLD encompasses a spectrum of disease severities from benign steatosis to non-alcoholic steatohepatitis, fibrosis and eventual cirrhosis. Due, at least in part, to an increasingly sedentary lifestyle and excessive caloric intake, the number of NAFLD cases has more than tripled in the last decades and NAFLD prevalence increased to an estimated 25% globally. NASH is present in 21% of NAFLD patients with prevalence rates between 3% and 5% of the general population. Common comorbidities associated with NASH are obesity (82%), peripheral insulin resistance (48%), metabolic syndrome (76%) and hypertension (70%). Importantly, no pharmacological therapy for NASH has yet been approved for clinical use and, partly as a consequence, NASH has become the major cause of liver transplantation. New tools are thus urgently needed to aid in discovery and development of promising drug candidates. Traditionally, genetic and diet-induced rodent models were used to study NAFLD, but due to important species differences in metabolic and inflammatory features, gene expression patterns and diets, these do not accurately mimic human NAFLD aetiology and progression. Thus, over the last years, there has been considerable interest in the development of human cell models that can emulate features of human NAFLD.

In spheroids, insulin resistance and steatosis can easily be mimicked by increasing insulin, glucose, fructose and free fatty acid (FFA) concentrations in the medium in a similar fashion as seen in vivo. Liver spheroids consisting of PHH and primary NPCs can moreover mimic the progression from steatosis to NASH in pathophysiological conditions purely driven by metabolic perturbations (elevated FFA levels) without the need to supply extrinsic cytokines. Similarly, fibrosis can be mimicked in spheroids containing the stellate cell line LX2 instead of primary cells or in liver organoids composed of hepatocyte-, stellate- and Kupffer-like cells differentiated from iPSC. However, comparative analyses and benchmarking studies regarding their pathophysiological relevance and added value for drug development have yet to be conducted.

NAFLD has a strong genetic component of which rs738409 in PNPLA3 (I148M) and rs58542926 in TM6SF2 (E167K) have the largest effects. The PNPLA3 I148M mutation is associated with increased lipogenesis, altered retinyl-palmitate lipase activity in hepatic stellate cells and altered lipid profiles, whereas TM6SF2 E167K causes
decreased lipoprotein secretion associated with hypocholesterolaemia and reduced cardiovascular disease risk. Spheroids generated from PHH of TM6SF2 E167K variant carriers showed increased steatosis while PNPLA3 I148M resulted in increased fibrogenesis, thus recapitulating effects of genetic risk factors and allowing for mechanistic studies and the development of stratified drug screening strategies.

Spheroids of PHH and primary human NPCs have furthermore been used for mechanistic studies into NASH pathobiology. Spheroid results demonstrated that IGFBP7, a non-inflammatory factor produced by liver macrophages, activates AKT and ERK signalling in hepatocytes, thus linking macrophage signalling to the induction of lipogenesis, gluconeogenesis and insulin resistance. We also showed that elevated FFA levels induce oxidative stress and miR-144 expression in liver macrophages, resulting in the functional downregulation of its antioxidant target NRF2. Interestingly, liver macrophage-specific silencing of miR-144 increased NRF2 activity and resulted in decreased reactive oxygen species (ROS) production upon FFA exposure. These results demonstrate the potential of liver spheroids in recapitulating communication between the different liver cell types and suggest the possibility to identify new classes of drug candidates that specifically modulate these interactions.

### 4.5 Infectious diseases

Human liver spheroids have been used to study factors involved in hepatic tropism of a variety of hepatotropic infectious diseases. Spheroids from both cell lines (HepG2, HC-04 and HepaRG) and PHH support the liver life cycle of *Plasmodium* malaria parasites, from infection with sporozoites to the release of hepatic merozoites that were capable of infecting erythrocytes. The authors furthermore show that spheroid data for inhibitory concentrations of the antiplasmodial drug candidate M5717 align reasonably well with in vivo data of a Plasmodium infection mouse model, thus indicating that 3D liver spheroids can provide an accessible high-throughput paradigm for the development of prophylactic and cidal antimalarial therapies.

Besides malaria, liver spheroids have been used for the study of a multitude of viral diseases, as well as for antiviral compound screening. To our knowledge, the first application of human liver spheroids for studies of infectious disease was in the context of hepatitis C virus (HCV) infection. HCV uses CD81 as the main but not only entry receptor and, uniquely among the hepatotropic viruses, requires binding of miR-122 to the HCV genomic RNA for replication. PHH spheroids showed stable expression of CD81 and release of viral particles, which was not seen in spheroids generated from HepG2 that feature low to absent CD81 levels. The authors did not evaluate miR-122 expression, as HCV miR-122 dependency was not known at the time of study. However, it later became clear that the lack of miR-122 expression rather than low CD81 levels limited studies of HCV infection in HepG2 cells, as miR-122 overexpression rescued the ability of HepG2 cells to support the entire HCV life cycle.

Additionally, human liver spheroids have been used to study hepatitis B virus (HBV) infection. In an elegant study using a protocol in which PHH are dedifferentiated into proliferating liver progenitor cells followed by redifferentiation post-expansion, the authors could show that redifferentiation in spheroid culture but not 2D monolayer culture resulted in a strong upregulation of the viral entry receptor NTCP (encoded by *SLC10A1*). The resulting spheroids were permissive to HBV infection and secreted newly produced virions. Furthermore, the authors could show antiviral effects of the reverse transcriptase inhibitor entecavir and the NTCP substrate tauroursodeoxycholic acid that blocks HBV binding.

Liver spheroids have also been used to study effects of SARS-CoV-2 infection, as liver injury is observed in up to 60% of severely ill COVID-19 patients. Human hepatocytes are permissive to SARS-CoV-2 infection in spheroids. While PHH normally express only low levels of the SARS-CoV-2 receptor ACE2, its expression is induced upon exposure to pro-inflammatory cytokines, particularly class I interferons, which is paralleled by increased infectivity. Interestingly, SARS-CoV-2 infection tuned the response of hepatocytes to inflammatory signalling, specifically affecting the expression of genes involved in coagulation and platelet activation, thus providing a potential link between liver infection and coagulation defects in COVID-19. The model was furthermore used to validate the antiviral properties of baricitinib, an oral JAK/STAT inhibitor approved for the treatment of rheumatoid arthritis, that had been suggested to inhibit both excessive cytokine signalling and viral endocytosis.

In PHH spheroids, baricitinib prevented virus-mediated effects on the host transcriptomic signatures and reduced infectivity at sub-micromolar concentrations. These findings aligned with curtailed inflammation, fewer serious adverse events and reduced mortality in patients receiving baricitinib plus standard-of-care in multiple trials and has resulted in the US Food and Drug Administration (FDA) emergency use authorization (EUA) of baricitinib in combination with remdesivir for COVID-19 in hospitalized adults requiring supplemental oxygen, invasive mechanical ventilation or extracorporeal membrane oxygenation.

### 5 CONCLUSIONS

In hindsight, it has taken a surprisingly long time between initial presentation and characterization to the widespread adoption of 3D liver spheroids by the scientific community. While
initial studies used only monocultures of hepatic cell lines, there are clear trends towards the use of primary cells and the inclusion of non-parenchymal cell types to mimic their physiological and pathophysiological interactions. Since spheroids retain the molecular and cellular phenotypes of the liver from which they originate, the system allows to recapitulate interindividual variations in liver function, susceptibility to disease and drug-mediated injury. As a consequence, the number of applications of such spheroid models is plentiful, including studies of acute and chronic drug toxicity, pharmacokinetics, drug target validations, mechanisms and pharmacological therapies of various liver diseases, analyses of human liver regeneration and, perhaps, studies of liver cell transformation, such as mechanisms behind viral-induced cell transformation and epithelial to mesenchymal transition.

The rapid increase in the number of papers in the field indicates that liver spheroids might become the new gold standard 3D liver culture system. However, standardization of protocols and methods, as well as careful evaluations of intra- and inter-experimental variability, are required to facilitate further dissemination. These considerations include the choice of culture media, time frames, endpoints, genetic background of the donors, PHH monoculture spheroids vs. multi-lineage co-cultures, as well as the number of intra- and inter-donor replicate experiments required to support a given conclusion. These decisions have to be reevaluated for each application; for instance, the increased simplicity of monoculture PHH spheroids over co-cultures might be warranted when evaluating hepatotoxicity or clearance, whereas co-culture spheroids are required for disease modelling. Furthermore, consistent genotyping and reporting of appropriate genetic polymorphisms of the utilized donors, for instance for variants in PNPLA3 or TM6SF2 for NASH applications or genetic variability in drug-metabolizing enzymes for clearance and toxicity studies, would be an important and easy-to-implement initiative to increase result reproducibility across studies.

While some multicentre trials have already been conducted in which PHH spheroids were compared to other hepatic cell models or culture methods, such as sandwich cultures or microfluidic chips, additional benchmarking studies, ideally conducted in an unbiased joint cross-sectoral setting, are needed. The increasing availability of such characterization and validation data can support informed decision-making by regulators and might eventually result in spheroids achieving regulatory acceptance for applications in translational pharmacology and toxicology.

ACKNOWLEDGEMENTS

MIS acknowledges grant support from the Swedish Research Council [2015-02760], the Swedish Cancer Society [17 0599], and the ERC-AdG project HEAPSPHER [742020]. VML receives support from the Swedish Research Council [2016-01153, 2016-01154 and 2019-01837], from the Strategic Research Programmes in Diabetes (SFO Diabetes) and Stem Cells and Regenerative Medicine (StratRegen) and from the EU/EFPIA/OICR/McGill/KTH/Diamond Innovative Medicines Initiative 2 Joint Undertaking (EUbOPEN grant number 875510), as well as from Merck KGaA and Eli Lilly and Company.

CONFLICT OF INTEREST

MIS and VML are co-founders and shareholders of HepaPredict AB. In addition, VML is co-founder and shareholder of PersoMedix AB and discloses consultancy work for Enginzyme AB.

ORCID

Volker M. Lauschke https://orcid.org/0000-0002-1140-6204

REFERENCES

1. Dixit R, David FS. Market watch: trends in pharmaceutical company R&D spending: 2005–2015. Nat Rev Endocrinol. 2017;16:376.
2. Hay M, Thomas DW, Craighead JL, Economides C, Rosenthal J. Clinical development success rates for investigational drugs. Nat Biotechnol. 2014;32:40-51.
3. Dowden H, Munro I. Trends in clinical success rates and therapeutic focus. Nat Rev Drug Discovery. 2019;18:495-496.
4. Seyhan AA. Lost in translation: the valley of death across preclinical and clinical divide – identification of problems and overcoming obstacles. Transl Med Comm. 2019;4:1-19.
5. Wysowski DK, Swartz L. Adverse drug event surveillance and drug withdrawals in the United States, 1969-2002: the importance of reporting suspected reactions. Arch Intern Med. 2005;165:1363-1369.
6. Downing NS, Shah ND, Aminawung JA, et al. Postmarket safety events among novel therapeutics approved by the US Food and Drug Administration between 2001 and 2010. JAMA. 2017;317:1854-1863.
7. Cook D, Brown D, Alexander R, et al. Lessons learned from the fate of AstraZeneca’s drug pipeline: a five-dimensional framework. Nat Rev Drug Discovery. 2014;13:419-431.
8. Harrison RK. Phase II and phase III failures: 2013-2015. Nat Rev Drug Discovery. 2016;15(12):817–818.
9. Lin C, Khetani SR. Advances in engineered liver models for investigating drug-induced liver injury. Biomed Res Int. 2016;2016:1829148.
10. Ewart L, Dehne E-M, Fabre K, et al. Application of microphysiological systems to enhance safety assessment in drug discovery. Annu Rev Pharmacol Toxicol. 2018;58:65-82.
11. Zhou Y, Shen JX, Lauschke VM. Comprehensive evaluation of organotypic and microphysiological liver models for prediction of drug-induced liver injury. Front Pharmacol. 2019;10:1093.
12. Zhang X, Jiang T, Chen D, Wang Q, Zhang LW. Three-dimensional liver models: state of the art and their application for hepatotoxicity evaluation. Crit Rev Toxicol. 2020;50:279-309.
13. Lauschke VM, Hendriks DFG, Bell CC, Andersson TB, Ingelman-Sundberg M. Novel 3D culture systems for studies of human liver
function and assessments of the hepatotoxicity of drugs and drug candidates. *Chem Res Toxicol*. 2016;29:1936-1955.

14. Lauschke VM, Zandi Shafagh R, Hendriks DFG, Ingelman-Sundberg M. 3D primary hepatocyte culture systems for analyses of liver diseases, drug metabolism, and toxicity: emerging culture paradigms and applications. *Biotecnol J*. 2019;14:e1800347.

15. Shen JX, Youhanna S, Zandi Shafagh R, Kele J, Lauschke VM. Organotypic and microphysiological models of liver, gut, and kidney for studies of drug metabolism, pharmacokinetics, and toxicity. *Chem Res Toxicol*. 2020;33:38-60.

16. Cox CR, Lynch S, Goldring C, Sharma P. Current perspective: 3D spheroid models utilizing human-based cells for investigating metabolism-dependent drug-induced liver injury. *Front Med Technol*. 2020;2:611913.

17. Elaut G, Henkens T, Papeleu P, et al. Molecular mechanisms underlying the dedifferentiation process of isolated hepatocytes and their cultures. *Curr Drug Metab*. 2006;7:629-660.

18. Lauschke VM, Vorrink SU, Moro SML, et al. Massive rearrangement of cellular MicroRNA signatures are key drivers of hepatocyte dedifferentiation. *Hepatology*. 2016;64:1743-1756.

19. Vorrink SU, Ullah S, Schmidt S, et al. Endogenous and xenobiotic metabolic stability and drug metabolism in long-term 3D spheroid cultures revealed by a combination of targeted and untargeted metabolomics. *FASEB J*. 2017;31:2696-2708.

20. Rowe C, Gerrard DT, Jenkins R, et al. Proteome-wide analyses of human hepatocytes during differentiation and dedifferentiation. *Hepatology*. 2013;58:799-809.

21. Heslop JF, Rowe C, Walsh J, et al. Mechanistic evaluation of primary human hepatocyte culture using global proteomic analysis reveals a selective dedifferentiation profile. *Arch Toxicol*. 2016;90:439-452.

22. Kiamehr M, Heiskanen L, Laufer T, et al. Dedifferentiation of primary hepatocytes is accompanied with reorganization of lipid metabolism indicated by altered molecular lipid and miRNA profiles. *Int J Mol Sci*. 2019;20:2910.

23. Kern A, Bader A, Pichlmayr R, Sewing KF. Drug metabolism in hepatocyte sandwich cultures of rats and humans. *Biochim Pharmacol*. 1997;54:761-772.

24. Orrenius S, Thor H, Rajs J, Berggren M. Isolated rat hepatocytes as an experimental tool in the study of cell injury. Effect of anaesthesia. *Forensic Sci*. 1976;8:255-263.

25. Landry J, Bernier D, Ouellet C, Goyette R, Marceau N. Spheroidal aggregate culture of rat liver cells: histotypic reorganization, bio-matrix deposition, and maintenance of functional activities. *J Cell Biol*. 1985;101:914-923.

26. Görlach A, Holtermann G, Jelkmann W, et al. Photometric characteristics of haem proteins in erythropoietin-producing hepatoma cells (HepG2). *Biochem J*. 1993;290:771-776.

27. Khalil M, Shariat-Panahi A, Tootle R, et al. Human hepatocyte cell lines proliferating as cohesive spheroid colonies in alginate markedly upregulate both synthetic and detoxificatory liver function. *J Hepatol*. 2001;34:68-77.

28. Xu J, Ma M, Purcell WM. Characterisation of some cytotoxic endpoints using rat liver and HepG2 spheroids as in vitro models and their application in hepatotoxicity studies. I. Glucose metabolism and enzyme release as cytotoxic markers. *Toxicol Appl Pharmacol*. 2003;189:100-111.

29. Parent R, Marion M, Furio L, Trepo C, Petit M-A. Origin and characterization of a human bipotent liver progenitor cell line. *Gastroenterology*. 2004;126:1147-1156.

30. Gunness P, Mueller D, Shevchenko V, Heinze E, Ingelman-Sundberg M, Noor F. 3D organotypic cultures of human HepaRG cells: a tool for in vitro toxicity studies. *Toxicol Sci*. 2013;133:67-78.

31. Messner S, Agarkova I, Moritz W, Kelm JM. Multi-cell type human liver microtissues for hepatotoxicity testing. *Arch Toxicol*. 2013;87:209-213.

32. Bell CC, Hendriks DFG, Moro SML, et al. Characterization of primary human hepatocyte spheroids as a model system for drug-induced liver injury, liver function and disease. *Sci Rep*. 2016;6:25187.

33. Yang H, Sun L, Pang Y, et al. Three-dimensional bioprinted hepatorgans prolong survival of mice with liver failure. *Gut*. 2021;70:567-574.

34. Goulart E, de Caires-Junior LC, Telles-Silva KA, et al. 3D bioprinting of liver spheroids derived from human induced pluripotent stem cells sustain liver function and viability in vitro. *Biofabrication*. 2019;12:015010.

35. Ramaiyahgari SC, den Braver MW, Herpers B, et al. A 3D in vitro model of differentiated HepG2 cell spheroids with improved liver-like properties for repeated dose high-throughput toxicity studies. *Arch Toxicol*. 2014;88:1083-1095.

36. Gaskell H, Sharma P, Colley HE, Murdoch C, Williams DP, Webb SD. Characterization of a functional C3A liver spheroid model. *Toxicol Res*. 2016;5:1053-1065.

37. Ramaiyahgari SC, Waidyanatha S, Dixon D, DeVito MJ, Paules RS, Ferguson SS. Three-dimensional (3D) HepaRG spheroid model with physiologically relevant xenobiotic metabolism competence and hepatocyte functionality for liver toxicity screening. *Toxicol Sci*. 2017;159:124-136.

38. Ott LM, Ramachandran K, Steno-Bittel L. An automated multiplexed hepatotoxicity and CYP induction assay using HepaRG cells in 2D and 3D. *SLAS Discovery*. 2017;22:614-625.

39. Hiemstra S, Ramaiyahgari SC, Wink S, et al. High-throughput confocal imaging of differentiated 3D liver-like spheroid cellular stress response reporters for identification of drug-induced liver injury liability. *Arch Toxicol*. 2019;93:2895-2911.

40. Hart SN, Li Y, Nakamoto K, Subileau E-A, Steen D, Zhong X-B. A comparison of whole genome gene expression profiles of HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. *Drug Metab Dispos*. 2010;38:988-994.

41. Nelson LJ, Morgan K, Treskes P, et al. Human hepatic HepaRG cells maintain an organotypic phenotype with high intrinsic stress response reporters for identification of drug-induced liver injury liability. *Arch Toxicol*. 2019;133:67-78.

42. Liu J, Li X, Xue R, et al. Liver extracellular matrices bioactivated hepatic spheroids as a model system for drug hepatotoxicity and CYP induction assay using HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. *Drug Metab Dispos*. 2013;41:988-994.

43. Elje E, Hesler M, Sundén-Pran E, et al. The comet assay applied to HepG2 liver spheroids. *Mutat Res*. 2019;845:403033.

44. Mandon M, Huet S, Dubreil E, Fessard V, Le Hégarat L. Three-dimensional HepaRG spheroids as a liver model to study human genotoxicity in vitro with the single cell gel electrophoresis assay. *Sci Rep*. 2019;9:10548.

45. Basharat A, Rollison HE, Williams DP, Ivanov DP. HepG2 (C3A) spheroids show higher sensitivity compared to HepaRG spheroids for drug-induced liver injury (DILI). *Toxicol Appl Pharmacol*. 2020;408:115279.

46. Choudhury Y, Toh Y-C, Xing J, et al. Patient-specific hepatocyte-like cells derived from induced pluripotent stem cells model pazopanib-mediated hepatotoxicity. *Sci Rep*. 2017;7:41238.
47. Baxter M, Withey S, Harrison S, et al. Phenotypic and functional analyses show stem-cell-derived hepatocyte-like cells better mimic fetal rather than adult hepatocytes. J Hepatol. 2015;62:581-589.
48. Zhao X, Zhu Y, Laslett AL, Chan HF. Hepatic differentiation of stem cells in 2D and 3D biomaterial systems. Bioengineering. 2020;7:47.
49. Ghoseh N, Küppers-Munther B, Asplund A, et al. Comparative transcriptomics of hepatic differentiation of human pluripotent stem cells and adult human liver tissue. Physiol Genomics. 2017;49:430-446.
50. Rashidi H, Luu N-T, Alwahsh SM, et al. 3D human liver tissue from pluripotent stem cells displays stable phenotype in vitro and supports compromised liver function in vivo. Arch Toxicol. 2018;92:3117-3129.
51. Holmgren G, Ulfenborg B, Asplund A, et al. Characterization of human induced pluripotent stem cell-derived hepatocytes with mature features and potential for modeling metabolic diseases. Int J Mol Sci. 2020;21:469.
52. Takayama K, Kawabata K, Nagamoto Y, et al. 3D spheroid culture of hES/hiPSC-derived hepatocyte-like cells for drug toxicity testing. Biomaterials. 2013;34:1781-1789.
53. Sirenko O, Hancock MK, Hesley J, et al. Phenotypic characterization of toxic compound effects on liver spheroids derived from iPSC using confocal imaging and three-dimensional image analysis. Assay Drug Dev Technol. 2016;14:381-394.
54. Tasnim F, Toh Y-C, Qu Y, et al. Functionally enhanced human stem cell derived hepatocytes in galactosylated cellulose sponges for hepatotoxicity testing. Mol Pharmacol. 2016;13:1947-1957.
55. Wang Z, Li W, Jing H, et al. Generation of hepatic spheroids using human hepatocyte-derived liver progenitor-like cells for hepatotoxicity screening. Theranostics. 2019;9:6690-6705.
56. Proctor WR, Foster AJ, Vogt J, et al. Utility of spherical human liver microtissues for prediction of clinical drug-induced liver injury. Arch Toxicol. 2017;91:2849-2863.
57. Richert L, Baze A, Parmentier C, et al. Cytotoxicity evaluation using cryopreserved primary human hepatocytes in various culture formats. Toxicol Lett. 2016;258:207-215.
58. Vorrink SU, Zhou Y, Ingelman-Sundberg M, Lauschke VM. Prediction of drug-induced hepatotoxicity using long-term stable primary hepatic 3D spheroid cultures in chemically defined conditions. Toxicol Sci. 2018;163:655-665.
59. Bell CC, Dankers ACA, Lauschke VM, et al. Comparison of hepatic 2D sandwich cultures and 3D spheroids for long-term toxicity applications: a multicenter study. Toxicol Sci. 2018;162:655-666.
60. Mizoi K, Hosono M, Kojima H, Oghihara T. Establishment of a primary human hepatocyte spheroid system for evaluating metabolic toxicity using darcabazine under conditions of CYP1A2 induction. Drug Metab Pharmacokinet. 2020;35:201-206.
61. Bell CC, Lauschke VM, Vorrink SU, et al. Transcriptional, functional, and mechanistic comparisons of stem cell-derived hepatocytes, HepaRG cells, and three-dimensional human hepatocyte spheroids as predictive in vitro systems for drug-induced liver injury. Drug Metab Dispos. 2017;45:419-429.
62. Hendriks DFG, Hurrell T, Riede J, van der Horst M, Tuovinen S, Ingelman-Sundberg M. Mechanisms of chronic fialuridine hepatotoxicity as revealed in primary human hepatocyte spheroids. Toxicol Sci. 2019;171:385-395.
63. Li F, Cao L, Parikh S, Zuo R. Three-dimensional spheroids with primary human liver cells and differential roles of kupffer cells in drug-induced liver injury. J Pharm Sci. 2020;109:1912-1923.
64. Bell CC, Chouhan B, Andersson LC, et al. Functionality of primary hepatic non-parenchymal cells in a 3D spheroid model and contribution to acetaminophen hepatotoxicity. Arch Toxicol. 2020;94:1251-1263.
65. Jiang J, Pieterman CD, Ertaylan G, Peeters RLM, de Kok TCM. The application of omics-based human liver platforms for investigating the mechanism of drug-induced hepatotoxicity in vitro. Arch Toxicol. 2019;93:3067-3098.
66. Lauschke VM. Toxicogenomics of drug induced liver injury – from mechanistic understanding to early prediction. Drug Metab Rev. 2021;1-8.
67. Mizoi K, Arakawa H, Yano K, Koyama S, Kojima H, Oghihara T. Utility of three-dimensional cultures of primary human hepatocytes (Spheroids) as pharmacokinetic models. Biomedicines. 2020;8:374.
68. Kanebratt KP, Janefeldt A, Vilén L, et al. Primary human hepatocyte spheroid model as a 3D in vitro platform for metabolism studies. J Pharm Sci. 2021;110:422-431.
69. Riede J, Wollmann BM, Molden E, Ingelman-Sundberg M. Primary human hepatocyte spheroids as an in vitro tool for investigating drug compounds with low clearance. Drug Metab Dispos. Resubmitted.
70. Leedale JA, Kyffin JA, Harding AL, et al. Multiscale modelling of drug transport and metabolism in liver spheroids. Interface Focus. 2020;10:20190041.
71. Hendriks DFG, Vorrink SU, Smutny T, et al. Clinically relevant cytochrome P450 3A4 induction mechanisms and drug screening in three-dimensional spheroid cultures of primary human hepatocytes. Clin Pharmacol Ther. 2020;108:844-855.
72. Smutny T, Hyrova L, Braeuning A, Ingelman-Sundberg M, Pavek P. Transcriptional and post-transcriptional regulation of the pregnane X receptor: a rationale for interindividual variability in drug metabolism. Arch Toxicol. 2021;95:11-25.
73. Jones BC, Rollison H, Johansson S, et al. Managing the risk of CYP3A induction in drug development: a strategic approach. Drug Metab Dispos. 2017;45:35-41.
74. Younossi Z, Anstee QM, Marietti M, et al. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. Nat Rev Gastroenterol Hepatol. 2018;15:11-20.
75. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Aliment Pharmacol Ther. 2011;34:274-285.
76. Younossi ZM, Tampi R, Priyadarshini M, Nader F, Younossi IM, Racila A. Burden of illness and economic model for patients with nonalcoholic steatohepatitis in the United States. Hepatology. 2019;69:564-572.
77. Møller FA, Sturla SJ. Human in vitro models of nonalcoholic fatty liver disease. Curr Opin Toxicol. 2019;16:9-16.
78. Soret P-A, Magusto J, Houset C, Gauteron J. In vitro and in vivo models of non-alcoholic fatty liver disease: a critical appraisal. J Clin Med. 2020;10:36.
79. Hundtemark J, Tacke F. How effective are nonalcoholic fatty liver disease models for drug discovery? Expert Opin Drug Discov. 2020;15:1237-1240.
80. Kozyra M, Johansson I, Nordling Å, Ulah S, Lauschke VM, Ingelman-Sundberg M. Human hepatic 3D spheroids as a model for steatosis and insulin resistance. Sci Rep. 2018;8:14297.
81. Kemas AM, Youhanna S, Zandi Shafagh R, Lauschke VM. Insulin-dependent glucose consumption dynamics in 3D primary
human liver cultures measured by a sensitive and specific glucose sensor with nanoliter input volume. *FASEB J*. 2021;35:e21305.

82. Hurrell T, Kastrinou-Lampou V, Fardellas A, et al. Human liver spheroids as a model to study aetiology and treatment of hepatic fibrosis. *Cells*. 2020;9:964.

83. Romualdo GR, Da Silva TC, de Albuquerque Landi MF, et al. Sorafenib reduces steatosis-induced fibrogenesis in a human 3D co-culture model of non-alcoholic fatty liver disease. *Environ Toxicol*. 2021;36:168-176.

84. Ouchi R, Togo S, Kimura M, et al. Modeling steatohepatitis in humans with pluripotent stem cell-derived organoids. *Cell Metab*. 2019;30:374-384.

85. Krawczyk M, Liebe R, Lammert F. Toward genetic prediction of nonalcoholic fatty liver disease trajectories: PNPLA3 and beyond. *Gastroenterology*. 2020;158:1865-1880.

86. Romeo S, Sanayal A, Valenti L. Leveraging human genetics to identify potential new treatments for fatty liver disease. *Cell Metab*. 2020;31:35-45.

87. Prill S, Caddeo A, Baselli G, et al. The TM6SF2 E167K genetic variant induces lipid biosynthesis and reduces apolipoprotein B secretion in human hepatic 3D spheroids. *Sci Rep*. 2019;9:686-712.

88. Pingitore P, Sasidharan K, Ekstrand M, Prill S, Lindén D, Romeo S. Human multilineage 3D spheroids as a model of liver steatosis and fibrosis. *Int J Mol Sci*. 2019;20:1629.

89. Morgantini C, Jager J, Li X, et al. Liver macrophages regulate systemic metabolism through non-inflammatory factors. *Nature Metabolism*. 2019;1:445-459.

90. Azzimato V, Jager J, Chen P, et al. Liver macrophages inhibit the endogenous antioxidant response in obesity-associated insulin resistance. *Sci Transl Med*. 2020;12:eaaaw9709.

91. Chua ACY, Ananthanarayanan A, Ong JJY, et al. Hepatic spheroids used as an in vitro model to study malaria relapse. *Biomaterials*. 2019;216:119221.

92. Arez F, Rebelo SP, Fontinha D, et al. Flexible 3D cell-based platforms for the discovery and profiling of novel drugs targeting plasmodium hepatic infection. *ACS Infect Dis*. 2019;5:1831-1842.

93. Zeisel MB, Fedana I, Fufi-Kremer S, Baumert TF. Hepatitis C virus entry into hepatocytes: molecular mechanisms and targets for antiviral therapies. *J Hepatol*. 2011;54:566-576.

94. Luna JM, Scheel TKH, Danino T, et al. Hepatitis C virus RNA functionally sequesters miR-122. *Cell*. 2015;160:1099-1110.

95. Chong TW, Smith RL, Hughes MG, et al. Primary human hepatocytes in spheroid formation to study hepatitis C infection. *J Surg Res*. 2006;130:52-57.

96. Narbus CM, Israelow B, Sourisseau M, et al. HepG2 cells expressing microRNA miR-122 support the entire hepatitis C virus life cycle. *J Virol*. 2011;85:12087-12092.

97. Fu G-B, Huang W-J, Zeng M, et al. Expansion and differentiation of human hepatocyte-derived liver progenitor-like cells and their use for the study of hepatotrophic pathogens. *Cell Res*. 2019;29:8-22.

98. Zhang C, Shi L, Wang F-S. Liver injury in COVID-19: management and challenges. *Lancet Gastroenterol Hepatol*. 2020;5:428-430.

99. Stebbing J, Krishnan V, de Bono S, et al. Mechanism of baricitinib supports artificial intelligence-predicted testing in COVID-19 patients. *EMBO Mol Med*. 2020;12:e12697.

100. Stebbing J, Sánchez Nievas G, Falcone M, et al. JAK inhibition reduces SARS-CoV-2 liver infectivity and modulates inflammatory responses to reduce morbidity and mortality. *Sci Adv*. 2021;7(1):eabe4724.

101. Richardson P, Griffin I, Tucker C, et al. Baricitinib as potential treatment for 2019-nCoV acute respiratory disease. *Lancet*. 2020;395:E30-E31.

102. Bronte V, Ugol S, Tinazzi E, et al. Baricitinib restrains the immune dysregulation in patients with severe COVID-19. *J Clin Invest*. 2020;140:6409-6416.

103. Kalil AC, Patterson TF, Mehta AK, et al. Baricitinib plus Remdesivir for hospitalized adults with Covid-19. *N Engl J Med*. 2020;384:795-807.

104. Ingelman-Sundberg M, Lauschke VM. Human liver spheroids in chemically defined conditions for studies of gene-drug, drug-drug and disease-drug interactions. *Pharmacogenomics*. 2018;19:1133-1138.

105. Oliva-Vilarnau N, Vorrink SU, Ingelman-Sundberg M, Lauschke VM. A 3D cell culture model identifies Wnt/β-catenin mediated inhibition of p53 as a critical step during human hepatocyte regeneration. *Adv Sci*. 2020;7:2000248.

106. Rubiano A, Indapurkar A, Yokosawa R, et al. Characterizing the reproducibility in using a liver microphysiological system for assessing drug toxicity, metabolism, and accumulation. *Clin Transl Sci*. 2020. [Epub ahead of print]

How to cite this article: Ingelman-Sundberg M, Lauschke VM. 3D human liver spheroids for translational pharmacology and toxicology. *Basic Clin Pharmacol Toxicol*. 2021;00:1–11. [https://doi.org/10.1111/bcpt.13587]