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PII: S2352-345X(22)00245-4
DOI: https://doi.org/10.1016/j.jcmgh.2022.11.008
Reference: JCMGH 1114

To appear in: *Cellular and Molecular Gastroenterology and Hepatology*
Accepted Date: 23 November 2022

Please cite this article as: Kaur S, Kidambi S, Ortega-Ribera M, Thanh Thuy LT, Nieto N, Cogger VC, Xie W-F, Tacke F, Gracia-Sancho J, *In vitro* models for the study of liver biology and diseases – advances and limitations, *Cellular and Molecular Gastroenterology and Hepatology* (2022), doi: https://doi.org/10.1016/j.jcmgh.2022.11.008.

In vitro models for the study of liver biology and diseases – advances and limitations

Short title: In vitro methods to study liver (patho)biology

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Financial support

S.K. is supported by the ASEAN grant (CRD/2019/000120) from Department of Science and Technology, India.

S.K. is supported, in whole or in part, by NIH grants 1R01AA027189-01A1, P20 GM104320 (to the Nebraska Center for the Prevention of Obesity Diseases Pilot Grant), P20 GM113126 (to the Nebraska Center for Integrated Biomolecular Communication), and Nebraska Research Initiative-Systems Grant.

L.T.T.T. received support from the Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS-22K08083, 22H03061) and a Grant for Research Program on Hepatitis from the Japan Agency for Medical Research and Development (AMED- JP22fk0210107).

N.N. received funding from the US Public Health Service Grant R01 DK111677 from the National Institute of Diabetes and Digestive and Kidney Diseases, R01AA025907 from the National Institute on Alcohol Abuse and Alcoholism, and US Veterans Administration Grant I01BX005093 from the Biomedical Laboratory Research & Development.

V.C.C is supported by the McKnight Philanthropic Bequest and National Health and Medical Research Council

W-F.X. is supported by the National Natural Science Foundation of China (82030021).

F.T. is supported by the German Research Foundation (DFG SFB/TRR 296 and CRC1382, Project-ID 403224013) and the German Ministry of Education and Research (BMBF DEEP-HCC consortium).

J.G.-S. is supported by the Instituto de Salud Carlos III (FIS PI20/00220, co-funded by the European Union), the CIBEREHD, the Swiss National Science Funds (320030_189252), the Novartis Foundation for Medical-Biological Research and the Foundation Suisse Contre le Cancer du Foie.

Disclosures

FT's lab received research grants from Gilead, Allergan, Bristol-Myers Squibb and Inventiva.

JG-S' lab received research funding from Gilead, Inventiva, Conatus, Surrozen, GAT Tx and BrudyLab.

S.K., S.K., M.O-R., L. T.T.T, N.N, V.C.C. and W.-F.X have nothing to disclose.

Word count: 4980

Abbreviations

iPSCs	induced pluripotent stem cells
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
LoC	liver-on-a-chip
CLD	chronic liver disease
HSCs	hepatic stellate cells
2D	two-dimensional
HBV	hepatitis B virus

LSECs	liver sinusoidal endothelial cells
ECM	extracellular matrix
3D	three-dimensional
PLA	polylactic acid
PLGA	poly (lactic-co-glycolic acid)
BEASTS	bio-engineered adhesive siloxane substrate with tunable stiffness
PDMS	polydimethyl siloxane
KLF2	krüppel-like factor 2
sc-RNA Seq	single cell RNA sequencing
PCLS	precision cut liver slices
HCC	hepatocellular carcinoma
PNPLA3	patatin like phospholipase domain containing 3
MS	mass spectrometry
PDGFβ	platelet derived growth factor beta
ECs	endothelial cells

Abstract

In vitro models of liver (patho)physiology, new technologies and experimental approaches are progressing rapidly. Based on cell lines, induced pluripotent stem cells (iPSCs) or primary cells derived from mouse or human liver as well as whole tissue (slices), such *in vitro* single- and multi-cellular models, including complex microfluidic organ-on-a-chip systems, provide tools to functionally understand mechanisms of liver health and disease. The International Society of Hepatic Sinusoidal Research (ISHSR) commissioned this working group to review the currently available *in vitro* liver models and describe the advantages and disadvantages of each in the context of evaluating their use for the study of liver functionality, disease modelling, therapeutic discovery and clinical applicability.

Keywords

Hepatic sinusoid, mechanobiology, omics, bioengineering, cirrhosis, NAFLD, NASH

1- Introduction

Liver disease represents one of the leading causes of death worldwide, and the incidence of some pathologies, such as non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH) and liver cancer, continues to rise [1]. Despite years of research, liver diseases still have limited treatment options in the clinic. This paucity of treatments is partly explained by the limitations of traditional *in vitro* tools and animal models that do not accurately mimic the clinical pathophysiology of diseases and have a low accuracy for drug discovery purposes. Indeed, several studies have shown that traditional cell culture methodologies do not reflect the complexity of a human liver *in vivo* and thus cannot predict drug sensitivity. In contrast, animal models differ in biology compared to human pathologies, which explains why promising therapies tested in animal models often fail when tested in humans and, unfortunately, the field of hepatology has numerous recent examples of failures in clinical phases [2]. With the advent of precision medicine, which offers much hope for individual patient outcomes, there is increased demand for robust and patient-specific tools to better improve our understanding and treatment of complex and multifactorial diseases such as liver diseases. Advances in vascular biology, microfluidics, and bioengineering have led to the development of sophisticated *in vitro* models that could fill this gap (Figure 1). In addition, omics techniques provide further insight to pre-clinical research in hepatology. In this review, we discuss the benefits and limitations of advanced *in vitro* research techniques that are presently being applied to the study of liver diseases and further critique how these tools may provide an insight into the prediction of patients' responses to a therapy.

2- Liver-on-chip & microfluidic devices

During the XXI century, the development of biology-inspired devices aimed at mimicking the sinusoidal niche integrating microfluidics led to the rapidly evolving liver-on-a-chip (LoC) technology [3]. The design of these in vitro liver-resembling tools, which have been

extensively reviewed in [4] and is out of the scope of the present review, are inspired in sinusoidal cell biology, architecture and hemodynamics but materialized under each research teams' eyes in terms of appearance, size, fabrication procedures, costs, and microfluidics integration, leading to significant variation in the finalized product.

Latest advances in the field include chronic liver disease-specific devices, LoC models designed to study key pathophysiological processes in the development of liver disease and to understand the interconnection with other organs-on-chip to better depict liver functions and systemic implications (Figure 2). Multi-organ chips, for instance, liver-, adipose- and gut-on-a-chip connected, may be particularly suitable to understand organ-crosstalk in chronic liver disease (CLD) such as NAFLD/NASH or cholangiopathies [5].

In the recent years, disease-focused LoC devices mimicking some of the landmark etiological characteristics of CLD have been developed. Fat accumulation in hepatocytes, occurring in NAFLD/NASH, has been represented in LoC under the combination of glucose and free fatty acids (usually a 2:1 ratio of oleic and palmitic acid) [6]. Antifibrotic compounds such as obeticholic acid, elafibranor [7], pioglitazone or metformin [8,9] showed promising results in reducing lipid droplets in these *in vitro* settings. Indeed, the anti-NASH agent lanifibranor efficiently reduced hepatocytic lipid accumulation [10], and improved human hepatocyte and hepatic stellate cells (HSCs) phenotype [11] in a LoC model but not in two-dimensional (2D) cell cultures, supporting the specific value of multicellular LoC devices over traditional mono-cell cultures. Alcohol-associated liver disease has been addressed in several publications focusing on its impact in sinusoidal cell biology during development [12] or recovery from alcohol (abstinence) either with perfused spheroid [13] or layered cultures [14]. Importantly, Ortega-Prieto et al [15] developed a model for hepatitis B virus (HBV) long-term infection in primary human hepatocytes which recapitulates virus-host interactions and its associated immune effectors. LoC using primary cells isolated using standardized protocols from pre-clinical models of CLD and from patients have also been developed [16,17]. These

specialized LoC settings may widen the current knowledge on disease dynamics and provide potential applicability as *in vitro* preclinical models for drug screening.

Even though LoC complexity has outstandingly increased since the initial models/prototypes, the intricacy of the whole liver is still underrepresented. In this regard, several scientists brought the attention and focused their studies into specific processes or structures. For example: the essential features of the bile duct containing primary mouse cholangiocytes [18], the unique vasculature organization of the liver [19], the sinusoidal zonation within LoC devices [20], neutrophil recruitment and interaction with liver sinusoidal endothelial cells (LSECs) after lipopolysaccharide stimulation [21] or drug metabolism and toxicity [22] are now embedded in available LoC systems.

Moreover, CLD has been extensively reported as a systemic syndrome with major extrahepatic implications [23]. Therefore, the combination of LoC devices has now evolved to the extent that disease specific models are being combined with others such as intestine, brain, kidney [24–26] or even metastasis niche-on-chip models [27] to recreate body-on-chip structures to further study gut-liver-brain axis, systemic drug clearance or exosome communication between the liver and the tumor microenvironment. However, alongside these advances in multi-organ and etiology-centered approaches, a lack of consensus in cellular sources and mechanobiological cues within the various LoC models remain as unsolved challenges in the field.

3- Liver scaffolds, matrices & other substrates

A key component for the engineering of *in vitro* liver models is the development of appropriate scaffold/matrix that recapitulates the hepatic microenvironment well-enough to result in realistic functional cells. Several factors affect the efficiency of a scaffold as a support for liver cell growth and function including porosity, pore size, biomechanical properties, and the scaffold design. To simulate the microenvironment of natural extracellular matrix (ECM), substrate design and biomechanical properties are of great

significance; hence, bioinspired and biomimetic approaches have been explored to model the healthy or damaged liver (Figure 3). Double layers of collagen have been used for years as a well-established 2D *in vitro* model for sandwich cultures of hepatocytes [28]. Recent studies have identified, and characterized, the hepatic matrisome comprising ECM signatures beyond collagen that can potentially provide matrix for *in vitro* systems to study liver diseases [29,30].

Scaffolds have been fabricated using natural polymers such as gelatin, elastin, silk fibroin, chitosan, chitin, fibrin, and fibrinogen or synthetic polymers like polylactic acid (PLA), poly(glycolic acid), polyhydroxyalkanoate, and poly (lactic-co-glycolic acid) (PLGA) [31]. Modified versions of biomaterials such as collagen incorporated PLGA have resulted in enhancement of hepatocyte survival and functions likely due to an increase in the bioactivity of the newly developed scaffolds [32,33]. Similarly, natural polymers such as silk fibroin have been modified with arginyl-glycyl-aspartic acid or RGD (an integrin-based cell adhesion motif), that has been reported to support the growth of functional hepatocyte clusters [34]. The modification with RGD may also support attachment of LSECs, known as endothelization of materials [35]. Efficient spheroid cultures of hepatocytes have been reported on highly porous hydrogel scaffolds composed of alginate and galactosylated chitosan [36]. Additionally, synthetic polymer thin films-based scaffolds allow organized hepatocytes culture and patterned co-culture of hepatocytes with non-parenchymal cells [37–39]. Recent interest in mechanical signaling has led to the development of scaffolds that can recreate liver stiffness in physiological and pathological conditions. In this context, heparin hydrogel has been developed to modulate stiffness and has demonstrated that hepatocytes cultured on a softer heparin hydrogel (10 kPa) retained five times higher levels of albumin production compared to those on a stiffer heparin gel (110 kPa) after 5 days [40]. Primary hepatocytes grown on modified polyacrylamide gels with cell adhesive ligands are shown to reduce albumin production and impair hepatocyte function with increasing stiffness [41,42]. Chang and co-

workers used polyacrylamide gels to tune the substrate stiffness and demonstrated that fibrotic levels of stiffness significantly inhibit hepatocyte-specific functions in part through the inhibition of the hepatocyte nuclear factor 4α transcriptional network mediated via the Rho/Rho-associated protein kinase pathway [43]. An innovative platform named BEASTS (Bio-Engineered Adhesive Siloxane substrate with Tunable Stiffness) based on a polydimethyl siloxane (PDMS) substrate in combination with polyelectrolyte multilayer film-coating technology was developed to engineer mechanically tunable substrates mimicking physiologic and pathologic liver stiffness [44–48]. More recently, 3D bioprinting has emerged for precise spatial positioning of both cells and biomaterials or bioinks such as alginate together in 3D complex geometries and providing mechanical support [49,50]. Nguyen et al have bioprinted hepatocytes and non-parenchymal cells in 3D architecture and developed models of drug induced liver injury [51]. Recent studies have printed liver cells along with a liver decellularized ECM bioink creating an environment for maximal cellular function [52–54]. With 3D bioprinting, vascular and biliary fluidic channels have also been successfully created in the LoC device format [53]. 3D bioprinting of spheroids and organoids represent the next level of technological advancement for creating the highly complex liver architecture [55].

4- Liver spheroids and organoids

As described above, over the last century, 2D cell cultures have been used as common *in vitro* models to study cellular responses to stimulations and allowing the construction of low-cost, simple and well-accepted models of liver disease. However, they do not precisely reflect the true physiological state of cells *in vivo* due to the absence of structural, mechanical, and biochemical cues, as well as the interaction between cells and extracellular matrices [56]. To overcome this limitation, novel 3D cell culture platforms including liver spheroid and organoid cultures are being created to better mimic the *in vivo* conditions [57–61]. 3D spheroids are produced via self-assembly, in which mono-dispersed cells form 3D microtissues called multi-cellular spheroids, and mimic natural

processes that occur during embryogenesis, morphogenesis and organogenesis [62]. 3D organoids derive from either pluripotent stem cells, neonatal tissue stem cells or adult stem cells/adult progenitors, in which cells spontaneously self-organize into properly differentiated functional cell types and progenitors, resembling their *in vivo* counterparts and recapitulating at least some functions of the organ [63].

In the field of studying liver diseases, recent innovation of hepatic 3D spheroids also offer a promising application via combination of 3D printing based techniques and HepG2 liver spheroid culture models to develop *in situ* quantitative evaluation and high-throughput monitoring of drug-induced hepatotoxicity [64]. HepG2 cell-laden hydrogel constructs were 3D printed in the shape of a cross on the mini-9-well plate which showed the of HepG2 liver spheroids embedded in the gelatin-alginate hydrogel. On the 6th day of culture, HepG2 liver spheroids exposed to varying concentrations of troglitazone and nefazodone were used to predict hepatotoxicity. This model provided a promising tool for screening and characterization of hepatotoxicity in a 3D spheroid-embedded hydrogel system that more closely resembles conditions in vivo.

In 2013, Takebe et al. first described the *in vitro* generation of 3D liver buds organoids from human iPSCs derived hepatic endoderm cells co-cultured with endothelial and mesenchymal lineages [59]. Interestingly, when these liver buds were ectopic transplanted at various sites including the cranium, subrenal capsule, distal-, and proximal-mesentery in immunodeficient mice, they were able to rescue the drug induced lethal liver failure model [61,65]. These studies have provided a promising new approach to study regenerative medicine and to translate these techniques for treating patients with end-stage liver failure [65]. Furthermore, single cell RNA sequencing (scRNAseq) data from human liver bud organoids revealed several aspects of heterotypic interlineage communication and organ development [66]. Interestingly, Shinozawa et al. reported a simple, robust, and high-throughput human liver organoid system to measure bile transport activity by live fluorescent imaging with large-scale screening and multiplexed

readouts. By using this system, the study analyzed the pathology of drug induced liver injury and provided the possibility to assess varying drug susceptibilities based on individual polymorphism at organoid resolution [67]. These approaches are undergoing rapid developments, allowing to establish human organoids from adult/fetal human liver or pluripotent stem cells and modelling different liver diseases [68,69,70,71]. Different types of liver organoid models from mice, humans, dogs, cats are now available for several monogenic liver diseases such as Alagille syndrome, cystic fibrosis, primary sclerosing cholangitis, Wilsons disease, HBV infection, steatosis or liver cancer amongst others [72–78]. The generation of organoids from adult patient liver tissues also retains the genetic background of the individual patient thus creating patient-specific disease models and enabling in-depth investigations of pathogenesis mechanisms underlying genetic diseases and cancer. In conclusion, induced liver buds and liver organoids provide a platform for cell-based therapy, liver disease models, drug screening which satisfy the demands of both basic and translational biomedical research.

5- Tissue-based approaches

Precision cut liver slices (PCLS) are a native liver-like *ex vivo* model with intact intercellular and cell-matrix interactions [79]. PCLS systems use *ex vivo* liver explants with a well-defined thickness and in comparison, to the primary hepatocytes that are short-lived and lose much of their function in culture, PCLS cultures have been maintained for 15 days under optimal conditions. Hepatocytes in slices retain their membrane and intracellular polarization, in contrast to isolated hepatocytes, which lose their anatomical polarity after isolation. PCLS cultures have been established both from murine and human livers (Figure 3).

Human tissue for PCLS are obtained from explanted, resected, or non-transplantable tissues from liver tumor patients undergoing transplantation or liver resection. Liver slices can also be obtained from patients with severe fibrosis and cirrhosis undergoing transplantation. These are usually obtained using Krumdieck (now ‘Alabama’) tissue

slicer to make liver slices with their diametric from 5-8 mm [79,80], their thickness at 250-350 µm [81]. These slices are then cultured with William's' E medium in regular tissue plates either in static, dynamic or bioreactor-based culture systems. In static conditions, PCLS cultures have a shorter lifespan (24-48 hours) due to hypoxia and increased cell death. To minimize hypoxic death, strategies such as the use of synthetic oxygen carriers, rocking or shaking cultures or perfusion bioreactors have been employed to provide better perfusion of oxygen and media components [82,83]. One study has reported PCLS *ex vivo* cultures with sustained viability for over a two-week period on a rocking platform [84]. Through microarray profiling of purified individual cells, this study illustrated that all liver cells undergo changes in their gene expression profiles until day 4 of PCLS cultures, however these changes seem to be stabilized from day 4 until day 15.

Recently, a study has cultured PCLS on a bioreactor platform at a flow rate of about 18 µL/sec, which imparted functional longevity to the system for about 6 days without any hepatocellular stress and fibrogenesis [83]. Using this system, the study also successfully modelled *ex vivo* liver fibrogenesis using transforming growth factor β1 and platelet-derived growth factor (PDGFβ) stimulation. In another similar culture platform, primary hepatocytes or liver stem cells have been cultured on ECM discs developed from a decellularized porcine [85] or rat liver [86].

Human PCLS cultures have proven indispensable for modelling of liver diseases and to the study of transport, metabolism, biotransformation of drugs, toxins and xenobiotics in both normal and diseased conditions [87–89]. They have also been employed to study ischemia/reperfusion damage in rodents and to evaluate the efficacy, specificity and toxicity of virus-mediated gene therapy agents [80,90].

With improved technological advancements and culture longevity, PCLS cultures of patient-specific tissues offers enormous potential for the characterization of patient-specific liver cellular heterogeneity and for the screening of novel anti-fibrotic and anti-tumorigenic drugs.

6- Mimicking the sinusoidal mechanobiology *in vitro*

LSECs, the second most abundant cell type in the liver, are a key players in maintaining hepatic homeostasis [91]. Importantly, LSECs differ from classical vasculature endothelium, as they lack an organized basement membrane and have cytoplasm that is penetrated by open fenestrae, making the hepatic microvascular endothelium discontinuous [92]. LSECs behavior is largely regulated by shear stress and mechanical stretch induced by blood perfusion and liver microenvironment stiffness changes derived from deposition of ECM [93–96]. The effect of these varying mechanical cues on LSECs is particularly interesting, however, this has not been extensively explored until recently. Employing *in vitro* culture models of LSECs with microfluidic setups revealed the effects of shear stress-derived effects. In a pioneering work from the Sessa and Groszmann labs, authors demonstrated that LSECs respond to increasing shear stress in the microenvironment by increasing nitric oxide (NO) synthesis [97]. Subsequent work defined the upstream signaling pathways, including the induction of the transcription factor Krüppel-like factor 2, in both healthy and cirrhotic LSECs [98]. A recent study by Shah and co-workers further elucidated the role of the mechano-sensitive pathways in LSECs which drive recruitment of circulating blood cells contributing to portal hypertension and fibrogenesis [99]. Using a Flexcell device, cyclic biaxial stretch on murine LSEC was modelled, and demonstrated transcriptional up-regulation of several chemotactic cytokines (CXCL1, CXCL2, and CCL2), neutrophil-extracellular traps activation from the recruited neutrophils and microthrombi formation contributing to fibrosis. More recently, a LoC device with microfluidics was used to mimic physiological and pathological pressures on primary LSECs culture [100]. Transcriptomic analysis revealed the detrimental effect of increased pressure on LSECs phenotype and allowed to identify LSEC-derived pressure-related genes as non-invasive biomarkers for portal hypertension. Altogether these data demonstrate that mechanical cues can cause

angiocrine and phenotypic changes in LSECs, leading to rapid alteration of HSCs phenotype and fibrogenesis [101].

In fibrotic livers, microvasculature remodeling contributes to increased ECM deposition and consequent raise in the intrahepatic vascular resistance [92]. Wells and colleagues demonstrated that increased stiffness induced activation of HSCs [102]. Juin and co-workers showed that increased ECM matrix rigidity increased the number of podosomes (actin-rich structures involved in motility and proteolysis) formed in LSECs suggesting that the cells responded to mechanical stress, however effect on LSEC function was not explored [103]. Impairment of hepatocyte and stellate cell function in response to high stiffness has been previously described in the literature [41,43,102,104]. In the context of liver-specific endothelial cells, a recent publication demonstrated that LSECs also dedifferentiate in high stiffness conditions, losing their capacity of producing vasoactive mediators such as NO and becoming capillarized as shown by the loss of their characteristic fenestrae. Interestingly, authors point out the tension between the cytoskeleton and the nuclear shape as process transducing the stiffness sensing into phenotypical responses in all sinusoidal cells [105]. Importantly, this last study also demonstrated that cirrhotic liver cells improve their phenotype when cultured in a healthy (non-stiff) environment, suggesting potential new avenues of therapy development. In an unpublished work, Kidambi and co-workers confirmed that LSECs are responsive to stiffness resulting in rapid capillarization (loss in fenestrae), loss of hyaluronic acid endocytosis, and higher cell adhesion molecules [46].

These advanced *in vitro* experiments point to an interesting and underexplored area of the role of mechanical stimuli on sinusoidal biology during physiological and pathological conditions. The key to unlocking the potential therapeutic avenues for sinusoidal dysfunction from these *in vitro* findings will be to integrate the data with *in vivo* functions.

7- “omics” for the study of liver cells

Advances in omics methods have led to discoveries in liver biology and pathology at the cellular, tissue and system levels. These methods have also facilitated holistic insight into CLD in the clinical setting, and are generating non-invasive diagnostic modalities for the distinct stages of liver diseases. This multi-omics approach consists of tracing the flow of information from transcriptomics, proteomics, metabolomics, scRNA-seq, single nucleus analysis, and interactomics. The key findings of these techniques are summarized herein.

Transcriptomics refers to the quantitative assessment of all coding and non-coding RNA transcripts and reflects cellular transcriptional activity. Transcriptomic profiling has resulted in various predictive modalities involving gene expression parameters, targeted measurements and miRNA panels with increased functionality in different chronic liver diseases [106,107]. Several studies have identified miR-122 as a potential diagnostic biomarker for chronic liver diseases. Most of them have shown that miR-122 alone or in combination with other miRNAs (e.g. miR-1290, miR-27, miR-192, miR-34, miR-99a) can accurately predict the presence of NAFLD or NASH, but they all perform inadequately when trying to differentiate NAFLD from NASH [108–110]. A recent study carried out a comprehensive transcriptomic analysis of primary LSECs during the progression of cirrhosis in which specific molecular signatures, novel biomarkers and therapeutic targets associated with LSECs were delineated [111].

Proteomics refers to the investigation of “proteome” - all proteins expressed by a cell. Several studies have investigated the hepatic proteome alone or in combination with the blood proteome, both in animal models or in humans with chronic liver diseases, aiming to answer fundamental pathophysiological questions [112,113]. Mann and co-workers assessed the levels and cellular distribution of 6000 liver proteins and 16000 phosphopeptides in the liver of mice developing hepatic steatosis due to high fat diet. This work produced important fundamental information about the reorganization of organelles, lipid accumulation and cellular dysfunction that occurs with nutrient overload

[112]. Xue and co-workers identified almost 220 proteins that are significantly different in patients with NAFLD compared to obese metabolically healthy individuals. The proteins that were identified to be increased in CLD were those involved in peroxisome proliferator-activate receptor-signaling and ECM-receptor interactions whereas the ones that were reduced were mainly localized in mitochondria and involved with oxidative phosphorylation [113]. Expanding on complications of the disease, the proteome of specific cells has also been examined. Helm and co-workers compared the proteome of hepatocytes monoculture and hepatocytes in organotypic rat liver models [114], and showed that when in a 3D liver model the predominant proteomic phenotype supports fatty acid metabolism and when hepatocytes are cultured in monoculture the proteome shifts to favor glucose metabolism. Additionally, they observed an increase in structural and migratory proteins (signaling hepatocyte dedifferentiation), in hepatocytes monoculture, highlighting the need for cell-cell and cell-ECM interactions for maintenance of functional hepatocytes. He and co-workers carried out a proteomic analysis between normal and dedifferentiated LSECs [115]. Dedifferentiation and loss of fenestrae in LSECs precedes the onset of fibrosis and is considered a crucial event in the pathology of liver diseases [92,116,117]. A comparison of the normal and dedifferentiated LSECs showed that in dedifferentiated LSECs the most enriched functional categories of proteins were those related to nucleotide, organic acid metabolism, oxidative stress, small molecular and lipid metabolism, cell death regulation and endocytosis while those down-regulated by dedifferentiation were transcription regulation, actin cytoskeleton reorganization, cell migration, immune system process, ribosome biogenesis, apoptotic process, angiogenesis, glycerophospholipid metabolism and cellular lipid metabolism.

Metabolomics refers to the investigation of small molecules and metabolic products, such as amino acids, fatty acids and carbohydrates. A growing number of studies have begun to study liver specific metabolomics in the context of develop and disease using both primary cells and cell culture models. Ishida and co-workers analyzed and compared

metabolites in fetal and adult hepatocytes from human donors [118]. They identified 211 metabolites in the hepatocytes. Specifically, the metabolites in the glycolysis/glyconeogenesis pathway, tricarboxylic acid cycle and urea cycle were lower in fetal hepatocytes than in adult hepatocytes. Tang and co-workers used nuclear magnetic resonance based metabolomics to investigate the metabolic alterations in hepatocytes caused by HBV infection [119]. They showed that HBV infection contributed to hepatocellular carcinoma (HCC) by upregulation of the glutamine-fructose-6-phosphate amidotransferase 1 -activated hexosamine biosynthesis and choline kinase alpha -activated phosphatidylcholine biosynthesis. Cheng and co-workers demonstrated using NMR-based metabolomic approach that HBV protein (HBx) disrupted the metabolism of glucose, lipids, and amino acids, especially nucleic acids [120]. Sanyal and co-workers performed metabolic profiling on Huh7 cells with patatin like phospholipase domain containing 3 (PNPLA3) siRNA silencing and overexpression using gas chromatography – mass spectrometry (MS) and liquid chromatography-MS metabolic profiling of Huh7 cells to investigate its role in HCC [121]. Silencing of PNPLA3 gene resulted in decrease in amino acid metabolism, suggestive of a catabolic response with extensive protein breakdown. Among the lipids, there was an increase in the levels of myoinositol, cysteine sulfinic acid, polyunsaturated fatty acids, lysolipids, and sphingolipids Overexpression of PNPLA3 mirrored metabolic changes in the opposite direction with an increase in the levels of cholesterol and lactic acid with a shift to anaerobic metabolism. Some of the metabolic signatures associated with the presence of PNPLA3 risk allele such as high cholesterol levels, very low density lipoproteins levels etc have also been associated with cardiovascular disease in patients with NAFLD [122]. These, and other studies[123], explain how the use of omics approaches could help to unravel novel phenotype and pathogenesis mechanisms associated with the presence of genetic polymorphisms in complex human liver diseases.

Single-cell, -nuclei transcriptomics using next-generation transcript sequencing (sc/snRNA-seq) is now emerging as a powerful tool to profile cell-to-cell variability on a genomic scale with broad implications for both basic and clinical research [124]. In a mouse model of liver fibrosis induced by carbon tetrachloride (CCl_4), Krenkel *et al* used freshly isolated HSCs for scRNA-seq and found that activation of HSCs and their trans-differentiation towards collagen-secreting myofibroblasts split into heterogeneous populations, characterized by αSMA, collagens, or immunological markers, while resting HSCs formed a homogenous population characterized by high PDGFR expression [125]. A similar scRNA-seq study using CCl_4 to induce advanced liver cirrhosis identified 6 clusters of liver endothelial cells (EC) populations including 3 clusters of LSECs which associated with zone-specific transcriptomic changes, 2 clusters of vascular ECs, and 1 cluster of lymphatic ECs [126]. Hepatotoxicity induced by 2,3,7,8-Tetrachlorodibenzo-p-dioxin also demonstrated the diversity of liver cells through the identification of 11 subtypes following pericentral, midzonal, and periportal hepatocyte subpopulations by snRNA-seq whose technique was more feasible than scRNA-seq in terms of application to frozen samples [127]. Recently, scRNA-seq was utilized to characterize mouse embryos at day E7.5 to E10.5, and Lotto et al provided a comprehensive atlas liver cell lineage detailing divergence of vascular and sinusoidal endothelia, hepatoblast specification, and the emergence of a distinct, migratory hepatomesenchymal cell type [128]. The most developed scRNA-seq data set is likely that established key immune cell populations in the liver, particularly from mouse models of NAFLD/NASH [129]. A series of elegant studies using scRNA-seq has provided unprecedentedly granular insights into hepatic immune cell heterogeneity, revealing striking alterations, particularly in myeloid cells and macrophages in liver diseases [130–134] , and into related extrahepatic compartments such as bone marrow [135] or adipose tissue [136] .

Regarding the cellular landscape of the human liver, scRNA-seq has also revealed the physiological heterogeneity of human liver cells [137,138], the fibrotic niche of human liver

cirrhosis including the identification of pathogenic subpopulations of TREM2+CD9+ macrophages, atypical chemokine receptor 1+ and plasmalemma vesicle associated protein+ ECs and PDGFRα+ collagen-producing myofibroblasts [139], and the immune microenvironment in the context of HCC [140]. Although sc/snRNA-seq remains an expensive and time consuming technique that requires skilled bioinformatics support, it is a valuable tool to characterize liver function and gene expression dynamics during liver disease, as well as to identify prognostic markers or signatures, and to facilitate discovery of new therapeutic targets [141]. A key challenge for all mentioned omics techniques is accurate data integration. For instance, the most granular insight into single-cell transcriptomes by sc/snRNA-seq techniques comes at the expense of isolating the cells (or nuclei) out of their cellular context [141]; therefore, spatially resolved modalities (e.g. multiplex immunostaining, spatial transcriptomics, imaging mass cytometry) are needed to complement these findings [142]. This has been convincingly demonstrated for immune cell populations, in which not only the immune cell phenotype (or single-cell transcriptome) but also their location within the hepatic microenvironment determines their most likely function during liver diseases [143] .

8- Conclusions and future directions

As described above, in recent years there has been a great advance in the availability and utility of *in vitro* systems for the study of the pathophysiology of the liver. Today we have a wide range of possibilities to better understand the behavior of cells and tissues in the laboratory, which combine harmoniously with those observations obtained in animal models. Although progress in the field of translational hepatology is evident, we must continue working to create more complete, reliable and cost-effective systems of human liver diseases. In the following lines we summarize some of the avenues of work that we should develop through collaborative, multidisciplinary work combining the academic and private sectors.

Liver-on-a-chip systems, which already reflect the multicellularity of the liver, should be improved by incorporating biomechanical stimuli typical of the disease under study, such as a specific matrix or sinusoidal pressure, and potentially the relevant immune cells. In addition, the incorporation of biochemical or biological parameter sensors would be of great help for real time cellular analysis in response to new drugs.

The great potential of 3D liver systems, which currently mostly use matrices of natural origin, has the great advantage of simulating the ECM of the human liver but, at the same time, complicates its standardization and global use. The development and validation of matrices with defined composition, perhaps including the most abundant components in adequate ratios, could assist with expanding their use. Similarly, experimental variables that mimic the biomechanics of the sinusoid (shear stress, pressure, stiffness...) should also be standardized, thus facilitating the comparison of results from different research groups.

The use of PCLS allows an understanding the hepatic response to new compounds but only for a limited period of time. It would be very beneficial to improve the viable incubation time, perhaps by combining several *in vitro* systems including slices, and the use of tissue from liver disease patients/models.

The field of omics applied to hepatology, and to the rest of biomedical disciplines, is immense and it is difficult to ensure currency of literature and use of the most advanced techniques. Analysis at the single cell level, which are already being prototyped using fixed tissue, will transform what we know today as spatial omics. However, tissue cartography requires significant financial investment and excellent experimental design. Therefore, public-private consortiums that include basic scientists and physicians would be of great interest for the sake of advancing knowledge.

Overall, the techniques described in this review and those that are on the horizon can greatly assist to understand liver diseases, develop new therapies and foster

personalized medicine in hepatology. Of course, we need to combine them in a virtuous way, including tissue/cells of human origin whenever possible, and improving the way we mimic human diseases *in vitro*. If future work is further developed by multidisciplinary teams, success is assured.

Figure legends

Figure 1. Schematic view of mostly used *in vitro* models in Hepatology. PCLS, precision cut liver slices. + low; ++ medium; +++ high.

Figure 2. From single cells to liver-on-a-chip and body-on-a-chip. HSCs, hepatic stellate cells; LSECs, liver sinusoidal endothelial cells.

Figure 3. Schematic overview of *in vitro* and *ex vivo* liver models using natural scaffolds including hydrogels, fiber-like structures generated by electrospinning or bioinks (A) and synthetic scaffolds such as microporous, 3D fibrous or bioengineered platforms (B) to generate organoids/spheroids (C), PCLS and bioreactors (D), and 3D liver sinusoid on a chip (E). Natural scaffolds obtained from hepatic tissues from various sources such as human, porcine and rat undergo decellularization using detergents. Synthetic scaffolds including polymer and hydrogel based in combination with novel methods like 3D printing provides the capability for tuning the properties of the material to recreate the liver microenvironment at stages of disease progression. Current 2D and 3D hepatic models include organoids and spheroids, tissue-based approaches such as PCLS and ex vivo bioreactors, and liver-on-a-chip, micropatterned co-culture models. ECM, extracellular matrix; PCLS, precision cut liver slices; BEASTS, Bio-Engineered Adhesive Siloxane substrate with Tunable Stiffness.

Acknowledgements

Authors acknowledge the International Society for Hepatic Sinusoidal Research (ISHSR, www.ishsr.net) for commissioning this review article and for continuously fostering research in the field of basic and translational Hepatology.

Figures of this work were created with biorender.com

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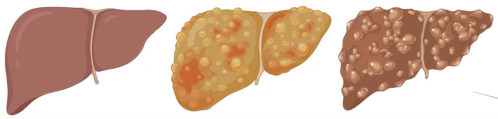

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	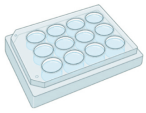		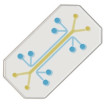		
Accessibility	+++	+++	+	+	++
Complexity	+	+	+++	++	+++
Reproducibility	+++	+++	++	++	++
Relevance	+	++	+++	+++	+++
Human-derived	Primary cells	Primary cells	Primary cells	Fresh tissue	Human-like cells
Biomechanical stimuli	Stiffness	Stiffness (bottom well)	Stiffness, controlled shear stress, pressure	Intrinsic matrix stiffness	Stiffness, perfusion