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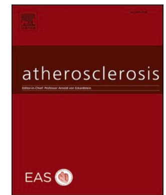

# 3D culture models to study pathophysiology of steatotic liver disease

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ABSTRACT

Steatotic liver disease (SLD) refers to a spectrum of diseases caused by hepatic lipid accumulation. SLD has emerged as the leading cause of chronic liver disease worldwide. Despite this burden and many years, understanding the pathophysiology of this disease is challenging due to the inaccessibility to human liver specimens. Therefore, cell-based *in vitro* systems are widely used as models to investigate the pathophysiology of SLD. Culturing hepatic cells in monolayers causes the loss of their hepatocyte-specific phenotype and, consequently, tissue-specific function and architecture. Hence, three-dimensional (3D) culture models allow cells to mimic the *in vivo* microenvironment and spatial organization of the liver unit. The utilization of 3D *in vitro* models minimizes the drawbacks of two-dimensional (2D) cultures and aligns with the 3Rs principles to alleviate the number of *in vivo* experiments. This article provides an overview of liver 3D models highlighting advantages and limitations, and culminates by discussing their applications in pharmaceutical and biomedical research.

## 1. Introduction

The liver plays a crucial role in maintaining lipid, protein, and glucose homeostasis. Concurrent with the global increase in obesity, steatotic liver disease (SLD) has become the most prevalent condition affecting this vital organ. The heterogeneity between SLD in humans and rodents has hindered the identification of specific therapeutic targets, leading to a lack of specific treatments for fatty liver. Over the past decade, several three-dimensional (3D) liver models have been generated with the goal of providing improved insights into pathophysiology and discovering novel, effective therapeutics. In this review, we will focus on hepatic 3D models, their application, and their prospective role in translational medicine.

## 2. Liver functional unit

The normal adult human liver constitutes approximately 2.5 % of body weight [1]. It is composed of several cell types, namely hepatocytes, biliary epithelial cells (cholangiocytes), hepatic stellate cells, resident macrophages (Kupffer cells), and liver sinusoidal endothelial cells. These cells interact with each other and with a broad spectrum of non-resident cells such as lymphocytes, natural killer cells, and NK-T cells [2]. Hepatocytes and cholangiocytes generate from the same liver embryonic progenitor cells, namely hepatoblasts, which differentiate into mature cell types depending on the local signals to which they are exposed to during liver development [3].

The adult liver is organized into hexagonal functional and structural units, namely lobules (Fig. 1A), and is supplied by the portal vein and the hepatic artery, which provide 65–70 % and 30–35 % of hepatic blood flow, respectively [1]. Each lobule is characterized by a branch of the hepatic vein at its center and by the portal triad, composed of branches of the hepatic artery, portal vein, and bile ducts, at its periphery [4]. Hepatocytes, organized into plates extending from the peripheral portal tracts to the central hepatic veins, are termed centrilobular when located near the hepatic vein, and periportal when closer to the portal tracts. In this model, each lobule is divided into three zones: zone 1 is the closest part of the parenchyma to the portal tracts, zone 2 is intermediate, and zone 3 is closer to the hepatic venule. The vascularization of each hepatocyte is guaranteed by a network of dense vascular sinusoids composed of fenestrated endothelial cells allowing the exchange of nutrients and oxygen while maintaining their barrier function. The oxygen-rich blood coming from the hepatic artery mixes

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along the length of the sinusoids with nutrient-rich blood from the portal circulation, creating a gradient of oxygen, nutrients, and waste that finally drains into the central vein. Kupffer cells are attached to the lumen of endothelial cells that are separated from hepatocytes by the space of Disse, in which quiescent hepatic stellate cells reside until they are activated by hepatic insults (Fig. 1B). Moreover, bile canaliculi, responsible for transporting bile from the liver to the duodenum, run between adjoining hepatocytes and merge into the terminal bile ducts within the portal tract. Indeed, hepatic functional anatomy is based on vascular and biliary relationships [1].

The liver is the primary site for detoxification and nutrient metabolism, and responsible for many primary physiological functions, including the breakdown and excretion of toxic products, immune system support, and the control of metabolites and proteins in the bloodstream. Because of these multiple roles, the liver is susceptible to a wide spectrum of toxic, metabolic, viral, and neoplastic insults. The majority of these hepatic lesions are silent and difficult to detect in the early stage. This is why the high incidence of chronic liver disorders has become a global health problem.

Amongst all hepatic conditions, metabolic dysfunction-associated steatotic liver disease (MASLD), formerly known as non-alcoholic liver disease (NAFLD), has emerged as the leading cause of chronic liver disease worldwide, and the percentage is projected to further increase due to unhealthy lifestyle choices and challenging diagnosis [5–7]. MASLD, a sub-category of steatotic liver disease (SLD) [8], encompasses patients with hepatic steatosis and at least one of five cardiometabolic risk factors. It is described as one of the primary risk factors for the development and progression of metabolic comorbidities such as hypertension, type 2 diabetes mellitus, insulin resistance, and hypertriglyceridemia [9] in the absence of excessive alcohol consumption.

Despite the development of pharmacotherapies for MASLD treatment, to date only one has been approved. This is likely due to MASLD heterogeneity, the presence of disease modifiers, and the genetic background of patients. In this context, preclinical research is the first step in identifying potential therapeutic targets. Preclinical models range from monolayer cell cultures of immortalized hepatic cell lines, usually obtained from tumor tissues and characterized by a high proliferative capacity but potential altered metabolism, to primary liver cells that, on the contrary, better mimic the morphology and function of the native liver but have a limited life, availability, and expansion capacity [10]. Co-culturing different cell populations in the same microenvironment can enhance mutual interactions between hepatocytes and other liver cells, better recapitulating *in vivo* physiological processes.

## 3. Murine models to study steatotic liver disease

Despite the widespread use of monolayer cultures, the gold standard for the study of SLD in the last decades has been *in vivo* dietary metabolic dysfunction-associated steatohepatitis (MASH) models. Amongst these, high-fat diet- (HFD) and western diet-fed murine models develop obesity, steatosis, and impaired fasting glycemia, while chronic exposure leads to early-stage fibrosis, MASH, and preneoplastic hepatic lesions [11,12]. The methionine- and choline-deficient (MCD) diet lacks the amino acids methionine and choline and causes the impairment of VLDL synthesis and secretion, leading to rapid hepatic triglycerides (TAG) accumulation followed by severe inflammation, fibrosis, oxidative stress, and apoptosis [13,14]. The choline-deficient ethionine-supplemented (CDE) diet quickly induces MASH and hepatocellular carcinoma (HCC) due to ethionine hepatocarcinogen properties [15]. However, its use in mice is limited due to weight loss and high mortality rate [16,17].

Mice fed a choline-deficient L-amino acid-defined (CDAA) diet slowly display liver inflammation and fibrosis, but not hepatic insulin resistance and peripheral insulin sensitivity [18]. To develop the most important hepatic features of MASH, such as hepatic inflammation, oxidative stress, lipid peroxidation, fibrosis, and hepatocellular ballooning, these diets are often complemented with cholesterol and fructose [19,20]. Moreover, due to differences in the lipid diet composition and the genetic backgrounds of species, strains, and genders, these models are difficult to standardize, and the data are hard to reproduce [21]. Therefore, with the advent of modern genetic manipulations, transgenic murine models that closely mimic the entire spectrum of SLD within a shorter time frame are increasingly used, particularly when associated with MASH-inducing diets. Amongst these, the *leptin*-deficient (*ob/ob*) and the *leptin receptor* (*db/db*) mice harbor homozygous point mutations for the gene encoding leptin or leptin receptor, respectively. These animals develop severe obesity, hepatic steatosis, hyperlipidemia, hyperglycemia, hyperinsulinemia, and insulin resistance [22,23], but these models must be fed a MASH-inducing diets or given injections of carbon tetrachloride to cause MASH [24].

*Mc4r*-deficient mice-fed HFD develop grave MASH that can easily progress to HCC [25], while mice overexpressing the sterol regulatory element-binding proteins (*SREBP1c*) and fed HFD display liver steatosis, MASH, increased visceral adipose tissue, and ER stress [26,27]. Noteworthily, LDL receptor-deficient ($Ldlr^{-/-}$) mice fed a high-fat/high-cholesterol diet have been used to study the onset and progression of MASLD, as they develop obesity, liver steatosis, inflammation, fibrosis and insulin resistance [28,29]. Recently, $Ldlr^{-/-}$ mice have been used to evaluate the efficacy of Fenretinide in preventing diet-induced obesity, steatosis and in improving insulin sensitivity [30]. In the last decades, other transgenic murine models, such as *peroxisome proliferator-activated receptor α* (*PPARα*) [31], *Phosphatase and tensin homolog* (*PTEN*) [32], *peroxisomal fatty acyl-coenzyme A oxidase* (*ACOX*) [33], and *methionine adenosyltransferase 1a* (*MAT1A*) [34] null mice, have been developed to closely recapitulate human MASLD features, but, to date, the ideal animal model recapitulating human MASLD has

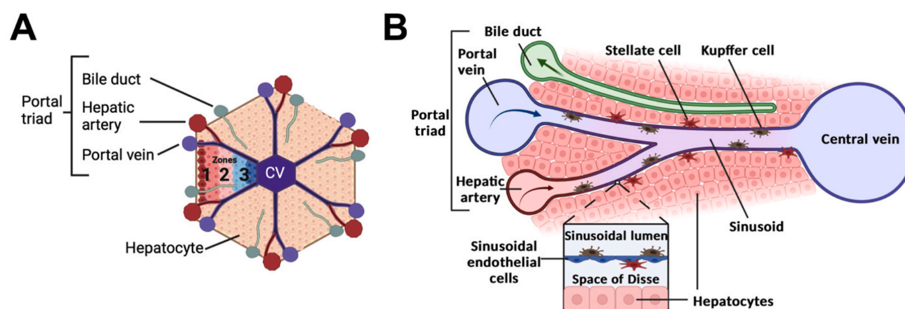


**Fig. 1.** The lobule is the liver functional unit.
(A) Lobules, the hepatic structural and functional units, are supplied by the portal triad, composed of a branch of the portal vein, hepatic artery and bile duct. The central vein (CV) is localized at the center of each lobule and is divided into three zones based on proximity to the CV. (B) Structural organization of liver sinusoids. The fenestrated epithelium, composed of sinusoidal endothelial cells, allows molecules to move from the sinusoidal lumen to the space of Disse in which stellate cells reside. Created with BioRender.com.

not been established.

Given that *in vivo* MASLD studies are laborious, time-consuming, and expensive, there is a need for *in vitro* models that can recapitulate human disease. To address this, the scientific community sought a new tool capable of complementing existing model systems and bridging the experimental gaps between *in vivo* and *in vitro* studies. Hence, liver organoid cultures, defined as artificially grown masses of hepatocytes that resemble miniature livers, emerged as an alternative *in vitro* system that recapitulates tissue physiology, architecture, and spatial organization, provides faster and robust outcomes, lends itself well to live imaging techniques, and is not affected by species-specific microbiota and pathogen composition.

## 4. Organoids

Organoid refers to a 3D cell culture technique that recapitulates the functionality, development, and pathological processes of the modeled organ. Human pluripotent stem cells have been co-cultured to assemble organoid-like tissues for various organs such as the intestine, kidney, brain, retinal, and liver, employing a variety of protocols, specific nutrients, and growth factors to help cells acquire their tissue identity (Fig. 2). Organoid generation methods primarily rely on two approaches: self-organization through cell sorting mediated by adhesive properties, and spatially restricted fate specification to establish 3D self-organized structures morphologically like those observed *in vivo*. In 2017, Nature Methods recognized organoids as the 'Method of the Year 2017,' describing them as a powerful tool that is still evolving to probe human biology and disease [35]. Several 3D-culture systems have been developed to generate structures resembling whole organs by using pluripotent and embryonic stem cells, hepatoblasts, and adult tissue-derived cells [36].

Cells comprising 3D organoids must be able to spatially self-organize themselves, reaggregate, and reconstruct the original architecture of an organ even after complete dissociation. In addition, these organ-like structures must include more than one cell type of the organ they model, and they must exhibit structural and functional features of the organ itself, such as contraction and secretion [37]. Indeed, organoids are 3D *in vitro*–derived tissues that recapitulate some properties of human organ physiology which are difficult to observe by using *in vivo* models. Consequently, organoids can represent a more accurate model of human pathophysiology than animal models do, although the lack of organ crosstalk and infiltrating immune cells are important disadvantages [38].

Organoids made of patient-derived induced pluripotent stem cells, or cells genetically modified to introduce the most common genetic variants involved in SLD, such as *PNPLA3*, *MBOAT7*, *TM6SF2*, *GCKR*, *HSD17B13*, *PSD3* [39–44], as well as rare mutations, must have the capacity to model degenerative conditions, tumor biology, tissue development, organogenesis, infectious disease, and genetic disorders (Fig. 3). These features allow the use of organoids in different fields, including gene therapy, regenerative medicine, drug testing and discovery, and more recently, organ replacement strategies [45].

The term "three-dimensional culture model" was used for the first time in 1989 [47] despite the first experiments date back to the beginning of the twentieth century. In a pioneer study Michalopoulos et al. showed that primary liver cells, derived from collagenase perfusion of rat liver, could be maintained in 3D culture in the presence of EGF, HGF, and the corticoid Dexamethasone [48]. More importantly, these 3D formations, which originate from totally dissociated hepatic cellular elements, recreate a structure similar to the histological organization of the liver in culturing extracellular matrices, such as Matrigel, a blend of laminin, collagen IV, entactin, and growth factors particularly used for evaluating angiogenesis and cellular differentiation [49], collagen matrices, composed of collagen fibers that provide mechanical stability and cell adhesion sites [50], hyaluronic acid, or various synthetic polymers. When grown in a 3D environment, embryonic stem cells (ESCs) and tissue-resident adult stem cells (AdSCs) can generate almost all human and mouse cell types by using *in vitro* differentiation protocols which involve the use of differentiation compounds such as dexamethasone, EGF, TGFα, TNFα, and oncostatin M [51]. In particular, hepatoblasts self-organize into 3D hepatocyte-like or ductal-like liver organoids expressing hepatocytes or ductal markers, such as *Hnf4a or Krt19* respectively, by regulating the exposure of these cells to different growth factors, cytokines, and signaling molecules [52].

Palakkan et al. differentiated human pluripotent stem cells (iPSC) into functional hepatocyte-like cells by exposing cells to a complex set of

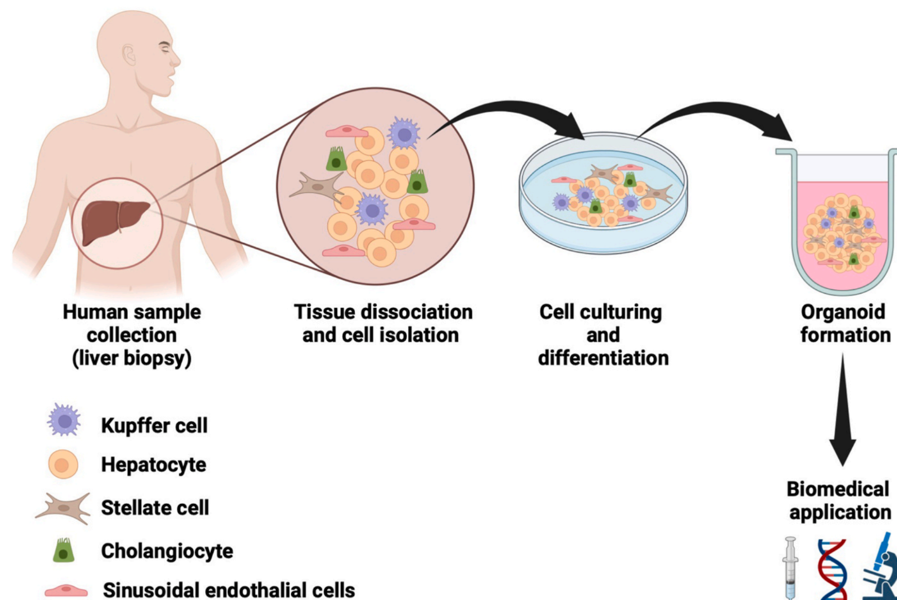


**Fig. 2.** From intact liver to hepatic organoids.
Human liver cells, once collected from surgical resection or needle biopsies of patients, are cultured with a defined supplemented medium to induce cell differentiation followed by three-dimensional organoid formation. Organoids are then used in a wide variety of applications, such as biomedical research, regenerative medicine, or drug testing. Created with BioRender.com.

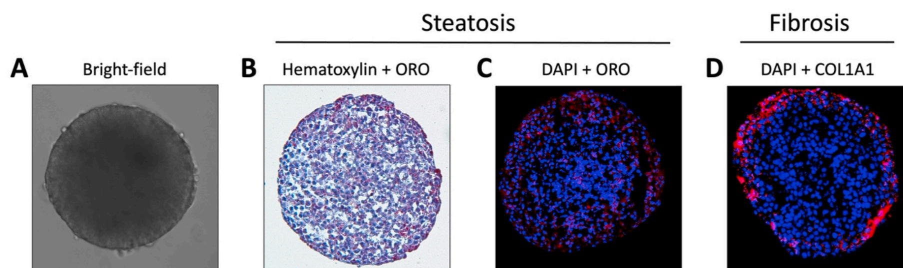


**Fig. 3.** Visualization of neutral lipids and collagen levels in multilineage 3D spheroids.
In the figure, a multilineage 3D model composed by human hepatoma cells (HEPG2) and immortalized hepatic stellate cells (LX-2) mimics liver steatosis and fibrosis. (A) Bright-field image of a spheroid after 7 days of co-culture in 3D; (B) hematoxylin and Oil Red O (ORO) staining, (C) DAPI (blue) and ORO fluorescent staining used to detect the neutral lipids in spheroids; (D) immunofluorescence staining of DAPI (blue) and Collagen Type I Alpha 1 Chain (COL1A1, red). Consistently with the localization of stellate cells in the space of Disse, LX-2 cells localize primarily in the periphery of the spheroids and facilitate the compactness of 3D spheroids themselves [46].

chemical inhibitors such as activin A and Wnt3A [53], while HNF4a and other factors induce the differentiation of fibroblasts into hepatocyte-like cells. Interestingly, Huch et al. showed that Lgr, the receptor for the Wnt agonist R-spondins, characterizes active dividing hepatic stem cells. Single mouse $Lgr5^{+}$ liver stem cells can be long-term cultured as epithelial organoids *in vitro*. This culture utilizes epidermal growth factor (EGF), Rspo14, Fgf10, HGF, and nicotinamide. Furthermore, these cells can be differentiated into functional hepatocytes *in vitro* by inhibiting Notch and TGF-β signaling. These pathways are two of the main ones implicated in biliary cell fate determination *in vivo*, thus inducing the expression of genes involved in liver maturation. Indeed, these hepatocytes secrete albumin, LDL, and accumulate glycogen *in vitro*. Organoids, namely budding cysts by the authors, were then transplanted into fumarylacetoacetate hydrolase ($Fah^{-/-}$) deficient mice in which the cells Fah $^{+}$ deriving from the organoids acquired a fully mature hepatocyte phenotype *in vivo* in the absence of any distinctive ductal expression [52].

Above all, organoids must reflect inter-donor variability. In fact, 3D *in vitro* models generated from primary human hepatocytes have proven to be excellent surrogates for human physiology by providing an *in vivo*-like cellular microenvironment consistent with the observed *in vivo* phenotype [54–56]. Broutier et al. described a near-physiological organoid culture system to form long-term expanding organoids by using primary human healthy liver cells. This approach was applied to make primary liver cancer (PLC) organoids from three PLC subtypes: hepatocellular carcinoma (HCC), cholangiocarcinoma (CC), and combined HCC/CC tumors. These organoids retained liver tissue function, histological architecture, the gene expression profile, and the genetic alterations of the corresponding parental tumor, allowing the identification of each tumor tissue and subtype, and suggesting that their tumor profile was not significantly modified even after long-term expansion in culture in the same medium conditions [57]. In addition, PLC-derived organoids showed a preserved metastatic potential following xenograft in immunocompromised mice. For these reasons, PLC-derived organoids can be considered as a novel personalized medicine approach for biomarker identification and drug-screening testing [57].

Consistently, Bell et al. showed that primary human hepatocyte (PHH) spheroids can reflect liver pathologies, such as cholestasis, steatosis, and viral hepatitis, as they are phenotypically stable and retain their inter-individual variability, morphology, viability, and hepatocyte-specific functions [55]. Hence, 3D spheroids may represent a useful model to better understand how closely the cells match their *in vivo* counterparts, to study the mechanisms of liver disease development and the impact of genetic background, and to offer a promising human representative tool for the development of clinically safe drugs. On these premises, Li et al. utilized patient-derived primary liver cancer organoid lines, obtained from distinct regions of each liver tumor. They tested 129 cancer drugs that *in vivo* had different responses in patients, possibly due to the intrinsic intratumor genetic heterogeneity, contributing significantly to the high intratumor drug response heterogeneity. Consequently, this leads to an elevated failure rate of chemotherapy. Following the treatment, they observed that the drugs were either ineffective or effective only in selected organoid lines, while only a few were pan-effective. This study highlights the necessity of a functional personalized oncology approach and the use of 3D culture as a part of the drug discovery process [58].

In the same years, Nuciforo et al. reported the generation of long-term organoids from hepatic needle biopsies of HCC patients with various etiologies and tumor stages [59]. HCC organoids retained the architecture and gene expression pattern of HCC, thus preserving the genetic heterogeneity of parental tumors. Moreover, once injected subcutaneously into immunodeficient mice, they gave rise to xenograft tumors that recapitulated the histopathological features and the tumor marker expression of the originating human tumors. These data confirmed the intratumor and interpatient drug response heterogeneity and the need for personalized oncology approaches to identify the best chemotherapy for each patient.

Recently, novel 3D *in vitro* models have been developed to study liver-heart axis as cardiovascular complications represent the major cause of death in SLD patients [60]. Moreover, the liver is mainly responsible for drug metabolism and the resulting compounds can cause off-target insults, including severe cardiac side-effects [61]. Therefore, to understand the cardio hepatic interaction dynamics, a pumpless heart/liver-on-a-chip (HLC) using human hepatocellular carcinoma cells (HepG2) and rat cardiomyocytes (H9C2) has been developed to study the cardiotoxicity of chemotherapeutic drugs, such as doxorubicin, which can compromise systolic and diastolic cardiac function [62]. In addition, human induced pluripotent stem cells (hiPSCs) were used to develop a liver microphysiological system (MPS) along with a cardiac MPS derived from the same hiPSC line, to predict drug-drug interactions, efficacy and toxicity in the same genetic background [63].

## 5. Difference between spheroids, organoids and buds

The generation of 3D cultures is currently in its infancy, but the field is rapidly evolving. Indeed, multiple methods for 3D cultures have been recently developed, such as microfluidic devices made of polydimethylsiloxane (PDMS), tissue-engineering scaffolds, microchips, ultra-low adherent plates, microwells made of non-adhesion hydrogels, embryoid bodies, or hanging-drop cultures. These new-generation methods have improved the longevity and differentiation state of cells composing 3D cultures, primarily varying in the types of cells employed. Stem cells, cancer cell lines, and primary cells have generally been utilized to develop liver organoids, aiming to model hepatic diseases. This approach facilitates the investigation of cellular and molecular mechanisms involved in cancer development and potential treatment options.

**Table 1**
Characteristics of spheroids, organoids, and buds.

| 3D *in vitro* model | Spheroid | Organoid | Bud |
|---|---|---|---|
| **Definition** | Cells are cultured in a scaffold-free environment, such as ultra-low adherent plates, that allow cells to aggregate by cell-cell-adhesion within 24–72 h [64] | Cells self-assemble into 3D grape-like structures with an internal lumen facilitated by a scaffolding matrix [65] | Cells self-assemble into 3D vascularized embryonic-like organs facilitated by a scaffolding matrix [66,67] |
| **Cell source** | Immortalized cell lines | Stem cells, cancer cells or primary cells | iPSC |
| **Characteristics** | Spheroid surfaces has high proliferation rate whereas the core holds more quiescent or necrotic cells due to limited access to oxygen and nutrients | Based on the growth factors and chemical inhibitors added to the matrix, hepatic primary cells can differentiate into hepatocyte-like or cholangiocyte-like cells, gain cell polarity and show specific functions [68,69] | Cells self-condense, self-organize, and once transplanted in immunodeficient mice perform liver-specific functions by connecting to the host vessels within 48 h of transplantation |
| **Applications** | Drug screening assays, offer valid alternatives to PHHs [70] | Genetic disorders, viral infections, drug testing, degenerative diseases [46,56,71–73] | Organ development, cell-based therapies, drug testing [69] |

Several terms, such as spheroids, organoids, and buds, have been used to indicate the same tool, even though differences between the three of them exist, as discussed in Table 1, Fig. 4.

## 6. Application of 3D cultures

The application of 3D cultures offers significant advantages in improving our understanding of liver pathophysiology, exploring molecular genetics, and identifying new drugs (Table 2).

Organoids made of human primary hepatocytes (PHHs) represent a sophisticated and physiologically relevant 3D *in vitro* model that recapitulates key aspects of liver tissue architecture and function. PHHs are sourced from liver donors intended for transplantation or from liver tissue resected during surgery [74]. Ethical and legal considerations govern the sourcing of this biological material, ensuring donor consent and compliance with established guidelines [75]. Organoids made of transformed tumor-derived cell lines or immortalized cells, such as HepG2, Huh7, HepaRG, Hep3B, or LX2, have widely been used in an extensive range of studies due to the ability of these cells to continuously proliferate, allowing the formation of long-term cultures and co-cultures [76]. In this respect, 3D co-cultures can be divided into two categories: direct and indirect. On the one hand, direct co-cultures involve mixing and culturing two or more different types of liver cells in a 3D environment to mimic *in vivo* hepatic physiology. On the other hand, indirect co-culture involves culturing two or more different types of cells with a physical separation system that does not allow physical contact between the cells, but signals are communicated through soluble secreted molecules [77,78]. In addition, organoids made up of hepatocytes and non-parenchymal cells (NPCs), such as Kupffer cells, liver sinusoidal endothelial cells, hepatic stellate cells, biliary cells, and pit cells, well resemble liver physiology and structure [79]. In this model, hepatocytes are mainly responsible for metabolism, detoxification and protein synthesis, whereas NPCs play indispensable role in hepatic development and homeostasis by secreting growth factors that regulate hepatocyte proliferation, DNA synthesis, and initiate reconstruction and reformation of matrix proteins [80]. Noteworthily, Lazzeri-Barcelo et al. developed intraocular liver spheroids to study liver physiology and disease progression [81]. Liver spheroids were engrafted on the iris of mice, they became vascularized, innervated and retained typical liver-like microstructures. Moreover, hepatocyte cell cycle activity, bile secretion and lipoprotein uptake were monitored by *in vivo* confocal microscopy, a high-resolution non-invasive imaging technique. Interestingly, when mice were fed a high-fat-high-fructose diet (HFHFrD) for 12 weeks, engrafted liver spheroids sensed metabolic changes in the recipient body and mirrored endogenous hepatosteatosis, suggesting that this novel tool can be used to monitor liver disease development [81]. However, spheroids were made of mouse cells and not human, and the question remains if they resemble mouse physiology more than human physiology. Recently, a 3D *in vitro* hepatic microphysiological system, named liver-on-a-chip, has been developed to microscopically recapitulate the liver microenvironment. These devices are made up of chambers containing hepatic and non-parenchymal cells interconnected via microfluidics which allow the physiological diffusion of nutrients, oxygen, and growth factors over the cells (Fig. 4) [82].

## 7. Limitations

3D cultures remain imperfect models in terms of experimental variability due to the lack of standard approaches and the need for different 3D systems for each cell type. The setting up of 3D cell culture models is still more expensive than 2D models. Despite the development of new standardized techniques, it is crucial to amortize costs to limit the use of *in vivo* animal models [96,97]. Moreover, cell-type representation, tissue architecture, and the availability of origin tissues are limitations that must be considered. The lack of vascularization, and thus the limited nutrient supply, are additional challenges. Another important deficiency is the absence of communication with other organs and with the infiltrating immune cells to mimic the *in vivo* cellular heterogeneity of the liver [98]. In addressing these challenges, Koike et al. have recently developed a novel 3D multi-organ differentiation approach to study multi-organ interactions. This approach creates human hepato-biliary-pancreatic organoids with distinct regional identities originated from human pluripotent stem cells [99]. In addition, various groups have generated human multi-lineage organoids with vascular networks by co-culturing the cells of interest with mesodermal progenitor cells or human endothelial cells [66,100]. They exposed liver cells to pro- and anti-inflammatory cytokines to study inflammation-associated liver dysfunction [101]. Tanimizu et al. generated hepatobiliary tubular organoids by co-culturing hepatocyte progenitors and cholangiocytes [102]. Ramli et al. and Ouchi et al. developed a novel approach to differentiate foregut endoderm cells into functional bile canaliculi or stellate and Kupffer cells [103,104] to study organogenesis and inflammation-induced fibrosis, respectively. Another limitation of organoids is that there is no straightforward translation related to sex. A recent study showed that the *PNPLA3 rs738409* variant has a larger effect in women [105]. To mimic women hormonal physiology, organoids were incubated with different estrogens and experimental studies showed that the interaction with the variants was due to a higher expression of the *PNPLA3* gene mediated by estrogens [105]. These findings highlight the need for standardized protocols to mimic sex differences present in humans.

## 8. Conclusion and future perspective

3D cultures represent a powerful tool to study physiological and pathological processes of the liver in health and disease (Fig. 5). 3D cultures have been used to address complex questions, to understand human molecular genetics, to increase the translation of finding in humans and to reduce the use of *in vivo* models. More studies are needed to further increase the complexity of these models by a) including

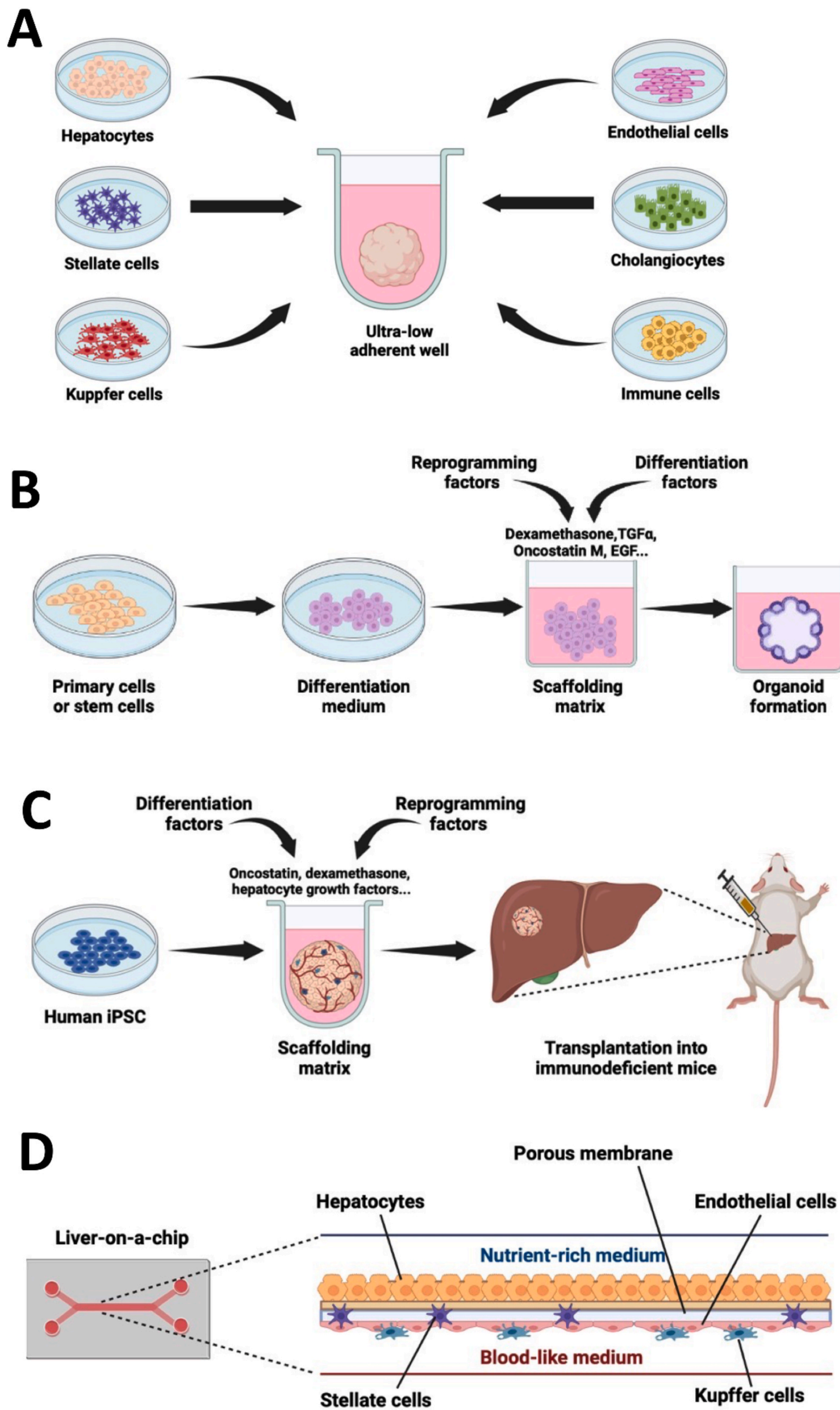


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**Fig. 4.** Main differences among three-dimensional cell culture systems.
(A) spheroids, composed of immortalized cell lines and made in scaffold-free ultra-low adherent plates; (B) organoids, usually composed of primary cells and grown in extracellular matrices; (C) organ buds, vascularized 3D cultures composed of iPSC, are grown in extracellular matrices and then transplanted into the livers of immunodeficient mice, where they are able to perform liver-specific functions; (D) liver-on-a-chip, a microfluidic liver device composed of two adjacent fluid channels separated by a porous permeable membrane integrated with primary hepatocytes, liver sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells that recapitulate the physiological structure of the human liver. Created with BioRender.com.

**Table 2**
Examples of the use of hepatic 3D *in vitro* models.

| 3D *in vitro* model | Cell source | Application | Characteristics |
|---|---|---|---|
| **PHH- organoids** | Primary human hepatocytes | Model for viral infections [83] | Infection induced by both recombinant virus and serum from HBV-infected patients.<br>Expression of HBsAg, HBeAg, and HBV core proteins, produced cccDNA and infectious HBV in the culture supernatant, indicating that liver organoids support the entire HBV replication cycle |
| | Primary human hepatocytes | Understanding molecular genetics and pharmacology of a specific target [40,84] | Primary cells from donors carrying different genotypes have been compared and recapitulate the human genetics<br>Antisense oligonucleotides targeting a specific gene were tested to assess the effect on intrahepatocyte neutral lipids |
| | Primary human hepatocytes | Model for drug metabolism [85] | Expression of cytochrome P450 and uridine diphosphate glucuronosyltransferase enzymes.<br>Metabolites identified in the spheroid cultures replicated the essential metabolites observed *in vivo* |
| **Organoids made of immortalized cells** | HepaRG and HSC | Drug-induced liver injury (DILI) [86] | Treating organoids with pro-fibrotic compounds.<br>Hepatic organoids exhibit fibrotic features such as HSC activation, collagen secretion and deposition |
| | Hep3B, HuH7, and HepG2 | Model for HCC [87] | Hep3B cell organoids display polarized 3D structures and expressed HepPar1, GS, and CK19.<br>Huh7 cell organoids express hepatocyte markers ARG1, A1AT, and CK18.<br>Organoids of HepG2 showed CDH1 and A1AT and heterogeneously express CK19 and AFP |
| | HepaRG | Model for MASLD [88] | HepaRG organoids differentiated *in situ* in a microfluidic perfusion device (SteatoChip).<br>Treating cells with FFAs led to the accumulation of fat, impaired glucose regulation and phosphorylation of Akt.<br>The metformin hydrochloride, pioglitazone hydrochloride and obeticholic acid reduce steatosis |
| **3D co-cultures** | Hepatocytes (HepG2) and HSCs (LX-2) | MASLD pathophysiology [71] | Depletion of *LPIAT1/MBOAT7* in hepatocytes elevates triglyceride synthesis fueled by high phosphatidylinositol turnover.<br>Triglyceride accumulation promotes liver fibrosis |
| | Hepatocytes (HepG2) and HSCs (LX-2) | Model for MASH [46] | Treating cells with FFAs led to the accumulation of fat and collagen.<br>Liraglutide or elafibranor treatment reduced anti-fibrotic properties |
| | Hepatocytes (HepG2) and HSCs (LX-2) | Drug screening for MASH [89] | Bergamot polyphenolic fraction decreases intracellular lipid content.<br>Increase in expression of ß-oxidation-related genes (*ACOX1*, *PPARA*, and *UCP2*) and the lipophagy-associated gene (*ATG7*) |
| **Organoids made up of hepatocytes and non-parenchymal cells (NPCs)** | hESC and iPSC | Modeling steatohepatitis [90] | Organoids generated from patients with genetic lysosomal enzyme deficiency expedited to steatohepatitis phenotype followed by *in vitro* rescue using clinically active FXR agonist.<br>Inflammatory and fibrotic liver disease due to the co-differentiation of multiple hepatic cells |
| | iPS-derived endothelial cells and primary mesenchymal stromal cells | Molecular genetics [91] | Increased expression of *ALB*, *CYP1A1*, *CYP1A2*, and *TDO2*.<br>Downregulation of Wnt and TGF-β signaling activity |
| | iPSC | Liver fibrosis and steatosis models [92] | Liver-specific functions, highly vascularized, and hepatic cellular spatial organization |
| **Liver-on-a-chip** | Caco-2[a] and HepG2 | Molecular mechanism of MASH [93] | Integrated-gut-liver-on-a-chip (iGLC).<br>Understanding how gut and liver interact in the milieu of MASLD.<br>FFAs treatment induce overexpression of genes associated with ER stress and the cellular response to copper ions.<br>Co-culturing Caco-2 and HepG2 provides protection against apoptosis, whereas monoculture of these cell lines exhibits induced apoptosis |
| | Hepatocytes (HepG2), LSECs (EAhy926[a]), HSCs (LX-2), and KCs (U937[a]) | Evaluating drugs for MASH [94] | Lipotoxic stress leads to MASH phenotypic traits.<br>Elafibranor reduces the hallmarks of MASH |
| | HCC (HCCLM3) HSCs (LX-2), and endothelial cells (HUVEC[a]) | HCC pathophysiology [95] | *LCN-2*, an iron-binding protein, helps in the remodeling of tumor microenvironments in the HCC-on-a-chip.<br>*LCN-2* ablation leads to anti-tumor effects |

[a] Non-hepatic cell origin.

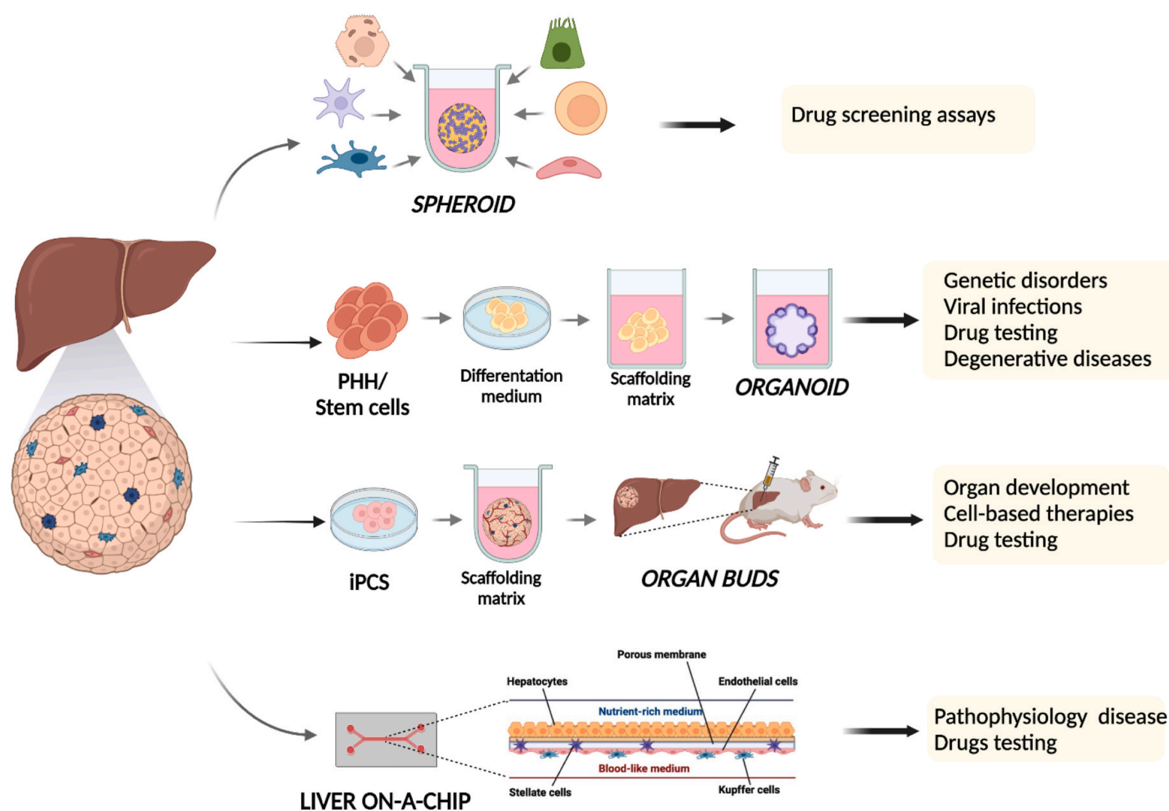


**Fig. 5.** Graphical abstract.
Three dimensional (3D) hepatic culture models derive from a large variety of cellular sources, are generated using different techniques, show different features and are used for a wide spectrum of applications including drug screening and the study of rare genetic disorders. 3D liver models represent a novel powerful tool to investigate physiological and pathological processes involved in liver disease.

vasculature structure and synovia, and b) assembling several organs at the same time.

## CRediT authorship contribution statement

**Andrea Caddeo:** Writing – original draft, Writing – review & editing, Visualization, Supervision. **Samantha Maurotti:** Writing – review & editing, Visualization. **Lohitesh Kovooru:** Writing – review & editing, Visualization. **Stefano Romeo:** Supervision, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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