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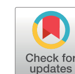
Perspective

From fatty hepatocytes to impaired bile flow: Matching model systems for liver biology and disease



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ABSTRACT

A large variety of model systems are used in hepatobiliary research. In this review, we aim to provide an overview of established and emerging models for specific research questions. We specifically discuss the value and limitations of these models for research on metabolic associated fatty liver disease (MAFLD), (previously named non-alcoholic fatty liver diseases/non-alcoholic steatohepatitis (NAFLD/NASH)) and cholestasis-related diseases such as primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC). The entire range of models is discussed varying from immortalized cell lines, mature or pluripotent stem cell-based models including organoids/spheroids, to animal models and human *ex vivo* models such as normothermic machine perfusion of livers and living liver slices. Finally, the pros and cons of each model are discussed as well as the need in the scientific community for continuous innovation in model development to better mimic the human (patho) physiology.

1. Introduction

Cholangiopathies including primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) [1], auto-immune hepatitis, IgG4-associated cholangitis and genetic defects in biliary transporters such as cystic fibrosis transmembrane conductance regulator (CFTR) increase the risk of cholestasis. This is a clinical condition in which toxic bile acids accumulate in the liver and cause amongst others irreversible liver injury due to decreased or obstructed bile flow in intrahepatic or extrahepatic bile ducts. Cholangiopathies, but also the metabolic syndrome: metabolic associated fatty liver disease (MAFLD) [2] have unclear aetiology and treatment is not always available or effective. The definition MAFLD replaces the outdated exclusion-based definition non-alcoholic fatty liver disease (NAFLD) and the international experts consensus panel further argues against a "dichotomous stratification" of non-alcoholic steatohepatitis (NASH) versus non-NASH [2,3]. As the new name more accurately reflects the (complex) aetiology of this disease, this nomenclature will likely be adopted broadly in the near future and therefore used throughout this review. It is of utmost importance that further research fills the current knowledge-gap regarding

Abbreviations: ASC, Adult stem cell; ANIT, α-Naphtylisothiocyanate; BDL, Bile duct ligation; BSEP, Bile salt export pump (*ABCB11*); CDAA, Choline-deficient L-amino acid; CFTR, Cystic fibrosis transmembrane conductance regulator (*CFTR*); CLC, Cholangiocyte-like cell; DDC, 3,5-Diethoxycarbonyl-1,4-dihydrocollidine; DIC, Drug-induced cholestasis; ER, Endoplasmic reticulum; FFA, Free fatty acid; FMT, Fecal microbial transfer; GWAS, Genome wide association studies; HCC, Hepatocellular carcinoma; HFM, Hollow fiber membrane; HLA, Human leukocyte antigen; HLC, Hepatocyte-like cell; HPC, Hepatic progenitor cell; hiPSC, Human induced pluripotent stem cell; ICP, Intrahepatic cholestasis of pregnancy; iHEP, Induced pluripotent stem cell-derived human hepatocyte-like cell; LGR5, Leucine rich repeat containing G protein-coupled receptor 5; LOC, Liver-on-a-chip; MAFLD, Metabolic dysfunction-associated fatty liver disease; MDR3, Multi drug resistance protein 3 (*ABCB4*); MHC, Major histocompatibility complex; MSC, Mesenchymal stem cell; NAFLD, Non-alcoholic fatty liver disease; NASH, Non-alcoholic steatohepatitis; NTCP, Sodium taurocholate co-transporting polypeptide (*SLC10A1*); Ostα-β, Organic solute transporter alpha and beta (*SLC51A/SLC51B*); PCTS, Precision-cut liver slices; PBC, Primary biliary cholangitis; PFIC, Progressive familial intrahepatic cholestasis; PHH, Primary human hepatocyte; PSC, Primary sclerosing cholangitis; VLDL, Very low density lipoprotein

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disease aetiology and helps to develop better therapies. Currently, models vary from immortalized cell lines and primary cell cultures to more advanced 3-dimensional (3D) organoid models and animal models. The decision which model to use is not always straightforward and is dependent on many aspects, varying from technical arguments such as the costs, reproducibility, ease in use and possibilities to perform (semi) high throughput analyses, to biological arguments such as anatomical and physiological characteristics.

In this review, we aim to create an overview of established and state-of-the-art models in liver disease research and discuss their advantages and disadvantages. More specifically, we will focus on hepatocellular and cholangiocellular models for cholestatic liver diseases and hepatocellular models for MAFLD. First, we will discuss *in vitro* models such as primary human hepatocytes, immortalized hepatic/cholangiocyte cell lines and stem cell-derived models leading to hepatocyte- and cholangiocyte like cells or organoids. Subsequently, novel advanced *in vitro* models, human *ex vivo* liver systems and finally (rodent) animal models to study cholestatic liver diseases and MAFLD will be discussed.

2. *In vitro* models for liver function

The majority of the liver mass is formed by hepatocytes. Although these cells perform many of the functions of the liver, it is evident that cholangiocytes and the non-parenchymal cells (stellate cells, endothelial cells and Kupffer cells) also largely contribute to liver (patho) physiology. Furthermore, the liver 3D anatomy largely contributes to its functional characteristics and this generates further diversity in cellular functions. For example, pericentral-based hepatocytes encounter different concentrations of metabolites compared to periportal hepatocytes. Similarly, cholangiocytes of a large bile duct face a biliary content that is distinct from those of the small bile ducts. This implies that the full complexity of the liver cannot be modelled with homogeneous *in vitro* models of individual cell types. Nevertheless, such model systems do allow for the investigation of specific cell-intrinsic aspects of liver function, in a cost/user friendly manner with high experimental control. Here, we focus on models for hepatocytes and cholangiocytes, while other liver cell types are only briefly discussed (Fig. 1).

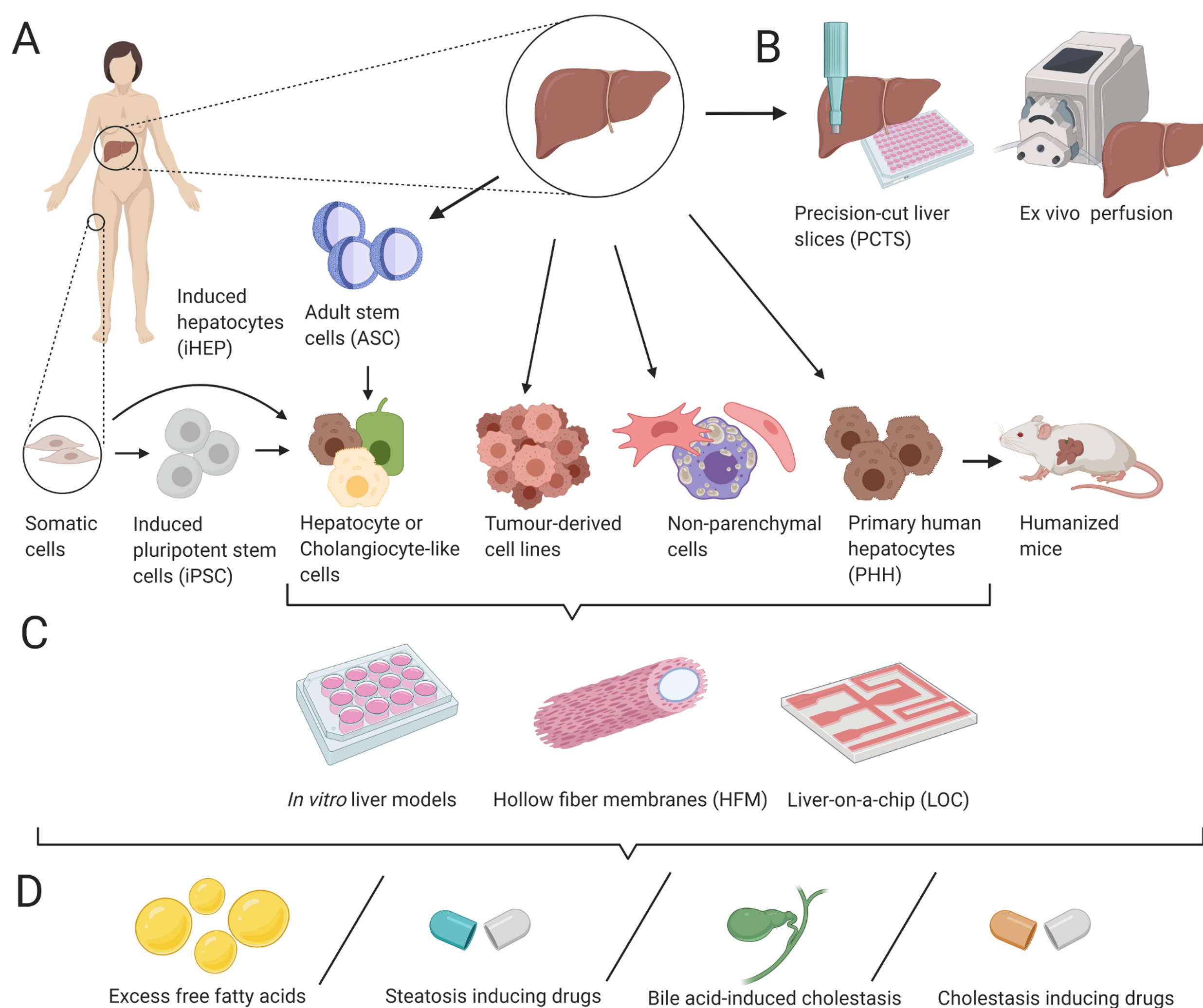

Fig. 1. Schematic overview of *in vitro* and ex vivo liver models and applications in hepatobiliary research (A) Different cell origins can be used to create hepatocyte and cholangiocyte models. *Ex vivo* systems (B) or more advanced *in vitro* models (C) in combination with treatments (D) can be used to model cholestatic liver diseases or MAFLD.

2.1. Primary human hepatocytes (PHHs)

The most obvious cell source to recapitulate fully matured hepatocytes *in vitro* would be primary human hepatocytes (PHHs). PHHs can be isolated through enzymatic digestion from either partial liver resection for therapeutic purposes, for example after removing a tumour [4], rejected livers unsuitable for liver transplantation or from liver segments after split liver transplantation [5]. Most of the isolation procedures use a two-step collagenase perfusion technique for the isolation of PHHs [6]. The obtained PHHs can be cultured using hepatocyte-specific media to optimize the preservation of the hepatic phenotype and are widely used in studies for metabolism and xenosensing [7]. Besides, PHHs can also be cryopreserved while maintaining these properties [8]. However, PHHs rapidly undergo dedifferentiation after isolation [9] and cannot be expanded upon culturing in standard 2D culture plates [10]. The dedifferentiation can be delayed by using a sandwich culture method where PHHs are maintained between two layers of collagen hydrogel [11-13].

The prediction of drug-induced cholestasis (DIC) predominantly relies on assessing the potential of compounds to inhibit bile salt export pump (BSEP) activity of PHHs in sandwich cultures [14]. Several well-known DIC compounds were tested on PHHs by using sandwich cultures [15,16]. Prerequisite for the analysis of DIC is that PHHs form canalicular networks using the sandwich culture which usually takes several days to form [17]. With respect to steatosis, free fatty acid (FAA)-exposed PHHs showed a dose-dependent increase in lipid accumulation with, amongst others, indications of endoplasmic reticulum (ER) stress and induced transforming growth factor beta-1 expression, indicative of a pro-fibrogenic response [18-21]. Another study involving PHHs showed that the long-term exposure to hyperglycaemia leads to accumulation of neutral fats in PHHs co-cultured with fibroblasts [22]. Although the use of PHHs for disease modelling has been hampered by the inability to propagate hepatocytes successfully, recently novel techniques allow the long-term culture of hepatocytes. One of these techniques is directed to the dedifferentiation of PHHs towards a hepatic progenitor cell (HPC)-like state for expansion and then differentiating them back toward the hepatocyte-lineage. This can be achieved either by using two small molecules, A83-01 and CHIR99021, in combination with hepatocyte growth factor (HGF) [23], or the manipulation of SIRT1 signaling [24]. Another PHH culture method relies on the inhibition of the dedifferentiation process resulting in a bi-phenotypic phase (in between PHHs and HPCs) by WNT3A addition and hypoxia [25]. Finally, PHHs can also be expanded without dedifferentiation using a mixture of five chemicals [9] or using a 3D organoid culture system [26].

There is also the possibility to use PHHs to create humanized chimeric mice [27]. Here, transgenic immune-deficient mice are used to generate chimeric animals harbouring PHHs. Two models, the albumin–uroplasminogen activator (uPA) transgenic mouse and the knockout of the fumarylacetoacetate hydrolase (Fah) gene, have been shown to have a high degree of human hepatocytes present in the mouse liver after transplantation [28]. Since virtually all CYP or Phase II conjugation pathways and transport proteins examined are expressed in the chimeric animals and polarisation of human hepatocytes does occur, these models will likely increasingly be used for steatosis and cholestatic modelling in the future [29].

2.2. Hepatic cell lines

Many different hepatic cell lines exist that can recapitulate liver function, established from either immortalised hepatocytes (e.g. Fa2N-4, THLE) or derived from tumours such as hepatocellular carcinoma or hepatoblastoma (e.g. HepG2, HepaRG, Huh7, Hep3B) [30]. The main advantage of these cell lines is their unlimited growth, the ability to easily alter the genome for disease modelling or screening purposes and the possibility to recapitulate (partial) liver function [12,31]. Limitations of these cell lines include lacking metabolism and the high prevalence of chromosomal abnormalities [31]. Moreover, these cell lines often have an immature gene-expression profile exemplified by the high expression of alpha-feto protein (AFP), a major serum protein synthesized during foetal development [32]. Since they are derived from a single clone and/or donor, cell lines are not representative for individual differences but can still aid in answering specific questions related to cholestasis and steatosis. Two of the most studied tumour-derived cell lines for *in vitro* modelling of hepatocytes are HepaRG and HepG2 [33]. Monolayer cultures of HepaRG demonstrate improved hepatocyte functionality compared to other models [34] and directly compared to HepG2s, which lack certain liver-specific functions. HepaRG cells are therefore more suited for *in vitro* modelling [35]. However, HepaRGs also express low levels of two essential CYP450 types 2D6 (CYP2D6) and 2E1 (CYP2E1), leading to a decreased suitability for studies involving specific metabolism [36]. Moreover, the HepaRGs overexpress CYP3A4 compared to PHHs which may lead to decreased sensitivity for some compounds in picking up toxicity [37]. HepaRGs have been shown to be sensitive to DIC (e.g. chlorpromazine) [38] which decreased expression of BSEP and multidrug resistance protein 3 (MDR3), leading to cholestasis. Increased fat accumulation has also been shown in HepaRGs with two compounds known to induce microvesicular steatosis (tolcapone and entacapone) [39] or with an access of FFAs [40].

HepG2s proliferate fast, can be differentiated into polarized cells with a clear canalicular membrane and can be used for toxicology- and disease modelling [41]. Although these cells lack liver-specific functional expression, such as CYP450 activity, HepG2s are commonly used in drug-efficacy tests [42]. HepG2s have been shown to be sensitive to glycochenodeoxycholicacid (GCDCA)-induced cholestasis [43], but the lack of sodium taurocholate co-transporting polypeptide (NTCP) expression in this cell line does not make it a suitable model for bile acid toxicity during cholestasis [44]. An artificially induced NTCP expression could overcome the absence of this bile acid transporter [45]. Steatosis can be achieved in HepG2s using high levels of FFAs to induce fat accumulation, and also downstream consequences of steatosis such as apoptosis and impaired insulin signaling could be studied in this cell model [46].

The vast majority of immortalised hepatocytes include an overexpression of an immortalisation gene (e.g. SV40 large-T antigen or telomerase) by means of transfection of transduction [47]. Although this provides a way to proliferate the hepatocytes *ex vivo*, the *in vivo*-like hepatic functionality is still largely lacking [47]. Immortalised hepatocytes have been used successfully in viral infection models but some studies also involved steatosis [48,49]. Both studies use an access of FFAs to induce fat accumulation.

The model of choice for immortalized human cholangiocytes is the H69 cell line, which is SV40 immortalized [50]. Other cell lines used for *in vitro* modelling of cholangiocytes such as CLCC1 are derived from cholangiocarcinoma and consequently somewhat less suitable to study normal cholangiocyte physiology.

2.3. Stem cell-derived models

Besides liver cancer cell lines and primary liver cells, the usage of stem cells followed by differentiation towards liver-specific lineages allows for novel opportunities in *in vitro* liver disease modeling such as for MAFLD and cholestasis. Stem cells are a novel, hypothetically unlimited, source of hepatocytes and bile duct epithelial cells alike, suitable for both drug screening as well as for gaining mechanistic understanding of liver disease. Stem cells can readily be expanded in culture due to high proliferative and self-renewal capacity. In addition, stem cells can be derived from specific patients which allows to study disease traits for personalized precision medicine. Furthermore, genome alteration and gene editing strategies in the stem cell state allow to study the effect of specific gene mutations on liver disease progression after differentiation. Major limitations of these models are related to costs, as the culture conditions are more demanding than those of cell lines, and the level of differentiation towards hepatocytes or cholangiocytes.

2.3.1. Human induced pluripotent stem cell (hiPSC) models

The discovery by Takahashi *et al.* (2007) [51] enabled great

opportunities in the usage of stem cells and resolved earlier ethical issues raised by embryonic stem cells, since they were the first to generate human induced pluripotent stem cells (hiPSCs) from somatic cells by using genetic engineering techniques. To generate hiPSCs, four transcription factors, namely OCT3/4, SOX2, C-MYC and KLF4, are transfected in human somatic cells, such as fibroblasts or epithelial cells resulting in cells which can grow indefinitely and show pluripotency [51]. The transfection of these factors in somatic cells was first achieved by using an integrating viral system such as retroviral vectors, but can now also be achieved using non-integrating strategies to improve safety for the usage of hiPSCs in clinical settings [52]. This enabled to establish pluripotent stem cells from specific patients non-invasively by obtaining somatic cells from blood, skin or urine. This allows to study disease mechanisms with certain genetic backgrounds. These hiPSCs are capable of being differentiated in all three germ lines, namely endoderm, mesoderm and ectoderm. Indeed, hiPSCs can be transformed into any cell type of interest.

2.3.2. hiPSC-derived hepatocyte-like cells (HLCs)

Reports have shown that hiPSCs can be efficiently differentiated into hepatocytes using growth factors and cytokines in a step-wise fashion [53-58]. In general, hiPSCs are first differentiated into definitive endoderm via stimulation with Activin A, a member of the TGF-β superfamily and mimics Nodal signaling [59]. Thereafter, hepatic progenitor cell (HPC) formation is initiated through the use of certain growth factors followed by differentiation into HLCs. However, relying on recombinant growth factors may hinder the differentiation reproducibility due to possible batch-to-batch variability and are costly. Recently, also other differentiation approaches have been established completely relying on small molecules to improve reproducibility and cost-effectiveness [60-62]. Other strategies incorporating overexpression of certain critical transcription factors, such as HEX or HNF1A, showed to improve the differentiation towards HLCs [63-67]. Despite the efforts and great progress that has been made regarding hepatocyte differentiation, often these HLCs still show fetal characteristics and do not yet fully resemble mature hepatocytes *in vivo*, possibly due to a sub-optimal differentiation strategy or the lack of liver-specific 3D architecture. To improve the maturity of HLCs, efforts have been made to improve the maturation phase of the differentiation process, either by improving medium composition [63], growing in a 3D environment [68-70], including multiple liver relevant cell types and utilizing microfluidics [71]. Up until now, great progress was achieved in optimizing the differentiation strategy of hiPSCs towards HLCs to serve as an *in vitro* model closely mimicking the liver *in vivo* and can be used for liver disease modeling purposes.

To obtain a better understanding of the development of particular liver diseases, such as MAFLD or cholestatic related diseases, HLCs can be used as an *in vitro* liver model to model such a disease, allowing to identify novel drug targets and perform drug screening. Indeed, Parafati *et al.* (2018) [72] were able to develop a MAFLD model for the early phase of the disease using hiPSC-derived HLCs which were treated with FFAs, palmitic and oleic acid, and ER stress inducer thapsigargin to elevate fatty acid accumulation. After exposure with the combination treatment, lipid accumulation and upregulation of MAFLD markers such as FGF21 was seen showing a steatotic transcriptomic phenotype. Moreover, a comparable triacylglycerols accumulation was seen as in steatotic human livers. The model was validated with obeticholic acid being a clinical stage drug for NASH, and showed its usability for drug discovery purposes against MAFLD.

Another study by Ouchi *et al.* (2019) [73] showed the development of a hiPSC-derived multi-cellular organoid model in combination with FFA treatment to recapitulate the development of steatohepatitis. Here, hiPSCs were differentiated as organoids where single-cell transcriptomics and FACS analysis revealed the existence of multiple cell types including hepatocytes, stellate cells and Kupffer cells. These organoids showed dose-dependent lipid accumulation and triglyceride production when treated with oleic acid. Upon FFA treatment, cytokine induction was seen mediated by the Kupffer cells and activation of stellate cells indicating both inflammation and fibrosis signaling. Although further model characterization is needed, combined this study showed its capability to cover multiple facets of the development of steatosis and study mechanisms for the treatment of liver disease.

2.3.3. hiPSC-derived cholangiocyte-like cells (CLCs)

Besides HLCs, hiPSCs can also be a source for cholangiocyte-like cells (CLCs) [74]. Similar to hepatocytes, intrahepatic cholangiocytes are derived from hepatoblasts, thereby having a common progenitor. By culturing them with specific growth factors key in cholangiocyte differentiation after the hepatoblast stage, cholangiocytes can be efficiently generated [75-77]. Indeed, Sampaziotis *et al.* (2017) [77] have shown to be able to differentiate hiPSCs towards CLCs using a 26-day differentiation protocol where hepatoblasts are differentiated towards cholangiocytes using FGF10, retinoic acid, ActivinA and EGF [77,78]. These hiPSC-derived CLCs showed expression of cholangiocyte markers keratin 19 (KRT19), keratin 7 (KRT7) and SRY-box transcription factor 9 (SOX9), lack of hepatocyte markers AFP and hepatocyte nuclear factor 4 alpha (HNF4A), and showed functional characteristics such as γ-glutamyl transferase and alkaline phosphatase activity. Similar to HLCs, here hiPSC-derived CLCs still express immature cholangiocyte markers indicating their immature state.

2.3.4. Human adult stem cells and organoid models

Another source of stem cells which could serve for modeling liver diseases such as MAFLD or cholestasis are the adult liver tissue-derived stem cells or progenitors. Upon injury, the liver has the great capability to regenerate through various described mechanisms depending on the type of damage [79]. One of these mechanisms is by the hepatocytes themselves being capable of self-renewal [80,81]. Alternatively, it can be mediated by a population of bipotent progenitor cells derived from cholangiocytes capable of proliferation and differentiation towards both hepatocyte and cholangiocyte fates [82-85].

2.3.5. Adult stem cell-derived HLCs

Indeed, Huch *et al.* (2015) [84] has shown to be able to grow bipotent progenitor cell organoids from human liver tissue dependent on the presence of EpCAM positive ductal cells. These organoids can be expanded and kept in culture for many months while maintaining genetic integrity. Single cell transcriptomics revealed that indeed these bipotent progenitor cells derive from bile duct cells and could give rise to both CLCs as well as HLCs. Although they show more dominantly cholangiocyte characteristics, these organoids can be differentiated with a two-step strategy for 15 days to enhance their hepatocyte maturation status, which increases albumin secretion, CYP3A4 activity and bile acid secretion. However, this does not yet result in fully functional mature hepatocytes and still some cholangiocyte markers remain expressed, suggesting a mixed population of cell phenotypes. Other studies have focused on culture expansion of hepatocytes themselves using modified organoid culture conditions. Indeed, recently it was demonstrated to be feasible to initiate organoids from primary hepatocytes. These primary hepatocyte organoids showed proliferation capability in culture and more closely retained the hepatocyte phenotype [86,87]. However, initiation was most effective from fetal liver tissue rather than adult liver and the growth rate declines already after a few months of culture. Therefore, these new primary hepatocyte organoid models show potential to serve as a model system to study liver diseases, although improvements in culture strategies are needed to allow for prolonged stability.

Adult stem cell liver organoids could well serve to study liver disease, especially since these organoids could be established from patient-specific material retaining stably their genetic background. Indeed, a study by Kruitwagen *et al.* (2017) [88] had applied liver-derived organoids to study hepatic steatosis. Here, liver organoids from multiple species were treated with FFAs. After treatment, lipid accumulation could be observed and revealed more insight in cellular lipid-coping strategies by testing the effect of specific small molecules. Liver-tissue derived organoids can also be used to study genetic-related liver diseases, such as alpha-1 antitrypsin deficiency (A1ATD) where accumulation of misfolded and aggregated A1AT in hepatocytes leads to ER

stress and liver injury [89]. Studies have shown that organoids derived from A1ATD patients could recapitulate pathology upon differentiation towards a more hepatocyte phenotype, showing A1AT aggregate accumulation and induction of ER stress signaling [84,90]. Organoids can also be applied to study liver cancer development. Indeed, Broutier *et al.* (2017) [91] have shown to be able to grow organoids derived from primary liver cancer tissues enabling to study liver tumorigenesis and applied for drug screening. Together this emphasizes the suitability of liver organoids for disease modeling, shedding more light on mechanisms of disease progression. These liver organoids have the advantage of resembling the genetic background of patients enabling to study specific diseases, maintain genetic stability during culture, robust expansion after initiation, self-assembling properties consisting of multiple liver cell types while no genetic manipulation is needed. However, many challenges remain. For example, to initiate liver-derived organoids, invasive retrieval of liver tissue is needed and differentiation does not yet reflect a fully mature hepatocyte status.

2.3.6. Mesenchymal stem cell-derived HLCs

Mesenchymal stem cells (MSCs) are another source of adult stem cells that could serve as a starting point for differentiation towards HLCs [92]. They can be retrieved from blood, adipose tissue or bone marrow, have self-renewal capabilities and are multipotent. Differentiation of MSCs towards HLCs encompasses either a one or two-step strategy of growth factor and cytokine exposure [93-95]. However, due to a lack of specific markers for MSCs, potentially there is a heterogeneous population of differentiated cells and increase in variability [96]. Also the tissue of origin may affect the differentiation capability towards HLCs [97]. Similar to the differentiation of hiPSCs, growing the differentiated MSCs in 3D combined with other relevant cell types during the maturation phase may improve the differentiation towards HLCs [98]. Overall, MSC-derived HLCs would also serve as a relevant model to study specific liver diseases such as MAFLD or cholestatic related diseases, having the advantage of a relatively easy access of cells, unlimited supply, capability of studying genetic background of specific patients and no genetic manipulation is needed. Although, MSC-derived HLCs may be more variable due to lack of specific markers for MSCs to retrieve an uniform cell population and do not yet reach full maturity of primary hepatocytes.

2.3.7. Adult stem cell-derived CLCs

Liver-derived organoids described by Huch *et al.* (2015) [84] show already cholangiocyte-like characteristics without any differentiation, which could serve as a model for intrahepatic cholangiocytes to study cholangiopathies. Upon cholangiocyte differentiation of these organoids, further cholangiocyte maturity can be established. Another study by Sampaziotis *et al.* (2017) [77] showed the capability of growing primary cholangiocyte organoids derived from isolation of primary extrahepatic cholangiocytes which closely resemble characteristics of mature cholangiocytes exhibiting expression of key cholangiocyte markers such as KRT7 and KRT19, and activity of GGT and ALP. Recently, Rimland *et al.* (2020) [99] compared cholangiocyte organoids derived from different locations along the biliary tract. Here, organoids derived from intra- or extrahepatic cholangiocytes showed all expression of cholangiocyte markers KRT7/19 and stem cell marker leucine rich repeat containing G protein-coupled receptor 5 (LGR5). In general, biliary markers were downregulated while cell cycle related genes were upregulated. In addition, only the intrahepatic cholangiocyte organoids showed bipotent differentiation capacity towards HLCs and CLCs, while the extrahepatic organoids only showed differentiation towards CLCs but not HLCs. Besides these intrahepatic and extrahepatic tissue-derived organoids, organoids could also be initiated from fresh human bile obtained from patients. Indeed, Soroka *et al.* 2019 [100] established bile-derived organoids from PSC patients. These organoids expressed cholangiocyte markers and are responsive to inflammatory stimuli, reflecting the pathology of PSC. Together, these cholangiocyte organoids can serve as a disease model for cholangiopathies from specific regions enabling to unravel underlying disease mechanisms and identify novel drug targets.

2.3.8. Direct transdifferentiation (iHEP cells)

Adult somatic cells can be converted into other cell types through a process termed direct transdifferentiation. Direct transdifferentiation of somatic cells can be achieved through an intermediary state of less differentiated cells or directly without any intermediate step [101]. Most commonly used cells for transdifferentiation into hepatocytes are fibroblasts from the skin but other sources such as pancreatic progenitor cells have also been described [102]. Usually transdifferentiation is achieved by overexpression of hepatocyte specific transcription factors and/or transcription factors important during embryonic hepatocyte development [103]. For overexpression usually lentiviral vectors are used carrying the transcription factors although the use of mRNAs has also been reported [104]. These induced hepatocytes or iHEPs usually have an immature phenotype but can be used in disease modelling [105]. In one study neonatal fibroblasts and human foreskin fibroblasts were transdifferentiated to iHEPs by overexpression of the transcription factors HNF4A, CEBPB, FOXA2, and MYC [106]. The iHEPs generated through this protocol have a hepatocyte-like phenotype, but display low growth rates and only short-term culture potential. A similar approach using a different combination of transcription factors (FOXA3, HNF1A, HNF4A) also resulted in HLCs with a poor proliferation, but the latter was overcome by overexpression of SV40 large T antigen [103]. DIC has been modelled in iHEPs and showed a highly similar response to nevaripine-induced cholestasis compared to PHHs [107].

3. Advanced *in vitro* models

Current liver models are usually based on a single cellular entity and often lack polarity, inter-cell communication and gradients/zonation. To circumvent these limitations and in order to recapitulate a more *in vivo*-like liver model, several approaches can be applied which include co-culture, spatial orientation or microfluidics. Even *ex vivo* approaches are used such as normothermic machine perfusion of livers or precision-cut liver. Several advanced *in vitro* models of fatty liver disease and cholestasis have been developed during the last decades. These include both scaffold-containing and scaffold-free systems, such as organ-on-chip systems [108].

3.1. Liver-on-a-chip (LOC)

In 2016, during the World Economic Forum, “Organs-on-chips” were amongst the top 10 emerging technologies and several reviews have been published describing the technology, principles and potential [109-111]. The vast majority of liver-on-chip (LOC) devices are directed towards hepatotoxicity [112,113], but several allow the analysis of fat accumulation in hepatic cells [114]. The LOC models were usually based on an excess of FFAs and lacked non-parenchymal cells limiting the analysis to end-point fat accumulation [114].

3.2. Hollow fiber membranes (HFMs)

Hollow fiber membranes (HFMs) have been used to study different organ systems *in vitro* including intestine and kidney [115,116]. The advantages of the HFMs include the addition of microfluidics and the epithelial polarisation allowing the analysis of *trans*-epithelial transport [117]. For *in vitro* modelling of liver HFMs cultured with HepG2s have been shown to increase the functionality of the cells [118]. With PHHs and supporting cells, HFMs have been used for drug biotransformation under static and dynamic conditions [119]. Although no cholestatic or steatotic models are described, bile acid transport has been described in a HFM system with cholangiocytes showing the feasibility of the system [120].

3.3. Spheroids

The 3D culture of single cell-type spheroids with PHHs have been

show to better preserve the mature hepatocyte phenotype during long-term cultivation which re-establish cell polarity and improve (duration of) liver function [26,121]. With regards to cholestasis, spheroids created with PHHs can be used to study the effect of compounds (e.g. bosentan and troglitazone) known to cause cholestatic injury [122]. Exposure of spheroids to these cholestatic inducing drugs in the presence of bile acids lead to oxidative stress, increased bile acid accumulation, induced death receptor 5 expression and ultimately cell death [122]. Also for steatosis modelling PHH spheroids can be used to study the accumulation of neutral lipids, for instance after treatment with drug-induced steatosis compound cyclosporine A [26] or excessive amounts of FFAs [123].

4. *Ex vivo* human liver systems

The idea to use tissue slices for research has been posted as early as the 1920s [124], however only in the 1980s an instrument for the retrieval of advanced tissue slices was developed that was able to cut standardised sections of (liver) tissue [125]. As such, human precision-cut liver slices (PCTS) represent an *ex vivo* liver model which retains the complex and multi-cellular architecture of the hepatic environment. Usually, they are generated by cutting freshly isolated liver into sections of approximately 8 mm [126]. This multicellular model can be used to investigate the mechanisms of liver injury and for the identification of novel therapeutic targets. Moreover, PCTS show organ- and pathology-specific differences in the regulation of genes and canonical pathways for drug metabolism and fibrosis [127]. Some limitations remain, as the PCTS need to be obtained from fresh healthy tissue which usually is the most distal part of a diseased liver after hepatectomy [128]. In addition, the ischemia during organ collection, mechanical stress due to slicing and culture conditions (usually high-oxygen conditions under continuous agitation) lead to a loss of tissue viability [127]. DIC in PCTS has been shown to affect the pathways affected in drug-induced cholestasis in human liver [129] and also a steatotic model using excess FFAs has been described [128].

Some of the limitations of PCTS could potentially be overcome by the use of modern perfusion systems. Recent developments in machine perfusion systems originally developed for liver transplantation could create a potential to use normothermic perfused whole livers as an *in vitro* model to study liver diseases [130]. The main benefit of using normothermic perfusion is that the liver is maintained in a fully functioning state *ex situ* for up to one week by providing oxygen and nutrition at 37 °C [131]. Although this technique is currently mainly performed to improve the quality of human donor livers deemed unfit for transplantation [132-134], normothermic machine perfusion with whole blood using porcine livers has already been used to study specific liver diseases [135,136]. These studies were mainly focussed on hepatotoxicity, however, studies using steatotic pigs, for instance through a high fat diet, showed the potential to use these *ex vivo* models to study steatosis [137,138]. Cholestasis has not yet been investigated using this approach, as far as we could ascertain.

5. Animal models for liver diseases

Using animal (mostly rodent) models in research has several advantages and disadvantages (Fig. 2). Rodent models are useful to examine the liver *in situ* and to investigate inter-organ relations or even behavioural consequences of liver disorders. On the other hand, housing and breeding can be expensive and time consuming, animal research is subject to ethical concerns and sometimes issues arise about the translatability to human diseases. Over the years, several reviews have been written about established cholestatic rodent models

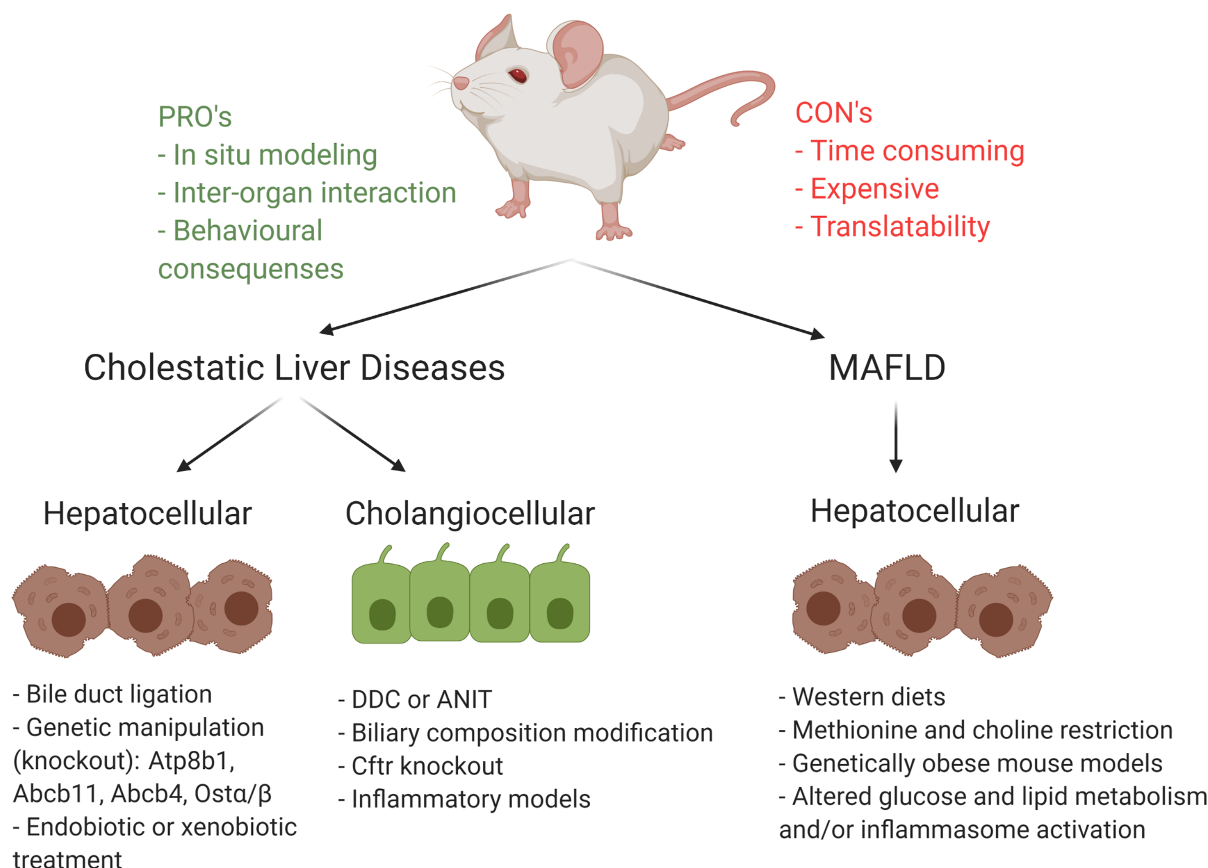

Fig. 2. Rodent models in hepatobiliary research. Numerous rodent models that reflect either hepatocellular or cholangiocellular origin of disease are available and encompass different aspects of cholestatic liver diseases or MAFLD.

[139,140]. Below, we aim to give an up-to-date overview of the current rodent models used in hepatobiliary research, focussing on cholestatic liver diseases and MAFLD. The section about cholestasis is subdivided based on location of the defective flow and subsequently in non-genetic approaches versus genetically modified animal models for disease.

Extrahepatic obstruction resulting in cholestasis is mimicked by performing a bile duct ligation (BDL), by surgically removing the gall bladder (this step is occasionally omitted) and ligating the common bile duct to fully disturb bile flow [141]. This is a relatively simple acute model for a total obstruction. Many of the clinical characteristics, such as jaundice, elevated bile salt levels and increased biochemical parameters of cholestasis are mimicked, but the onset is very acute and the severity of the phenotype often prevents long term follow-up. This can be overcome using a more advanced procedure with partial BDL which has been shown to cause obstructive cholestasis but has slightly milder symptoms such as reduced necrotic areas in the liver [142,143]. Injections of rhesus rotavirus soon after birth induces biliary atresia mimicking cholestasis in Balb/c mice [139,144]. Administration of the toxic isoflavonoid, biliatresone, induces a biliary atresia phenotype in zebrafish [145]. Finally, Marrone *et al.* (2019) [146] measured a significant reduction in bile flow 72h after femoral vein injection with *Salmonella* typhimurium lipopolysaccharide (LPS) in rats, showing that LPS can also be used to induce cholestasis. However, the origin of the blockade is somewhat unclear.

Intrahepatic cholestasis can be subdivided into cholestasis with a hepatocellular or cholangiocellular origin. Examples of hepatocellular-derived cholestasis include intrahepatic cholestasis of pregnancy (ICP), genetic causes leading to intrahepatic cholestasis and cholestasis due to drugs, alcohol, viral infection or hepatocellular cancer, whereas PBC and PSC are examples of cholangiocellular cholestasis.

5.1. Animal models for hepatocellular cholestasis

Experimental models for disturbances in bile flow with a hepatocellular aetiology can roughly be divided into 2 categories: chemically induced or linked to a monogenetic gene defect. Models for the latter are largely driven by human genetics identifying gene-defects that are postulated to be causatively linked to cholestasis. Here, we limit the discussion to models for progressive familial intrahepatic cholestasis (PFIC) and for Solute Carrier Family 51 Subunit Alpha/Beta (*SLC51A/SLC51B*) deficiency. Recently, Li & Dawson (2019) [171] extensively summarized animal models to study bile acid biosynthesis, transport and metabolism with both genetic and non-genetic models. Pharmacological inhibition of BSEP also leads to cholestasis with an hepatocellular origin. Most commonly used experimental approach is the use of a high dose of oestrogens. Particularly in rats this induces impaired bile flow and is sometimes used as a model for ICP. The aetiology of the latter disease is indeed linked to hormones that peak in the third trimester of pregnancy, but not unequivocally restricted to oestrogen.

5.1.1. Progressive familial intrahepatic cholestasis (PFIC)

PFIC is clinically subdivided in 5 different types, each caused by a different genetic mutation [147,148]. Symptoms caused by PFIC1 usually develop in the first months of life and originate from a malfunctioning ATPase Phospholipid Transporting 8B1 (ATP8B1) protein, due to mutations in the *ATP8B1* gene [149]. *Atp8b1* deficient mice have been used as a suitable model for intrahepatic cholestasis and PFIC, although the onset of disease requires additional dietary challenge with cholate [150]. PFIC2 also develops shortly after birth, and is associated with a mutation in the ATP-binding cassette subfamily B member 11 (*ABCB11*) gene encoding BSEP protein, facilitating bile salt export across the apical membrane [151]. Bsep deficient mice are less frequently used to model cholestasis since deletion of *Abcb11* does not automatically result in severe cholestasis, most likely due to the more hydrophilic composition of the bile acid pool in mice compared to human [152,153]. In contrast, *Bsep* knockout mice are protected against acquired cholestatic liver injury induced by ligation of the common bile duct or prolonged 3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) feeding as bile acid detoxification/hydroxylation is induced in these mice [154].

For the third PFIC type, PFIC3, the origin of the disease can be found in a genetic mutation in ATP binding cassette subfamily B member 4 (*ABCB4*), coding for MDR3 (in humans) and MDR2 (in rodents). MDR2/3 is responsible for the secretion of phospholipids in bile, thereby amongst others reducing the toxicity of bile salts [155]. *Abcb4* deficient mice are used as a rodent model for PFIC3 [155-157]. However, the lack of phospholipids in bile induces cholangiocyte damage, fibrosis and ultimately cholangiocarcinoma. Therefore, the *Mdr2* knockout mouse is a genetically modified mouse that is frequently used to study liver fibrosis and sclerosing cholangitis [155,156,158]. PFIC4 and PFIC5 are discovered more recently and associated with mutations in genes Tight Junction Protein 2 (*TJP2*) and Nuclear Receptor subfamily 1 Group H member 4 (*NR1H4*), respectively encoding the tight junction protein 2 and the nuclear receptor for bile acids FXR [159]. Rodent models to study these genetic diseases therefore mainly consist of *Tjp2* and *Nr1h4* knockout mice (the latter eventually cross bred with the *Shp* knockout mice, where Small Heterodimer Partner (SHP) is a downstream target of FXR signaling) [160].

5.1.2. Organic solute transporter alpha and beta (OSTα/β) deficiency

OSTα-β is a heteromeric transporter consisting of alpha and beta subunits encoded by *SLC51A* and *SLC51B* and plays a crucial role in the enterohepatic circulation of bile salts [161]. OSTα-β facilitates transport of bile salts across the basolateral membrane of enterocytes into the portal circulation, thereby effectively contributing to the recycling of bile salts from the ileum. In addition, OSTα-β is also increasingly expressed in hepatocytes during cholestasis to limit the toxic effects of accumulating bile acids [162]. The interaction between both subunits is crucial, in order to form a functional complex at the plasma membrane [163]. Absence of one of the subunits results in instability and finally degradation of the protein. Deficiency of OSTα in patients has been shown to result in liver fibrosis, cholestasis and congenital diarrhoea [164]. However, *Osta* knockout mice did not show increased fecal bile salt secretion and showed even protection against cholestasis-induced liver damage in a BDL model [165]. This protection could be related to the decreased total bile acid pool due to repressed bile salt formation in the liver [166] and increased renal excretion of bile salts [165]. Similar to OSTα deficiency, OSTβ deficiency in human was characterised by chronic diarrhoea, slightly elevated serum liver enzymes and histological signs of cholestasis [167]. An *Ostβ* knockout mouse model is not yet published and under development.

5.2. Animal models for cholangiocellular cholestasis

Cholangiocellular diseases with a clear genetic aetiology, particularly monogenetic cholangiopathies can be studied using genetically modified animal and cellular models that phenocopy the disease. This is further outlined below and reviewed in detail by Fabris *et al.* (2019) [168]. For acquired cholangiopathies, with a less direct causal genetic link, several models have been postulated. Biliary blockade of bile flow can be chemically induced by administration of various compounds including α-Naphtylisothiocyanate (ANIT) and DDC. DDC-diet induced cholestatic rodent model is already extensively described by Pose *et al.* (2019) [140]. ANIT can be administered orally and DDC is usually added to the diet [169]. DDC treatment results in biliary obstructions in the larger bile ducts whereas ANIT induces damage in the smaller bile ducts [139]. The importance of the intestinal microbiome in development of biliary damage has been demonstrated by Ten Isaacs *et al.* (2020) [169]. Cholestasis-inducing agent ANIT and DDC-diet induced cholestasis in germ-free mice but did not cause increased liver enzyme concentrations in blood plasma or necrotic areas in the liver. From origin germ-free mice enriched with wild-type microbiota on the other

hand did have these effects, drawing attention to the crucial role of intestinal microbiota during cholestasis [169].

Although these models have provided detailed insight particularly in hepatic adaptations upon cholestasis, in general these models seem to divert significantly from biliary diseases such as PBC and PSC with respect to aetiology and presentation. This is hampering translational research and therefore better models are required. In our view, genetically modified models already provide a better basis and are likely further refined in the near future to provide models that mimic the human situation with respect to the role of the immune system, biliary composition and microbial contribution to disease development.

5.2.1. Monogenetic models for cholangiopathies

Fabris *et al.* (2019) [168] recently proposed that the knowledge acquired in the last decade about genetic and congenital cholangiopathies has also led to a better understanding of the mechanisms of acquired cholangiopathies. In line with this line of reasoning, they suggest to consider models for these monogenetic disorders also for acquired biliary disorders and particularly advocate *Cftr* deficient mice as a suitable model for PSC. Lack of CFTR increases the sensitivity of epithelial Toll-like receptor 4, that sustains the secretion of pro-inflammatory cytokines and consequently leads to peribiliary inflammation in response to gut-derived products. We agree with their notion that a combination of an inflammatory component with biliary composition alteration seems the best strategy and provide alternative/ additive strategies to achieve this below.

5.2.2. Biliary composition modifiers

One of the main reasons why animal models sometimes poorly mimic human cholestatic liver disease is the difference in biliary composition. Two main discrepancies are i) the relative amounts of bicarbonate, which is much higher in humans and ii) the hydrophobicity of bile salts, with the majority of mouse bile salts formed by taurine-conjugated muricholate, a very hydrophilic compound, whereas human bile mainly contains glycine-conjugated chenodeoxycholate (CDCA) and glycine conjugated cholate, which contributes to a more toxic biliary milieu [170].

Currently, two distinct genetic strategies exist to induce bile salt-induced cholangiocyte damage. The first is the *Abcb4* knockout model, which is introduced above. These mice do not have phospholipids in bile, rendering it more cytotoxic, and these mice have biliary damage leading to various aspects of PSC. Therefore, this genetic model is sometimes considered as a PSC mimicking system although PSC patients do not have any defect in biliary phospholipid transport. Therefore, this system is mainly useful to study events downstream of biliary damage, and not so much the aetiology. Nevertheless, the *Mdr2* knockout mouse in combination with other genetic modifications or treatments is regularly used to study PSC pathogenesis. This way, studies have outlined the importance of the histamine pathway [172], filament protein vimentin [173], monoacylglycerol lipase [174] and transforming growth factor β2 [175] in PSC. Second is a more recent model using mice that lack CYP2C70, the enzyme responsible for sequential 6β-hydroxylation and C7-epimerization of CDCA and thus the formation of hydrophilic muricholate [176,177]. This can be combined with deficiency for CYP2A12, the enzyme responsible for 7α-rehydroxylation in mice. These mice have a more human-like bile acid pool composition (although the preference for taurine conjugation remains) and chronic liver inflammation with an obvious ductular reaction, despite normal (or even elevated) phospholipid excretion in bile.

5.2.3. Putative novel models

It is increasingly clear that the microbiome and its interplay with dietary components plays an important role in the aetiology of cholestasis and its consequences on liver function [178]. For example, microbial composition determines the bile acid composition (and vice versa), with obvious consequences on bile toxicity and signaling. PSC patients had decreased intestinal microbial diversity while specific pro-inflammatory bacteria were more abundant [179]. Future developments in controlling and modifying rodent microbiota in animal research facilities will no doubt contribute to the developments of better animal models and lower inter-facility variation in disease presentation.

The immune response to microbial products is largely determined on host genetics and might explain why some individuals are more prone to develop acquired cholangiopathies. Genome wide association studies (GWAS) in patients with PSC, PBC but also MAFLD have highlighted the importance of a diverse set of genetic variations [180-187]. The most obvious association of gene variants with disease has been found in genes associated with the human leukocyte antigen (HLA) complex [182,185]. The role of inflammatory cells in PSC has been outlined by several studies [179,188,189]. T cells can directly affect bile acid metabolism and thereby limit bile acid induced injury [188]. There is also an increased amount of pro-inflammatory cytokine IL-17 producing T cells found surrounding intrahepatic bile ducts of PSC patients [179]. Additionally, a potential role for the intestinal microbiome in T cell differentiation has been described. Due to the extensive difference between the human and mouse immune system, this insight is not easily exploited to design improved models for PBS or PSC. However, models with a modified immune response could be valuable. For example, cholangiocyte damage results in recruitment of predominantly pro-inflammatory macrophages via the CCL2 pathway. When this response was disrupted in mice, macrophage recruitment to the liver was decreased, together with the amount of liver fibrosis and serum bile acid concentrations [189]. Similarly, biliary senescence can be induced leading to local inflammatory response mimicking certain aspects of sclerosing cholangitis [190].

Genetic variants associated with PSC have also been found in non-immunology related genes such as Doublecortin Domain Containing 2 (*DCDC2*) [191], kinesin family member 12 (*KIF12*) and Protein Phosphatase Mg^{2+}/Mn^{2+} dependent 1F (*PPM1F)* [192]. DCDC2 is normally expressed in cilia of cholangiocytes and mutations in *DCDC2* in neonatal sclerosing cholangitis patients leads to complete depletion of functional DCDC2 and absence of primary cilia [193]. Physiologically, mutations in *DCDC2* therefore result in disturbed cholangiocyte homeostasis which might be caused by disturbed Wnt signaling [194]. KIF12 has previously been associated with cholestasis [195]. Three patients described harboured mutations in *KIF12* and showed symptoms such as liver fibrosis, inflammatory cell infiltration and bile duct loss. It is however not precisely known how *KIF12* contributes to sclerosing cholangitis, although the authors propose that KIF12 might play a role in bile duct epithelial cell function via hepatocyte nuclear factor 1 β (HNF1β) [192,195]. Similar to *DCDC2* and *KIF12*, *PPM1F* mutation is associated with primary cilia dysfunction since it is also known to regulate kinesin mediated transport [192]. A novel PSC rodent model might therefore include the dysfunctional primary cilia of cholangiocytes in order to accurately reflect human pathogenesis.

Animal studies can be used to investigate a possible causal contribution of genes identified in GWAS studies and lead to better animal models for cholestasis with a cholangiocellular aetiology. A study using *Fut2* knockout mice shows how challenging this can be. *FUT2* is risk gene for PSC, but these knockout mice did not fully mimic PSC pathogenesis [196]. Instead, a subset of *Fut2* knockout mice were found to have extremely high serum bile salt concentrations, although they did not develop cholestasis. The mice with elevated bile salt levels displayed portosystemic shunting, although also signs of biliary damage were found when the mice were challenged with a hydrophobic bile salt in the diet.

PBC is also associated with mutations in a broad set of genes. The disease is likely an auto-immune disease and many genetic mutations found in GWAS are associated with the immune system. Pathogenic pathways that are described in PBC are primarily involved in inflammation, fibrosis, oxidative stress, cell proliferation, signaling and apoptosis [197]. Arenas *et al.* (2019) [198] found that reduced

expression of Anion Exchange Protein 2 (AE2), involved in biliary bicarbonate secretion, in PBC patients is most likely caused by promotor hypermethylation. The relevance of AE2 in development of PBC has been shown in the *Ae2a,b* knockout mouse, which develops damaged cholangiocytes as a result of bile salt induced injury [199]. However, this model steps away from the original idea where PBC is described as an auto-immune disease. The NOD.c3c4 mouse on the other hand follows the autoimmune aetiology [200,201]. The NOD.c3c4 mouse develops antibodies against the pyruvate dehydrogenase complex, specifically the lipoyl domain of the E2 subunit (PDC-E2), which is in line with the typical production of mitochondrial antibodies against PDC-E2 in PBC patients [200,201]. In addition to this model, an IL-2 receptor α (IL-2Rα) knockout mouse and a mouse lacking TGFβ signaling in T cells specifically have been shown to feature PBC characteristics [202-204]. The lack of TGFβ signaling in T cells or global IL-2Rα signaling caused lymphocyte infiltration around biliary tracts and an increase in pro-inflammatory cytokines IFN-γ, TNF-α and IL-6 in the mouse serum, findings similar to PBC patients. E26 transformation specific sequence 1 (ETS-1) is a transcription factor involved in, amongst other, T cell proliferation and differentiation and has been associated with PBC [205]. Furthermore, SNPs have been found in the major histocompatibility complex (MHC) region, specifically antigen presenting [206], and nuclear factor-κB subunit 1 (*NFKB1*), while array datasets have shown that C–C motif chemokine ligand 5 (CCL5), interleukin 7 receptor (IL7R), TNF receptor superfamily member 1 A (TNFRSF1A) could also play a role in PBC [207]. These genes have immunoregulatory functions. NFKB1 encodes for a subunit for the nuclear factor-κB (NF-κB) dimeric complex acting as a transcription factor which has a pivotal role in regulating immune metabolism. Additionally, Tumor Necrosis Factor Receptor Superfamily Member 1A (TNFRSF1A) is a membrane receptor, amongst other expressed in T cells, that binds TNFα and can thereby activate NF-κB. CCL5 recruits T cells towards inflamed tissue while IL7R is important for both B and T cell development [208,209]. In addition, CCL5 and IL7R mediate T cell apoptosis. Since most genes are involved in T cell regulation, this might be an interesting target for a novel PBC model. Finally, PBC was more likely to develop in patients with a mutation in the gene coding for muscarinic acetylcholine receptor type 3 as described by Greverath *et al.* (2019) [210].

Taken all together, there is still a lot unknown about the pathogenesis of many cholestatic disorders. Nevertheless, it is likely that a rodent model can be used reliably to investigate novel therapeutic options consists of a combination of genetic mutations/variations and/or modifications in bile acid composition and microbiota.

5.3. Metabolic associated fatty liver disease

In 2016, Ibrahim and colleagues defined the ideal animal NASH model as a “model that encompasses all the defining features of the human condition, including obesity, insulin resistance, steatohepatitis, and fibrosis” [211]. Therefore, over the years several animal models have been developed based on genetic modifications, treatments consisting primarily of diets or a combination of both [211-214]. Challenging aspect of creating an accurate rodent model for MAFLD is to model both the metabolic and histopathological phenotype and to reflect the disease heterogeneity. Patient stratification is expected to be increasingly important in future clinical trials. Similarly, current animal models do not cover all aspects of disease, with regard to aetiology as well as testing novel therapeutic options and only a combination of models encompasses the full scope of MAFLD.

Treatment of mice with diets high in fat, cholesterol and fructose is a relatively easy way to induce obesity and a fatty liver in mice, and therefore often used to study the onset and treatment of steatosis in MAFLD [212,215]. The severe liver pathological phenotype of human NASH is not directly reproducible with a high fat diet in mice alone, as inflammation is mostly lacking likely due to insufficient onset of lipotoxicity [216]. Addition of high fructose and cholesterol to the high fat diet is used to induce steatohepatitis and fibrosis in mice [217] but still very time consuming. Therefore, it was postulated by Tsuchida *et al.* (2018) [218] to add weekly intraperitoneal dosing of low concentrations of carbon tetrachloride (CCl_4), as an accelerator. Another strategy is to inject mice with streptozotocin shortly after birth. Streptozotocin severely damages pancreatic islets, causing diabetes type 1, and contributes to the development of hepatic steatosis when fed a high fat diet [219]. Furthermore, there seems to be clear contribution of the genetic background of the mice as Asgharpour *et al.* (2016) [220] showed that mice derived from a stable isogenic cross between C57BL/6J and 129S1/SvImJ mice recapitulated multiple aspects of human MAFLD when fed a high fat diet with ad libitum consumption of glucose and fructose whereas the parent strains did not.

Another frequently used mouse model is based on dietary depletion of methionine and choline or only choline [211,221]. Methionine and choline are amongst others crucial for production and secretion of very low density lipoprotein (VLDL). Impairment of these pathways results in lipid accumulation in the liver which together with increased oxidative stress results in a phenotype with steatohepatitis and fibrosis. Nevertheless, this model lacks metabolic features such as insulin resistance and is even accompanied by weight loss. A similar phenotype is observed in methionine adenosyltransferase 1A (Mast1a) deficient mice. These genetic/dietary approaches may provide suitable models for “lean MAFLD”, that is the presence of steatohepatitis without obesity, a disease presentation more prevalent in Asia [3]. The choline-deficient L-amino acid (CDAA) diet on the other hand consists of similar constituents as the choline deficient diet, however the proteins are substituted by a mixture of L-amino acids [211,213]. The metabolic effects seen as a result of the CDAA vary, as Kodama *et al.* (2009) [222] do not find altered insulin sensitivity after a 20-week CDAA diet in both male and female C57BL6/J mice, whereas Miura *et al.* (2010) [223] did find increased insulin resistance in male C57BL/6J mice after a 22-week CDAA diet. This model however greatly represents the histopathological phenotype seen in advanced MAFLD, by expressing features such as steatohepatitis, fibrosis and finally carcinoma [213]. This may reflect some of the considerable sexual dimorphism of MAFLD with male predominance at earlier disease state and increased disease frequency in postmenopausal women [224].

Ob/ob or db/db mice have a genetic mutation in the leptin or leptin receptor gene resulting in increased food intake, causing amongst others severe obesity, hyperlipidaemia and insulin resistance [211]. Liver injury or severe steatohepatitis such as seen in NASH is usually not present and therefore requires additional treatments such as activation of the inflammasome and other interference with macrophage responses, toll like receptors or JNK signaling [211]. For example, Handa *et al.* (2016) [225] have shown that dietary iron supplementation in db/db mice leads to a severe NASH histopathological phenotype in the liver, accompanied by immune cell activation.

A less common but interesting genetic model for steatohepatitis is the foz/foz mouse, which has a mutated Alström syndrome protein 1 (*Alms1*) gene, previously associated with obesity, and has been characterized with steatosis, obesity, diabetes, high cholesterol and insulin resistance [212,226]. Combination of the foz/foz mouse with a high fat diet resulted in severe steatohepatitis, thereby generating a more accurate reflection of the human NASH physiology. Another obese mouse model is the KK-Ay/a mouse, harbouring a heterozygous mutation in the Agouti gene and therefore lack hypothalamic suppression of appetite. KK-Ay/a mice are obese, insulin resistant and possess steatotic livers [227]. Additional treatment of KK-Ay/a mice with methionine and choline depleted diet has been shown to further induce hepatic fibrosis, steatohepatitis and immune cell infiltration [228]. As fat plays a major role in development of NASH, it might not be surprising that impaired fatty acid oxidation increases the risk on developing fatty liver, fibrosis and finally hepatic steatosis. Therefore, several genetic mouse models aim to disrupt fatty acid oxidation, such as fatty acyl-CoA oxidase (Aox)

deficient mice [211,229]. These mice lack the primary enzyme required for peroxisomal β-oxidation, leading to fatty acid accumulation and mild hepatic steatosis. Hepatocellular carcinomas developed spontaneously between 10 and 15 months of age [230].

A major downside of gene-knockout models is that the pathway that is genetically inactivated could be also therapeutically relevant, leading to ineffective treatment in the knockout model. For example, ob/ob mice will not be a suitable model to test novel therapeutic modalities that engage with leptin signaling. Perhaps this limitation can be overcome by exploiting gene modifications and not total knockout models and by focusing on genes with a causal role in MAFLD. Human GWAS data has revealed genes associated with MAFLD. An overview and detailed discussion of these genes is provided by Anstee *et al.* (2016) [180] and Krawczyk *et al.* (2020) [231]. Involvement of these genes and pathways might explain human disease aetiology and therefore lead to a translatable mouse model that entails both the metabolic and histopathological phenotype. First, Karrar *et al.* (2019) [232] have shown that several alleles from both HLA class 1 and class 2 genes were associated with development of MAFLD and NASH, thereby highlighting the importance of the immune system in development of disease. Second, and not surprisingly, genetic pathways involved in glucose and lipid metabolism are associated with MAFLD. For example, variants in the gene coding for adiponectin, which is involved in glucose homeostasis and fatty acid metabolism has been associated with MAFLD [233]. Patatin-like phospholipase domain-containing protein 3 (PNPLA3) mediates triglyceride hydrolysis in adipocytes and is a downstream target of transcription factor peroxisome proliferator-activated receptor-γ (PPARγ). PNPLA3 is highly associated with MAFLD development, as has been shown in multiple independent studies [180,181,234,235]. James *et al.* (2019) [236] also confirmed that variants in the genes glucokinase regulatory protein (*GCKR*), *PNPLA3*, the lyosophosphatidylinositol-acyltransferase MBOAT7, Transmembrane 6 Superfamily Member 2 (*TM6SF2*) and Tribbles pseudokinase (*TRIB1*) are associated with MAFLD development [180,181,186]. TRIB1 functions via the MAPK pathway and therefore regulates cell proliferation, differentiation and apoptosis while dysfunctional TM6SF2 has previously been associated with MAFLD due to impaired VLDL secretion [237].

Finally, gut microbiota likely play a role in several of the factors underlying MAFLD, including composition of bile salts, intestinal permeability, altered immunity and presence of microbe-derived metabolites [238]. Using fecal microbial transfer (FMT) protocols several animal studies have demonstrated the contribution of the microbiome to the onset of steatosis and steatohepatitis [238]. However, a consensus on how to implement and standardize such approaches to create improved mouse models for MAFLD has not been reached yet. To summarize, it seems likely that a translatable mouse model for MAFLD entails both the metabolic and histopathological characteristics by combining an obese mouse model with additional treatment to activate the inflammasome, or by combining multiple models, each focusing on one of the causal factors in this multi-hit metabolic associated disease. This could be achieved with established rodent models as described above, however novel genetically modified mouse models based on human GWAS data and/or FMT could also be of use. Whether both metabolic and histopathologic characteristics are required in the mouse model or not, is fully dependent on the specific type of research and research questions involved.

6. Conclusion

In this review, we have extensively discussed *in vitro*, *ex vivo* and *in vivo* models specifically for MAFLD and cholestasis related diseases such as PBC and PSC. The availability of different types of models each with their own advantages and disadvantages creates a lot of opportunities for hepatobiliary research but also raises questions about correct use for a specific research goal. We summarized and clarified distinct approaches and therefore aim to be a helpful tool in deciding on the model that best suits your research question.

CRediT authorship contribution statement

Roni F. Kunst: Writing - original draft, Writing - review & editing. **Marije Niemeijer:** Writing - original draft, Writing - review & editing. **Luc J.W. van der Laan:** Writing - review & editing. **Bart Spee:** Visualization, Writing - original draft, Writing - review & editing. **Stan F.J. van de Graaf:** Conceptualization, Writing - review & editing.

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