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Review

In vitro and *in vivo* translational models for rare liver diseases☆

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ABSTRACT

A challenge in developing effective treatments is the modeling of the human disease using *in vitro* and *in vivo* systems. Animal models have played a critical role in the understanding of disease pathophysiology, target validation, and evaluation of novel therapeutic agents. However, as the success rate from entry into clinical testing to drug approval remains low, it is critical to have high quality and well-validated models reflective of the disease condition. Additional experimental models are being developed based on functional *in vitro* 3D tissue models such as organoids and 3D bioprinted tissues. Because these 3D tissue models mimic closer the architecture, cell composition and physiology of native tissues, they are now being used as screening platforms in drug discovery and development and for tissue transplant in regenerative medicine. Here we review the current state-of-art of *in vitro* and *in vivo* translational models for the development of therapies for rare diseases of the liver.

1. Introduction

The liver is a complex organ that is essential for numerous functions that maintain cellular homeostasis in the human body. It is also susceptible to a wide range of human diseases that occur when processes become impaired or injury occurs due to infection or other toxic insults. Although the injury and underlying causes of the different liver diseases vary, the stages and damage to the organ are consistent in the overall disease process, progressing in four stages from the initial insult to fibrosis, advanced liver disease, and organ failure (Fig. 1) [1]. Even though the stages are consistent, there is variability in age of onset, severity, phenotype, and progression associated with each type of liver disease. For patients with severe liver disease, the only treatment is liver transplantation, and as of May 2018, almost 14,000 patients are on the transplant waiting list, and only 20% (2664) have received a liver [2]. While for a few, liver transplantation offers the possibility for a cure or treatment, there is a huge unmet need for the development of new treatments that can intervene at the earlier stages of liver disease before organ damage is irreversible and a patient's only hope for survival is liver transplantation.

Critical to the development of effective treatments for human disease is the translation of knowledge from basic research through pre-

Abbreviations: AAT, alpha-1-antitrypsin; AATD, alpha-1-anti-trypsin deficiency; ALB, albumin; α-SMA, alpha smooth muscle actin; ALF, acute liver failure; ASO, antisense oligonucleotide; ATP7B, copper-transporting beta ATPase; AVV, adeno-associated virus; CCl_4, carbon tetrachloride; CEs, cholesteryl esters; CDS, coding sequence; Cu, copper; CN-1, Crigler–Najjar Syndrome Type 1; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9 nuclease; 4DM, Drug Discovery, Development and Deployment Map; ECM, extracellular matrix; ER, endoplasmic reticulum; ERT, enzyme replacement therapy; FAH, fumarylacetoacetate hydrolase; FDA, Federal Drug Administration; FPN, ferroportin; C6P, glucose-6-phosphate; C6PC, glucose-6-phosphatase; G6Pase-α, glucose-6-phosphatase α; G6PT, glucose-6-phosphate transporter (G6PT); GRT, gene replacement therapy; GSD-1, glycogen storage disease type I; HCC, hepatocellular carcinoma; HAMP, hepcidin antimicrobial peptide; HH, hereditary hemochromatosis; HFE, high (iron) Fe; hiPSCs, human induced pluripotent stem cells; HO, hepatic organoids; HT-1, Hereditary Tyrosinemia Type 1; HVE, hemojuvelin; IL2rg, Interleukin 2 receptor gamma; JAG1, jagged1; KRT19, keratin 19; LAL, liposomal acid lipase; LALDs, liposomal acid lipase deficiencies; LEC, Long-Evans Cinnamon; LOs, liver organoids; miRNA, micro-ribonucleic acid; MTX, methotrexate; NDR, nodder; NOTCH2, neurogenic locus notch homolog protein 2; PK/PD, pharmacodynamic/pharmacokinetic; NTBC, nitisinone; PI S, protease inhibitor S; PI Z, protease inhibitor Z; PiZZ, protease inhibitor ZZ; PI M, protease inhibitor M; POC, proof of concept; 2D or 3D, two or three dimensional; rAAV, recombinant adeno-associated virus; Rag2, recombinate activation gene 2; rh, recombinant human; RNAi, ribonucleic acid interference; SERPINA1, serine protease inhibitor family A member 1; siRNA, silencing ribonucleic acid; SOC, standard of care; TGFβ, transforming growth factor beta; TMD, transmembrane domain; tx, toxic milk; TRF2, transferrin receptor 2; UTR, untranslated region; UGT1A1, uridine diphosphate glucuronosyltransferase 1A1; WD, Wilson's Disease

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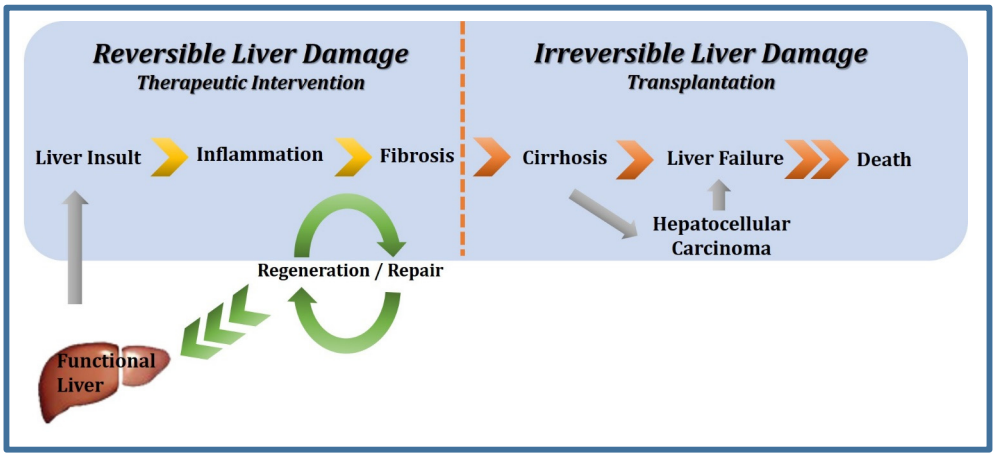

Fig. 1. Stages of liver disease progression.

clinical to clinical phases of drug development. Translation is an interactive and complex process that is not accurately captured in a one dimensional, linearly sequential process, as is often outlined in chevron diagrams. Recently, a 'Drug Discovery, Development and Deployment Map' (4DM) of the translational process was assembled (Fig. 2) [3, 4] as an attempt to capture the overall roadmap, connectivity and interactivity between all the components of the drug development ecosystem. This map highlights the complexity and challenges in the process of discovering new medicines, including bottlenecks in need of innovation.

One of the important steps and challenges in translation is the appropriate modeling of human disease using both *in vitro* and *in vivo* systems. Animal models have played a critical role in the understanding of disease pathophysiology, target identification/validation, and *in vivo* evaluation of novel therapeutic agents [5]. In drug development, animal models are also used to bridge the translational gap to clinic by addressing important parameters of *in vivo* pharmacology of the drug, including efficacy, mechanism of action, establishing the pharmacodynamic/pharmacokinetic (PK/PD) relationships, estimating clinical dosing regimens, and determining safety margins and toxicity.

It is generally accepted that the majority of animal models are not able to fully recapitulate all aspects of the human clinical condition. It is estimated that up 70% of new drug candidates fail in Phase 2 and of those that survive to Phase 3, another 50% fail, the majority of which is due to a lack of safety and efficacy [6, 7]. One of the largest hurdles to a higher success rate in drug development is the low clinical predictability of the existing disease models. In most cases, the positive efficacy results observed in *in vivo* preclinical studies using animal models that do not reproduce in clinical development. The differences in disease phenotypes are often attributed to physiological differences in species and/or genetic background [5, 8]. Notably, in a comparative analysis of liver transcriptomes between human, mouse, and rat, it was found that the expression profile of homologous genes was significantly different suggesting there are divergences in liver function [9].

To overcome limitations of *in vivo* models, there is now considerable efforts towards the development of *in vitro* systems that mimic native human tissues for drug testing. Experimental models under development use human induced pluripotent stem cells (IPSCs), 3D tissue models such as spheroids, organoids, and bioprinted tissues, and attempt to recreate the physiological complexity in native tissues which is not available in traditional 2D cell models in a dish. One of the major advantages of these approaches is that the process of "reverse" translation is engaged starting with clinical samples from patients, and then creating a model system to test drug candidates which are more relevant for therapeutic development. These new cell technologies will provide a combination of different pre-clinical models that may eventually provide more robust pre-clinical data that will enable a better selection of drugs candidates with better prediction of human responses in the clinic [5], for example, a mouse liver disease model complemented by an *ex vivo* human liver cell culture or a 3D printed liver tissue using human iPSC cells.

It is critical to have high quality and well-validated models that are reflective of the human disease condition, as determined by using the relevant disease markers, when designing and testing novel treatments in pre-clinical studies so that the results are predictive of efficacy in humans. *In vitro* and *in vivo* models with high predictive power will provide data to enable more effective decision-making during the selection of drug candidates, and hopefully have a higher probability of becoming approved medicines. Furthermore, a higher translational value could be better achieved when combining these systems with other approaches such as quantitative systems pharmacology, biomarkers, efficacy endpoints, natural history studies, and or other clinical data.

Here we will focus on reviewing the state of existing *in vitro* and *in vivo* models used to study a select group of inherited rare liver diseases, including alpha-1-anti-trypsin deficiency (AATD) and hereditary hemochromatosis (HH), the two most common inherited liver diseases. In addition, the review will examine how closely the models mirror the human disease, and their use and translatability in any previous or currently ongoing therapeutic development efforts. This review will not address liver diseases where the primary cause is cancer, autoimmune, infections, alcohol-drugs (prescribed and illicit) and diet-induced as well as idiopathic in etiology.

2. Rare diseases of the liver

There are over 7000 rare diseases, but only ~600 (~10%) have approved therapies. A disease is rare, by definition, if it affects fewer than 200,000 individuals in the US, and although individual diseases may be rare, collectively, they contribute to a significant portion of morbidity, particularly in pediatric populations affecting over 30 million people worldwide. There are many challenges to conducting clinical development for rare diseases, including small numbers of patients with the disease, few disease experts, and limited resources available for their study. Approximately 80% of rare diseases are genetic in origin but only a small percentage have ongoing basic, translational or clinical research. Although most people are aware of common liver diseases such as cancer and hepatitis, there are over 100 different types of liver diseases, and many of them are rare inherited errors of liver metabolism, and many affect pediatric populations [10].

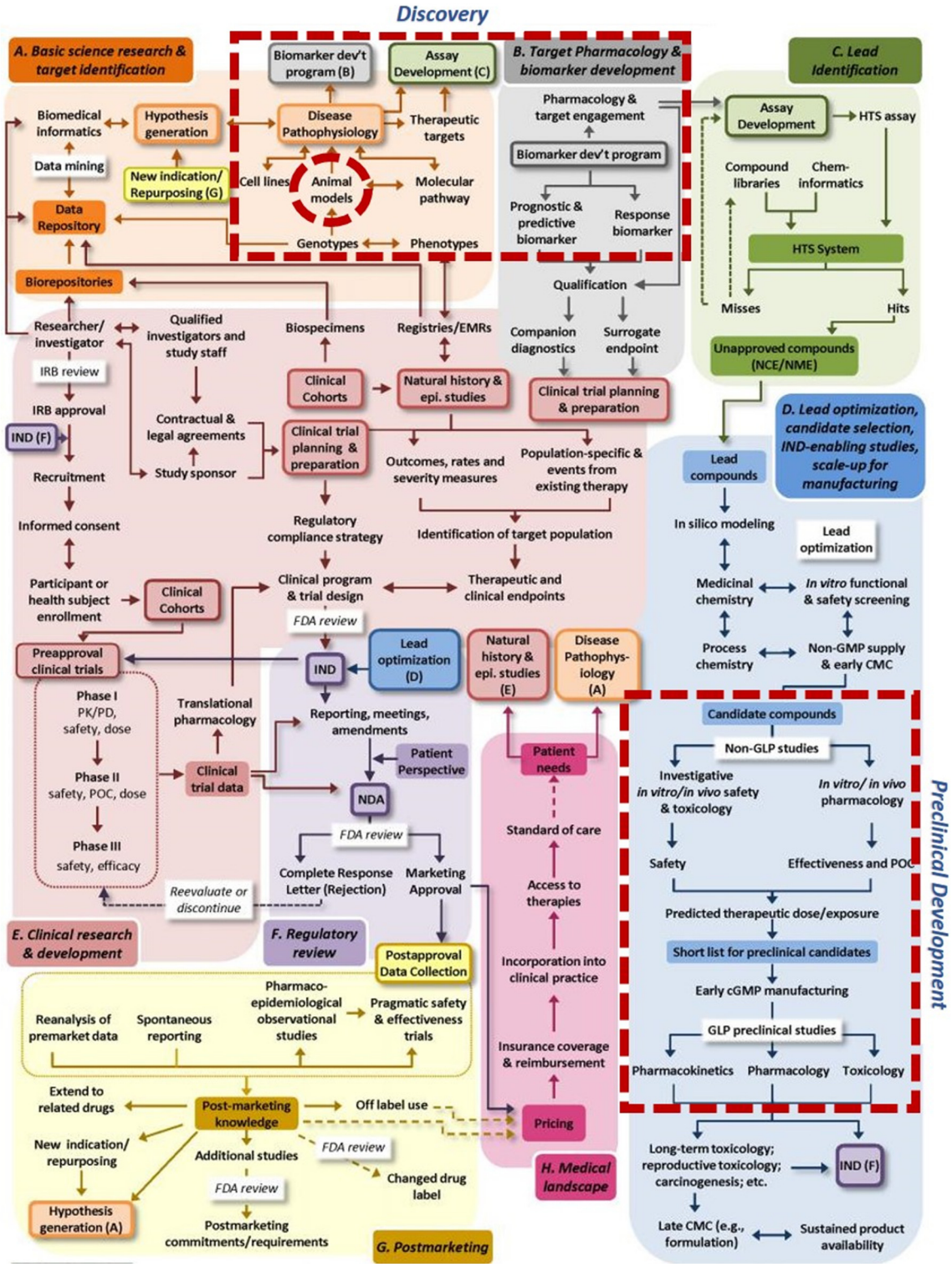

Fig. 2. The role of disease models in the therapeutic development process. Sections outlined in red dashed boxes highlight the importance of disease models and the stage at which they are incorporated into the discovery and preclinical stages of a very complex drug development process. 4D map is adapted and modified from Wagner JA, Dahlem AM, Hudson LD, Terry SF, Altman RB, Gilliland CT, DeFeo C, and Austin CP. Drug Discovery, Development and Deployment Map (4DM): Small Molecules and is licensed to the public under the Creative Commons Attribution-Share Alike 4.0 license. Available at https://ncats.nih.gov/translation/maps [3, 4].

2.1. Animal models of liver diseases

2.1.1. Alagille Syndrome

Alagille Syndrome (ALGS) is a rare autosomal dominant disorder with complex and variable multi-organ system involvement, exhibiting abnormalities in the liver, but the heart, eyes, ears, vertebra, face and in some afflicted individuals, vascular and renal defects are involved as well [11, 12]. ALGS affects between 1 in 30,000 to 1 in 70,000 individuals. The liver is the organ that is primarily associated with increased morbidity and early mortality and unlike the architecture observed in a normal liver, individuals afflicted with ALGS are phenotypically characterized by a reduced number of bile ducts that

clinically translates into chronic cholestasis, a condition where the flow of bile from the liver to the small intestines is either reduced or halted. This results in the build-up of waste such as bilirubin, bile salts, toxins and cholesterol that are normally eliminated *via* the bile which then leads to liver damage. Patients experience jaundice and severe itching known as pruritus from the toxic build-up, in the liver [13]. Interestingly, for some ALGS patients, an improvement in jaundice and pruritus is observed around school age, but unfortunately, for others the liver disease progresses leading to liver damage and cirrhosis (Fig. 1). There is currently no therapeutic available to intervene in the disease process, but gene mutations associated with this disorder have been identified in the NOTCH signaling pathway affecting either a ligand of NOTCH, known as JAGGED 1 or one of its receptors, NOTCH2 [14–17]. The majority of mutations identified to date in Alagille patients, whether missense mutations resulting in frameshifts or deletions, are found in *JAG1*, and only affect one allele of the gene. This indicates that the amount of the normal gene product, produced from the unaffected allele is insufficient to prevent disease manifestation and points to a reduction in gene dosage or haploinsufficiency as the underlying molecular cause of ALGS.

The development of animal models of this disease has been challenging, starting with the embryonic day 10 (E10) lethality of the *Jag1* homozygous null mouse, $Jag1^{dDSL/dDSL}$ [18] and by a lack of the characteristic Alagille phenotype, with the exception of the ocular abnormality in the related *Jag1* heterozygotes, $Jag1^{+/dDSL}$ [18]. These null mice were engineered by deletion of 5 kb of genomic sequence near the 5′-end of the *Jag1* gene that corresponds to the C-terminal region of the DSL domain required for Notch ligands to interact with the cognate receptor. Subsequently, a murine double mutant cross ($Jag1^{dDSL/+}$ $Notch2^{del1/+}$) between the viable heterozygous $Jag1^{dDSL/+}$ and one hypomorphic *NOTCH2* allele, $Notch2^{del1/+}$ was also generated [19]. However, all of the aforementioned models, while helpful in advancing the understanding of ALGS, were either not viable or phenotypically and or genotypically not reflective of the human condition. More, recently, however, two newly published mouse models based on modification of the background strains of existing mouse models appear to recapitulate the human liver disease (Table 1). The first model was achieved by backcrossing the original heterozygous $Jag1^{+/dDSL}$ mouse that existed on a mixed genetic background [18] by > 10 generations on to a pure C57BL6 background [20]. For the purposes of distinction in this review, will refer to the modified background variant as C57BL6 $Jag1^{+/-}$. The second model is derived from an existing mutant mouse with an amino acid substitution, p.H268Q in *JAG1*, known as the Nodder mouse, $Jag1^{+/Ndr}$ [21] which interestingly, like the $Jag1^{dDSL/dDSL}$ [18], the homozygous Nodder mouse, $Jag1^{Ndr/Ndr}$ was also embryonic lethal. This original heterozygous Nodder mouse which existed on a pure C3H background was bred to a mixed C3H/C57BL6 background that subsequently generated viable homozygotes, C3H/C57BL6 $Jag1^{Ndr/Ndr}$ [22]. Andersson et al. [22] pointed out the similarities in phenotypic characteristic compared to the human disease including that in some adult C3H/C57BL6 $Jag1^{Ndr/Ndr}$, similar to a subset of ALGS patients, a compensatory mechanism kicks in resulting in an increase in bile ducts and normalization of liver function. The authors also discussed the shortcomings of both these new background-optimized models indicating that the pure bred C57BL6 $Jag1^{+/-}$, although genotypically mimics the *JAG1* haploinsufficiency typical of ALGS, does not phenotypically capture all of the classical features of liver disease, for example, clinical presentation of jaundice. Alternatively, the mix bred C3H/C57BL6 $Jag1^{Ndr/Ndr}$ appears to phenotypically mimic the ALGS more than any previous model but genotypically does not mirror the haploinsufficient paradigm distinctive of this genetic defect. These observations highlight some of the challenges that the innate differences between the rodent and humans pose when attempting to fully model the human disease. Nonetheless the existence of these two optimized models for ALGS will most likely open the door for further understanding of the disease pathogenesis, development of therapeutics and relevant biomarkers that have the potential for clinical translation. These initial models will also serve as the basis for the development of further disease models for ALGS, including large animal models whose hepatology is closer to humans.

2.1.2. Alpha-1-anti-trypsin deficiency (AATD)

Alpha-1-antitrypsin deficiency (AATD) is a rare genetic autosomal recessive disorder that results in both a loss and toxic gain of function resulting from mutations in the alpha-1-antitrypsin (AAT) gene, *SERPINA1*. The protein product, alpha-1-antitrypsin (AAT), is an acute phase plasma glycoprotein primarily produced in hepatocytes and functions to inhibit degradation of extracellular matrix in the lung by neutrophil elastase. The loss of function is responsible for the lung phenotype due to the deficiency of the AAT protein while the toxic gain of function results from the accumulation of mutant AAT protein entrapped in the hepatocyte endoplasmic reticulum (ER). Many variations/mutations in the AAT gene, *SERPINA1*, have been identified and are classified as normal or deficient. The PI M variant is considered normal based on AAT levels in serum which does not correspond to a heightened risk of getting lung or liver disease. The most commonly occurring deficient variants are referred to as PI S and PI Z. Affected PI S patients have a mild decline in serum AAT levels. The PI Z variant however, predominates, accounting for the majority of incidence of known AAT deficient alleles, resulting in a dramatic reduction in serum AAT levels. The PI Z variant arises from an amino acid substitution, p.E342K in the AAT protein resulting from a single base pair adenine to guanine substitution in *SERPINA1*. This single amino acid change results in intracellular polymerization of the mutant AAT protein that remains trapped in the ER, where it aggregates, forming inclusion bodies that gives rise to enlarged liver cells. In some affected individuals, this protein accumulation results eventually in hepatocyte death progressing from the stages of early inflammatory and fibrotic liver changes (see Fig. 1) to eventual cirrhotic damage with a high incidence of hepatocellular carcinoma or sudden onset liver failure and certain death [23].

This toxic gain of function phenotype in the human liver and severe pathology is recapitulated in a transgenic mouse model, designated as PiZ which harbors the human Z-AAT variant at high levels of expression [24], corresponding to 16 copies of the human transgene in one transgenic variant and 8 copies in another. This PiZ mouse model shows protein aggregation in the ER of liver cells, exhibits, fibrosis, cirrhosis and subsequent HCC (detailed in Table 1). Mice harboring 16 copies reflect the pathology at a younger age while those carrying 8 copies have a delayed pathology but whose serum AAT levels mirror the human carriers of the homozygous PiZZ mutation. This mouse model has been extensively utilized in the development of multi-modal therapeutics to treat this disease, ranging from traditional small molecules through cell and various gene therapy approaches including siRNA and antisense oligonucleotides (ASO) [25]. To date, some of these approaches such as ASO [26], dual silencing AAT-PiZ miRNA/miRNA resistant AAV AAT-PiM [26–28], CRISPR/Cas9 genome editing [29–31], hepatocyte transplantation [27] have established preclinical POC. A number of these investigational agents such as siRNA and gene replacement have progressed beyond preclinical development and have been or are currently in clinical trials (Table 2) [27, 32, 33]. The fact that there are a wide variety of therapeutic approaches in the pipeline for finding a treatment or cure for this disease indicates that the transgenic PiZ mouse is a well validated animal model that may have predictive value in translating findings from the preclinical to the clinical development phase. For other disorders lacking such tools, this model provides an example of the importance of establishing a validated disease model so there is a high degree of confidence that the findings will be relevant to inform clinical development.

2.1.3. Hereditary hemochromatosis (HH)

Hereditary hemochromatosis (HH) is a group of autosomal recessive

Table 1
Comparison of liver and associated disease phenotype in animal models and human.

Liver disease	Disease models	Model phenotype	Human phenotype
Alagille Syndrome	*Mouse* (*C3H/C57BL6*) *Jag1*$^{Ndr/Ndr}$ [22]	• Failure to thrive; partial neonatal mortality • Neonatal ductopenia; rescued in adults with ↑, but still fewer, well-formed bile ducts; bile duct functions improve in adult mice, but persistent absence of hepatic arteries • Neonatal jaundice; resolves in surviving adult mice • Liver organoids collapsed in culture due to structural instability	• Highly variable without consistent genotype to phenotype correlation • Paucity of bile ducts associated with chronic cholestasis ○ Jaundice, pruritis, xanthomas • Failure to thrive • Cirrhosis and liver failure • Cholestasis and associated symptoms improved in a subset of patients by school age
	Mouse (*C57BL6*) *Jag1*$^{+/-}$ [20]	• Variably expressed phenotype • Partial perinatal/neonatal mortality • Growth retardation • Impaired intrahepatic bile duct development • Reduce number of bile ducts • Widespread ductular reactions and ductopenia • Mild bilirubinemia; no overt jaundice	• Liver organoids from patient-derived cells collapse in culture [121, 122]
Alpha-1 antitrypsin deficiency	Mouse Tg PiZ variant [24] Mouse Z#2 (line discontinued) [135, 136]	• ↓ hAAT-Z secretion • ↑ hAAT-Z accumulation of protein in hepatocyte ER intrahepatocytic globules, low grade necrosis/ inflammation/regeneration, mild steatosis • Absence of fibrosis, cirrhosis • HCC in mice > 1 year in globule-devoid hepatocyte [137–139] • Growth delay [140] • No consequential COPD – due to normal circulating wild-type mouse AAT	• ↓ Serum AAT-Z protein • Polymerization and accumulation of AAT-Z in liver hepatocytes • Neonates - increase liver enzymes, persistent unconjugated hyperbilirubinemia (jaundice), cholestatic neonatal hepatitis, hepatosplenomegaly, nutritional issues • Older children – chronic liver disease, cirrhosis, portal hypertension, no HCC • Adults – livers rarely cirrhotic but with similar incidence of HCC as other liver disease. Acquire consequential COPD
Crigler Najjar Syndrome	Gunn Rat [105, 106]	• Jaundice due to severe hyperbilirubinemia • Phototherapy similar in human can tune severity and increase lifespan by decreasing levels of bilirubin	Human phenotype depends on the extent of UGT1A1 enzyme activity
	Mouse Ugt$^{-/-}$ [109]	• Jaundice due to severe hyperbilirubinemia • Death within 2 weeks from kernicterus • Phototherapy similar in human can tune severity and increase lifespan by decreasing levels of bilirubin	• Jaundice due to severe hyperbilirubinemia • Accumulation of bilirubin in brain leads to encephalopathy followed by more chronic kernicterus • Liver failure from chronic disease • Phototherapy decreases severity and increases lifespan by decreasing levels of bilirubin
Glycogen storage disease - type 1	Mouse *G6pc*$^{-/-}$ [68, 70]	Constitutive KO • No detectable G6pase activity • Slow growth rate • Hypoglycemia -seizures • Hyperlipidemia -↑ plasma triglycerides, cholesterol • ↑ Uric acid; plasma lactic acid not elevated • Hepatomegaly -↑ glycogen storage in hepatocytes Liver-specific conditional KO • Marginal effect on weight gain (KO is adult onset conditional) • Hyperlipidemia • Lactic acidosis, uricemia • Hepatic steatosis • Hepatocellular adenoma	Type 1a • Decrease growth rate • Fast-induced hypoglycemia - seizures • Hepatomegaly • Hyperlipidemia, Hyperuricemia, Hyperlactacidemia • Growth delay • Hepatocellular adenomas or carcinoma • Hepatic steatosis Type 1b – all of the above for Type 1a plus: • Neutropenia • Splenomegaly • Nephromegaly
	Dog *G6pc*^{M121I} [71, 72, 141]	• ↓ G6Pase activity in liver • Failure to thrive • Fast-induced hypoglycemia • Hepatomegaly - glycogen accumulation	
	Mouse *G6pt*$^{-/-}$ [73]	• ↓ G6pase activity • Growth delay • Fasting hypoglycemia with seizures • Hyperlipidemia - ↑ triglycerides, cholesterol • ↑ Uric acid, lactic acid • Glycogen accumulation in liver and kidney – hepatomegaly/nephromegaly • Delayed bone/spleen development - neutropenia, leukopenia	

(*continued on next page*)

Table 1 (*continued*)

Liver disease	Disease models	Model phenotype	Human phenotype
Hereditary hemochromatosis	Mouse *Hfe*$^{-/-}$ [35] Mouse *Hfe*$^{0/0}$ [36] Mouse *Hfe*$^{-/-}$ [41] Mouse *Hfe*$^{y/y}$ [41]	• ↑ Tsat • ↑ Liver iron content • ↓ Splenic stores • ↑ Iron deposition in liver hepatocytes • Iron overload - ↑ Plasma iron, ↑ Tsat ↑ Ferritin	• ↑ SF - males: SF > 300 μg/L; females: SF > 200 μg/L • ↑ Tsat - males > 50%; females > 40% • Hepatic fibrosis with SF > 1000 μg/L & elevated liver transaminase • ↑ Liver Fe
	Mouse *Hamp*$^{-/-}$ [39]	• ↑ Liver iron levels • Iron overload - ↑ serum iron and ferritin levels • ↑ Tsat • ↓ Reticuloendothelial iron • ↑ Iron deposition in pancreas and heart • ↓ Splenic iron	• No ↑ in splenic iron • Fibrosis, cirrhosis and potentially HCC can occur if left undiagnosed or not managed properly with phlebotomy • Hyperglycemia - bronzed diabetes
	Mouse *TfR2*$^{-/-}$ [37]	• ↑ Liver iron levels • Iron overload - ↑ serum iron and ferritin levels • ↑ Tsat • ↓ Splenic iron	• Arthropathy • Cardiac toxicity
	Mouse *Hve*$^{-/-}$ [38]	• ↑ Liver iron levels • Iron overload - ↑ Iron serum and ferritin levels • ↑ Tsat • ↓ Reticuloendothelial iron • ↑ Iron deposition in pancreas and heart • ↓ Splenic iron • No hepatic fibrosis, cardiomyopathy, or diabetes observed	
Liposomal acid lipase deficiency	Mouse *Lal*$^{-/-}$ [99]	• LAL enzyme KO • Normal at birth but develop enlarged livers at 4 weeks • Hepatosplenomegaly with accumulation of triglycerides and cholesteryl esters by 8 weeks • Phenotype lacks the severity of Wolman disease • Normal development to adulthood with organ involvement that progresses with age that more resembles the survivability of human CESD, lifespan 8 months	Wolman disease • Severe neonatal onset with hepatosplenomegaly that leads to progressive liver failures with fibrosis and cirrhosis and death at 1–2 years. Cholesterol ester storage disease (CESD) • Later onset with hepatosplenomegaly • Progressive accumulation of cholesterol esters and to a lesser extent trigylcerides in lysosomal hepatocytes
Tyrosinemia	Mouse *Fah*$^{-/-}$ [78, 79]	• Neonatal lethal with severe hepatic dysfunction • + NTBC treatment-corrects liver function and abolishes neonatal lethality • After removal of NTBC-progression to liver failure phenotype similar to type1 human disease, and HCC • Liver fibrosis • Regenerative potential of hepatocytes	Type 1 acute infantile • Not lethal *in utero* • Symptoms develop in 1st few weeks of life with failure to thrive and severe liver involvement • Progresses fast to liver failure, hepatocellular carcinoma (HCC) and death in infancy
	Mouse *Fah*^{5961SB} [81]	• Phenotype same as Mouse *Fah*$^{-/-}$	
	Rat *Fah*$^{-/-}$ [94]	• Phenotype similar to mouse but more severe liver injury by clinical chemistry and cirrhosis but HCC not reported	Type 2 chronic
	Rabbit *Fah*$^{-/-}$ [88]	• Liver phenotype similar to rodents and pigs • Fibrosis but no cirrhosis observed	• Onset ~6 months, progressive • Liver and renal tubular dysfunction
	Pig *Fah*$^{-/-}$ [86, 87]	− NTBC treatment embryonic lethal + NTBC treatment- born normal, then after removal of NTBC • Failure to thrive-weight loss and muscle wasting • Severe liver injury • Elevated tyrosine and succinylacetone levels in urine • Hepatocellular damage and abnormal clinical chemistry • Liver cirrhosis • Renal tubal damage • Regenerative potential of hepatocytes	• HCC and death at < 10 years. Both forms: • Liver fibrosis and cirrhosis • Elevated tyrosine and succinylacetone levels in urine • Hepatocellular damage and abnormal clinical chemistry • Renal tubal damage • Livers contain nodules of FAH+ hepatocytes from somatic reversion of FAH gene mutation • Regenerative potential of hepatocytes
Wilson's Disease	Rat Long-Evans Cinnamon (LEC) [51, 52] Mouse Toxic Milk (*Tx*) [53–55] Mouse Jackson Lab Toxic Milk (T*x-j*) [56] Mouse *Atp7b*$^{-/-}$ [57, 58]	Recently reviewed in detail [50]	Recently reviewed in detail [50]

Abbreviations: AAT-Z, alpha-1-antitrypsin Z-variant; ATP7B, ATPase copper-transporting beta; CESD, Cholesterol ester storage disease COPD, chronic obstructive pulmonary disease; Cu, copper; FAH, fumarylacetoacetate hydrolase; G6CP, glucose-6-phosphatase-α; G6PT, glucose-6-phosphate transporter (G6PT); hAAT-Z, human alpha-1-antitrypsin Z-variant HCC, hepatocellular carcinoma; HVE, hemojuvelin; HAMP, hepcidin antimicrobial peptide; HFE, high (iron) Fe; JAG1, jagged1; KO, knockout; LAL, liposomal acid lipase; NDR, nodder; NOTCH2, neurogenic locus notch homolog protein 2; NTBC, nitisinone; SERPINA1, serine protease inhibitor family A member 1; SF, serum ferritin; TRF2, transferrin receptor 2; Tsat, transferrin saturation; UGT1A1, uridine diphosphate glucuronosyltransferase.

disorders that result in iron overload from increased absorption and accumulating stores in several organs including the liver where the hepatocytes are the primary site of iron storage in the body and are also responsible for regulating iron homeostasis. The resulting iron accumulation over time can lead to significant organ damage up to and including cirrhosis of the liver. There are multiple genes involved in the

Table 2
Rare liver disease models and therapeutic approach.

Liver disease	Affected genes	Disease models	Therapeutic approaches	Stage of development
Alagille Syndrome	*JAG1* *NOTCH2*	Mouse (C3H/C57BL6) *Jag1*$^{Ndr/Ndr}$ [22] Mouse (C57BL6) *Jag1*$^{+/-}$ [20] Liver organoids [121, 122]	**Regenerative medicine** • Liver organoids	**Liver organoids**-transplantation Discovery/Pre-Clinical [122]
Alpha-1 antitrypsin deficiency	*SERPINA1*	Mouse Tg PiZ variant [24] Liver organoids [122]	**Gene therapy and RNAi** • siRNA • miRNA • CRISPR/Cas9 *in vivo* gene editing • ASO • GRT **Small molecule** • Chemical chaperones • Autophagy inducers • Polymerization disrupters **Regenerative medicine** • Bioprinted liver tissues • Liver organoids • Stem cells/hepatocytes	**siRNA** ALN-AAT – Ph1/2 (terminated) – (Alnylam) NCT02503683 **siRNA** ALN-AAT-002 – Preclinical (Alnylam) **siRNA** ARC-AAT – Ph 1 (terminated) – (Arrowhead) NCT02363946 [33] **siRNA** ARO-AAT – Ph1 (Recruiting) – (Arrowhead) NCT03362242 **GRT** rAAV1-CB-hAAT – Ph1 (Completed) – (University of Massachusetts) NCT00430768 **GRT** rAAV1-CB-hAAT – Ph2 (Completed) – (Applied Genetic Technologies) NCT01054339 **GRT** rAAV2-CB-hAAT – Ph2 (Completed) – (University of Massachusetts) NCT00377416 **GRT** ADVM-043 – Ph1 (Recruiting) - (Adverum Biotechnologies) NCT02168686 **miRNA/GRT** APB-101 – Preclinical (Apic Bio) [27, 28, 142] (refs) **CRISRPR/Cas9** - Preclinical (RNA Therapeutics Institute, Editas Medicine, Astrazeneca) [29, 31, 143] **ASO** – Preclinical (Ionis Pharmaceutical) [26, 144] **Bioprinted liver tissue** – NovoTissues® – Preclinical (Organovo) [96]
Crigler Najjar Syndrome	*UGT1A1*	Gunn Rat [105] Mouse Ugt$^{-/-}$ [109]	**Gene therapy** • GRT **Regenerative medicine** • Stem cells/hepatocytes	**GRT** AT342 – rAAV8-hUGT1A1-Ph1/2 (Recruiting) - (Audentis Therapeutics) NCT03223194 **GRT** GNT0003 –Ph1/2 (Recruiting) (Genthon) NCT03466463 **GRT** rAAV-pAlB-hUgt1A1– Preclinical [110] **Hepatocyte transplantation**-not active, NCT01345578
Glycogen storage disease - type 1	*G6PC* *G6PT (SLC7A4)*	Mouse *G6pc*$^{-/-}$ [68, 70] Dog *G6pc*^{M121I} [71, 72, 141] Mouse *G6pt*$^{-/-}$ [73]	**Gene therapy** • GRT **Small molecule** • Autophagy inducers	**GRT** DTX401 (AAV8G6PC) – Phase 1/2 - (Ultragenyx Pharmaceutical, Inc.) NCT03517085 **GRT** – Preclinical (extensively reviewed by [65–67, 74]) **Small molecule** – Preclinical [145]
Hereditary hemochromatosis	*HFE* *HAMP* *TFR2* *HVE*	Mouse *Hfe*$^{-/-}$ [35] Mouse *Hfe*$^{0/0}$ [36] Mouse *Hfe*$^{-/-}$ [41] Mouse *Hfe*$^{y/y}$ [41] Mouse *Hamp*$^{-/-}$ [39] Mouse *TfR2*$^{-/-}$ [37] Mouse *Hve*$^{-/-}$ [38]	**Gene therapy and RNAi** • siRNAi • ASO **Protein therapy** • Peptide mimetics **Small molecules** • Inhibitors	ASO, siRNA, peptide mimetics and inhibitors either in preclinical development or Ph1 (recently reviewed in detail) [44]
Liposomal acid lipase deficiency	*LAL*	Mouse *Lal*$^{-/-}$ [99]	**ERT** **Gene therapy** • GRT	**ERT** Sebelipase alfa (rhLAL) – Approved 2015 (Alexion) **GRT**– Preclinical [103, 104]
Tyrosinemia	*FAH*	Mouse *Fah*$^{-/-}$ [78, 79] Mouse *Fah*^{5961SB} [81] Rat *Fah*$^{-/-}$ [94] Pig *Fah*$^{-/-}$ [86, 87]	**Gene therapy** • GRT • CRISPR/Cas9 *ex vivo* gene editing **Small molecule** • Inhibitor **Regenerative medicine** • Bioprinted liver tissues • Stem cells/hepatocytes	**GRT** LV-FAH - Preclinical [93] **GRT** *ex-vivo* CRISPR/Cas9 *ex-vivo* editing-AAV-HT-Preclinical [95] **Small molecule** nitisinone (NTBC, Orfadin®) - Approved 2002 **Bioprinted liver tissue** NovoTissues® – Preclinical (Organovo) [96]
Wilson's Disease	*ATP7B*	Rat Long-Evans Cinnamon (LEC) [51, 52] Mouse Toxic Milk (*Tx*) [53–55] Mouse Jackson Lab Toxic Milk	**Gene therapy** • GRT **Small molecule** • Structural correctors	**GRT** AAV8-AAT-ATP7B - Preclinical [62] **Cu chelator** WTX-101 – Ph2 (Active) - (Wilson Therapeutics) NCT02273596 [146] **Cu chelator** methanobactin – Preclinical [60] **Cell therapy** hepatocytes – Preclinical [147]

(*continued on next page*)

Table 2 (*continued*)

Liver disease	Affected genes	Disease models	Therapeutic approaches	Stage of development
		(Tx-*j*) [56] Mouse *Atp7b*$^{-/-}$ [57, 58]	• Chemical chaperones • Cu chelator • Transcriptional activators **Protein therapy** • Cu chelator **Regenerative medicine** • Stem cells/hepatocytes	

Abbreviations: ASO, antisense oligonucleotide; ATP7B, ATPase copper-transporting beta; Cu, copper; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/*CRISPR*-associated protein-9 nuclease; ERT, enzyme replacement therapy; FAH, fumarylacetoacetate hydrolase; G6CP, glucose-6-phosphatase-α; G6PT, glucose-6-phosphate transporter (G6PT); GRT, gene replacement therapy; HVE, hemojuvelin; HAMP, hepcidin antimicrobial peptide; HFE, high (iron) Fe; JAG1, jagged1; LAL, liposomal acid lipase; LV, lentivirus, miRNA, micro-ribonucleic acid; NDR, nodder; NCT number, ClinicalTrials.gov Identifier; NOTCH2, neurogenic locus notch homolog protein 2; Ph, phase; rAAV, recombinant adeno-associated virus; rh, recombinant human; RNAi, ribonucleic acid interference; SERPINA1, serine protease inhibitor family A member 1; siRNA, silencing ribonucleic acid; TRF2, transferrin receptor 2; UGT1A1, uridine diphosphate glucuronosyltransferase.

pathway regulating iron homeostasis and mutations have been identified in several resulting in HH [34]. The affected genes involved in HH has been classified into four types with Type 1 resulting from mutations in the high (Iron) Fe (*HFE*) gene which encodes the hereditary hemochromatosis protein that regulates hepcidin, a peptide hormone that regulates iron homeostasis. The most frequently occurring mutation identified in *HFE* is the homozygous p.C282Y amino acid substitution, occurring in the majority of HH patients. The remaining types, grouped together as non-*HFE* include Type 2A caused by mutations in *HJV* that encodes the hemojuvelin gene product and Type 2B with mutations in *HAMP* that encodes the hepcidin protein. Type 3 mutations are found in the transferrin 2 receptor protein gene, *TFR2*. Collectively, Types 2 and 3 are very rare and often are associated with a juvenile form of hemochromatosis. Additionally, Type 4 results from mutations in the region of *SLC40A1 (FPN)*, the ferroportin gene where the hormone, hepcidin binds, also leading to an iron overload phenotype. Several homozygous knockout mouse models, *Hfe*$^{-/-}$ [35], *Hfe*$^{0/0}$ [36], *TfR2*$^{-/-}$ [37], *Hjv*$^{-/-}$ [38], *Hepc1*$^{-/-}$(*Hamp*$^{-/-}$) [39] reflective of the different types of HH have been generated, corresponding to the aforementioned affected genes [39, 40] including a *Hfe* mouse (*Hfe*$^{y/y}$) harboring the common p.C282Y variant [41]. See Table 1 for comparison between the various animal models and the human disease phenotype.

Interestingly, it is important to highlight that the characteristic phenotype that this group of inherited disorders have in common, regardless of which gene is mutated, is a downregulation in the iron regulatory liver hormone, hepcidin [42] and therefore the current targeted approach to therapeutic development for HH does not directly focus on the individual HH affected gene in humans in the iron homeostasis pathway but instead is geared towards upregulating the levels of the hormone hepcidin that is suppressed in both the HFE and non-HFE forms of HH by whatever means necessary. This approach has widened the scope of druggable targets that regulate hepcidin expression and activity as well as the generation of animal models to demonstrate the validity of the target to address a therapeutic development approach that extend beyond the genes/proteins directly affected in HH to include others that regulate the affected HH genes including hepcidin itself, in essence, creating a “two degrees of separation” targeting [40, 43]. Nonetheless, the animal models created to mirror the disease genotypes identified in affected humans, in particular, the Hfe$^{-/-}$ remains the mainstay of *in vivo* model for preclinical proof of concept (POC). Several companies and academic researchers have advanced development candidates that have either already been validated preclinically or are in Phase 1 studies in the clinic (recently summarized by Casu et al. [44]). Therapeutic approaches range from hepcidin peptide mimetics to gene therapy *via* silencing of the Tmprss6 gene using siRNA or ASO. TMPRSS6 is one of those indirect targets that negatively regulates hepcidin production [45]. Table 2 summarizes the therapeutic development approach and the animal model(s) of HH used to generate preclinical POC.

2.1.4. Wilson's Disease

Wilson's Disease (WD) is a rare autosomal recessive disorder of inborn error of copper metabolism that results in the accumulation of toxic levels of copper initially in the liver and eventually the brain with accumulating stores of the metal. If left untreated, liver damage ensues followed eventually by neurological dysfunction [46, 47]. Current approved standard of care includes administering zinc salts and copper chelators, *e.g.* penicillamine that are approved for treatment of WD [48]. These therapies are far from curative and have many unpleasant toxic side effects such as hypersensitivity reactions in some individuals that make them unattractive for lifelong treatment. As such, non-adherence renders disease management ineffective and the sequelae of liver and neurologic dysfunction progresses. WD results from hundreds of pathogenic mutations in the ATPase copper-transporting beta (*ATP7B*) gene [49] whose protein product is a transmembrane P-type ATPase and is estimated to affect 1 in every 30,000 individuals worldwide. Specifically, in the liver, ATP7B functions in a dual role for maintenance of copper homeostasis, regulating its transport out of the liver into the plasma for delivery to the rest of the body by loading and activation of the copper transport protein, ceruloplasmin, as well as, *via* disposal of excess Cu into the bile [47]. There are currently four rodent animal models of WD, three that arose from spontaneous mutations and the fourth genetically engineered to mimic the human disease. Medici et al. recently authored a detail review of the animal models and included a comparison to the human clinical presentation [50]. Briefly, the first of the naturally occurring disease model is the Long-Evans Cinnamon (LEC) rat [51] with a partial 1300 bp deletion in the 3′ end of rat *Atp7b* spanning regions of the coding sequence (CDS) and the untranslated region (UTR) [52]. These rats exhibit abnormal accumulation of copper with some animals progressing to severe hepatitis and necrosis with early death while the remainder progress to fibrosis and eventual HCC [51]. It is noteworthy, however, that the progression to HCC is rare in WD human patients. The remaining spontaneous rodent models are the variant Toxic milk (tx) murine models, where pups require nursing from a control dam to ensure survival, due to the lack of copper in mutant dam's milk. This does not occur in human WD mothers. The other Toxic milk variant from Jackson Laboratory is designated tx-j. The tx mouse [53, 54] harbors the c.4066 A > G mutation [55] in the mouse homolog of the human *ATP7B* gene that results in the amino acid substitution, p.M1356V and, tx-j [56] has the recessive point mutation c.2135 G > A that results in p.G712D amino acid substitution, predicted in transmembrane domains, TMD8 and TMD2, respectively. Despite some species differences between the mouse and

humans, the phenotype of the disease is similar in many respects mirroring the accumulation of copper in the liver followed by a slow disease progression leading up to fibrosis and eventual cirrhosis. Lastly, the knockout mouse, *Atp7b*$^{-/-}$ [57, 58] genetically engineered with multiple stop codons in exon 2 also exhibit a similar phenotype to the human and the other two spontaneous murine models, except that it is also susceptible to liver tumors like the LEC rats. While not the focus of this review, it is also important to note that all these WD models either do not exhibit neurological symptoms or if they do, it is slight in nature.

Therapeutic development for WD (Table 2) has evolved from the traditional efforts geared towards chelation of copper and removal with non-specific chelating agents to more targeted small molecule approach designed to specifically bind Cu [59]. Preclinical POC was demonstrated for the molecule, bis-choline tetrathiomolybdate (WTX101) and is in the clinic for human testing. A methanotrophic proteobacterial peptide, methanobactin also appears to have a high affinity for copper with preclinical studies appearing to indicate that unlike other traditional chelating agents, this peptide also goes one step further and demonstrates the ability to clear the metal from the mitochondrial stores, preventing hepatocyte death and liver failure and extending life of the LEC rat model [60, 61]. It is important to point out that mitochondrial stores of copper in WD are not addressed by standard of care (SOC) chelating agents and the preclinical POC studies for this peptide was able to show that with acute treatment in LEC rats undergoing fulminant liver failure, the ability of this protobacterial peptide to remove mitochondrial copper reversed imminent liver failure and death. Gene therapy approaches including replacement of the defective gene [62] or various approaches used to generate and transplant normal hepatocytes [63] are currently in development utilizing the current catalog of available animal models. These models that appear to be proving their worth in studies conducted so far with new technological approaches to therapeutic intervention for WD.

2.1.5. Glycogen storage disease type I (GSD-1)

Glycogen storage disease type I (GSD-I), also known as von Gierke disease is a group of autosomal recessive disorder due to impaired glucose homeostasis resulting in the inability of the liver to convert the substrate glucose-6-phosphate (G6P) into glucose to address the body's immediate need. What occurs is that most, if not all G6P produced in the final steps of gluconeogenesis and glycogenolysis is instead converted to glycogen and lipids which excessively build up and are stored in the liver, resulting in hepatomegaly. The liver also experiences a build-up in neutral lipids that leads to steatosis, inducing further exacerbation of the existing hepatomegaly [64]. There are two subtypes of GSD-I [65]. The first, GSD-Ia, the most prevalent subtype of GSD-I, occurs in 80% of affected individuals and arises from mutations in a liver/kidney/intestines-specific glucose-6-phosphatase α (G6Pase-α), resulting in a deficiency of the G6Pase-α enzyme [66]. Subtype GSD-Ib is derived from mutations in the ubiquitous glucose-6-phosphate transporter (G6PT) that also renders the protein absent and accounts for 20% of the affected population [66]. Both the transporter and the enzyme work in concert to maintain glucose homeostasis between meals *via* uptake of the substrate G6P by the G6P transporter from the cytoplasm to the lumen of the ER followed by conversion to glucose by the enzyme, G6Pase α. Glucose is then released into the blood to maintain homeostasis. If either the transporter or the enzyme is absent or in short supply, one of the long-term consequence of GSD-1 in affected individuals beyond the immediate hypoglycemia after a brief fast is the excessive accumulation of glycogen stores in the liver that over time leads to hepatomegaly and severe liver damage, ultimately resulting in hepatic adenomas with the potential for malignant transformation to hepatocellular carcinomas.

Several animal models of GSD-Type 1 have been previously identified or generated [67]. For GSD-Ia, they include a constitutive GSD-Ia knockout mouse (G6pc$^{-/-}$) [68], two conditional *Gp6c* null mouse models [69, 70], and a spontaneous mutant dog model [71, 72] (now Maltese-Beagle mix) with a naturally occurring missense mutation, G6pc^{M121I}, originally identified [72] in a Maltese breed but later crossbred with beagles to preserve the progeny survival (Table 1). For GSD-Ib, a single transgenic knockout model, G6pt$^{-/-}$ exists [73]. Noteworthy is that even with significant dietary management, the constitutive knockout murine and mutant canine models of the GSD-I phenotypes have a limited survival of approximately 3 months. Table 1 provides a comparison between the human clinical phenotype and the disease models. In spite of the survival limitations of the available model, progress has been made in therapeutic development for this disease with the primary approach geared towards somatic gene therapy as protein replacement therapy for both these affected multi-TMD would be inherently challenging. Briefly, for GSD-Ia, the use of murine, canine and human G6PC or murine and human G6PT with a combination of viral, exogenous or endogenous gene promoters and use of existing variations of the adenoviral or adeno-associated virus (AAV) vectors for packaging have been investigated [49, 67, 74–76]. Table 2 summarizes the therapeutic approach and the animal model used to test the therapy under investigation including the current phase of development based on publicly available information as of the date this review was submitted. Noteworthy is the recent listing (NCT03517085) in clinicaltrials.gov for a Phase 1 study for the investigational agent DTX401 (AAV8G6PC).

2.1.6. Hereditary Tyrosinemia Type 1 (HT-1)

Hereditary Tyrosinemia Type 1 (HT-1) is an autosomal recessive disorder with an incidence of about 1 per 100,000 individuals. It is characterized by a patient's inability to metabolize the amino acid tyrosine due to a deficiency of the enzyme fumarylacetoacetate hydrolase (Fah) that leads to a buildup of toxic metabolites. The disease has a severe liver involvement and there are 2 forms; acute infantile onset in first few months with fast progression and liver failure and death before age one, some with HCC; and the other more chronic form with onset after 6 months, progressive, with life expectancy < 10 years and both liver and renal tubular dysfunction, and death usually caused by HCC. Current treatment is a nitisinone (NTBC, Orfadin) which inhibits the buildup of downstream metabolites, and a tyrosine restrictive diet or liver transplantation [77].

The mouse model was first developed to study Tyrosinemia disease pathophysiology and therapeutic development, by knocking out the FAH gene, starting with deletion of exon 5 to make the Fah$^{\Delta exon5}$ mouse, Fah$^{-/-}$ [78–80], and later by a point mutation to make Fah^{5961SB} [81]. These homozygous mice die as neonates with severe hepatic dysfunction but when treated with NTBC show complete correction of the liver phenotype, including normalization of the abnormal gene expression, with 100% survival [79]. NTBC treatment has provided a way for this mouse model to be utilized as a pre-clinical model for the human liver disease. It was found that patient livers contained nodules of FAH+ hepatocytes that arose by somatic reversion of FAH gene mutation, and the pathophysiology was further elucidate in the Fah$^{-/-}$ mice. The null mice were transplanted with WT hepatocytes [82] and it was found that the FAH+ cells proliferated and replaced most of the liver, and the animals remained healthy and survived, providing a proof of principle that liver repopulation can effectively cure a metabolic disease [80]. The selective advantage for FAH+ hepatocytes also led to an FAH mouse with a “humanized” liver. An FRG immune deficient null mouse model, Fah$^{-/-}$/Rag2$^{-/-}$/IL2rg$^{-/-}$ was repopulated with human FAH+ hepatocytes [83, 84]. Even though the mouse has answered many questions and recapitulates many aspect of the human liver disease, there are limitations, and the model is missing a critical phenotype of the disease, cirrhosis. The information gained from the mouse model has led to the development of a Fah null mutation (Fah$^{-/-}$) rat, rabbit, and pig model (Table 1) [85–88]. The larger size of these models offers advantages to better study liver drug metabolism and toxicity, and for example, an immunodeficient Fah null pig could allow access to large amounts of hepatocytes, a “cell factory”,

or even a "human organ factory" for drug screening and tissue or organ transplantation. The FAH animal models have had a huge impact in the translational landscape and provide a roadmap from mouse to humans, and from basic biology to therapeutic development, including stem cells [84, 89], gene therapy [90–95] and modeling liver tissue and tissue transplantation approaches [96] (Table 2).

2.1.7. Lysosomal acid lipase deficiencies (LALDs)/Wolman disease

Lysosomal acid lipase deficiencies (LALDs) are inherited autosomal recessive disorders caused by mutations in the lysosomal acid lipase (LAL) enzyme that hydrolyzes cholesteryl esters (CEs) and triglycerides (TGs) in the lysosomes [97]. There are two distinct clinical phenotypes in humans, Wolman disease and cholesteryl ester storage disease (CESD) that generally correlate with the amount of residual LAL activity. Wolman disease has an estimated worldwide incidence of 1 per 350,000 newborns and is a severe infantile onset, with massive accumulation of CEs and TGs, in the lysosomes, particularly in the liver, and leads to death before 1 year of age. CESD is a milder, later onset disease, with estimated incidence is 2.5 per 100,000 newborns, and characterized by the accumulation of only CE. The age of onset can be from childhood to early adulthood with hepatic fibrosis, cirrhosis, hyperlipidemia, and accelerated atherosclerosis, but patients can live to middle age with liver disease and cardiovascular failure being the most common causes of death [98].

The mouse and human LAL enzymes are 75% identical and have 85% similar amino acids and the mouse LAL mRNA and protein expression patterns are similar to the tissue and cellular involvement found Wolman disease patients, including high expression in hepatocytes. The main animal model developed to study the pathophysiology and the development of therapeutic approaches is the *Lal*$^{-/-}$ mouse model, a homozygous null (knockout) mice that does not express LAL mRNA, LAL protein, or any residual enzyme activity [99, 100]. This mouse biochemically resembles human Wolman disease with significant accumulation of triglycerides and cholesteryl esters in several organs, such as liver and spleen but the phenotype lacks the severity of Wolman disease and has normal development to adulthood with organ involvement that progresses with age that more resembles the survivability of human CESD (Table 1). As a model for LAL deficiencies, it is important to keep in mind that the lack of the full Wolman disease phenotype may be due to species differences in lipid metabolism and/or in lipoproteins as a function of genetic background. This mouse model has been used pre-clinically to support the recently FDA approved LAL enzyme replacement therapy, sebelipase alfa (Kanuma) in 2015 [101] (Table 2). The proof of concept studies showed that recombinant human LAL disease reversed the lipid accumulation in hepatocytes and liver injury, and lowered plasma lipid levels was shown in mice with advanced disease [102]. LAL is also a possible candidate for liver-directed gene therapy [103, 104], and has been tested in the mouse model in some *in vitro* and *in vivo* studies of adenovirus-mediated gene therapy but not in the clinical setting.

2.1.8. Crigler–Najjar Syndrome Type 1 (CN-1)

Crigler–Najjar Syndrome Type 1 (CN-1) is an autosomal recessive, ultra-rare disease (incidence estimated at 1 in 1 million individuals) caused by mutations in the uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) gene that leads to a complete absence of UGT1A1 activity. The role of UGT1A1 enzyme is to conjugate glucuronic acid to bilirubin, to increase solubility, and therefore excretion from the body. Bilirubin is a breakdown product of red blood cells and accumulation of its unconjugated form leads to toxicity, especially in the brain with neurological damage known as kernicterus, leading to speech impairment, ataxia, and death if untreated. Current standard of care is phototherapy (12 h/day) which becomes less effective with age so that the only therapeutic option is transplantation. Interestingly, in less severe forms like type 2 (Gilbert's Syndrome) with residual enzyme activity of 10–35% and bilirubin levels below 4 mg/dL, patients remain healthy.

Before the human disease was described in 1952, the first animal model, the Gunn Rat, was discovered in 1934 with the appearance of a yellowish discoloration of the skin from jaundice. The model showed bilirubin toxicity and was later found to lack UGT1A1 activity due to a premature stop codon in the gene, similar to humans [105] (Table 1). The model is very well characterized, commercially available, and has contributed greatly to an understanding of the pathophysiology of the disease and for *in vivo* testing of new therapeutic treatments such as hepatocyte transplantation and gene therapy approaches, utilizing decreases in the levels of unconjugated bilirubin as a measureable biomarker of efficacy [106, 107]. The rat highlights how a well validated animal model can be a valuable tool to support translation and preclinical development; for example, a hUGTA1A-modRNA (a modified messenger RNA encoding for UGTA1A) as a lipoparticle formulation in which an *in vivo* quantitative systems pharmacology (QSP) model was developed to capture the pharmacokinetic/pharmacokinetic relationship for this therapeutic candidate that did not fit traditional linear PK due to the complexity of the mRNA/lipid components and behavior *in vivo*. It was benchmarked using previously measured and well calibrated Gunn Rat bilirubin kinetic data, and was used to make first-in-human dose projections for efficacy [108]. Unfortunately, due to safety concerns the hUGTA1A-modRNA has not entered clinic testing. In 2008, a null *Ugt*$^{-/-}$ mouse model was developed with a point mutation identical to the Gunn rat [109]. These mice have similar disease aspects of postnatal lethality, high levels of bilirubin, and jaundice, with death by kernicterus within 2 weeks after birth. Similar to the use of NTBC to rescue FAH null mice in Tyrosinemia, the Ugt$^{-/-}$ mice can be treated with phototherapy from birth to 8 or 15 days, to tune the disease severity by decreasing the bilirubin levels and increasing life span. This mouse model was subsequently used to study a promoterless gene targeting approach [110]. There are other Ugt$^{-/-}$ mice developed that also silence the UGTA1A enzyme activity that are supporting current AAV directed gene therapy programs to restore the UGT1A1 protein. Two are in active Phase 1/2 clinical trials [111–113] (Table 2), and the results will offer a comparison between the animal model and humans to have a better understanding of their predictive value for Crigler Najjir Syndrome, in particular the correlation of protein levels and enzyme activity to clinical benefit.

2.2. In vitro models of liver diseases: stem cells and 3D tissue models

The conduct of *in vivo* pharmacological studies is resource, cost, and time intensive which limits the number of animals and compounds that can be tested. When these limitations are coupled to the fact that animal models are not always predictive of drug response in human disease, it highlights the need for more predictive human *in vitro* assay models for drug discovery and development. The use of physiologically relevant cells with native and diseased cellular background should provide an assay platform to identify candidate compounds in more native systems. However, it is difficult to obtain human liver tissue and maintain primary hepatocytes in cell culture, an obstacle to studying liver disease mechanisms and developing new therapies. The use of human induced pluripotent stem cell (hiPSC) technology has enabled an alternative venue to generate human liver disease models as "disease in a dish" assay platforms. Human iPS cells have been generated for many of the rare inherited liver diseases [114, 115].

In the last few years, there has also been an increased interest in the development and use of *in vitro* 3D models of human tissues as physiological relevant assay platforms for drug discovery and development [116, 117]. The expectations are that, because these tissue models include many of the different relevant cell types and extracellular components of the tissues, drug responses will be more akin to those observed in animals and humans. A continuum of technical approaches are being used to produce functional *in vitro* 3D models of tissues, each with increased morphological and physiological complexity: spheroids are cell aggregates with a rounded shape that lack a micro-anatomy

matching native organ patterns; organoids are cell aggregates, not necessarily rounded, with a micro-anatomy mimicking the native organs, and in most cases generated with mixtures of different cell types dissociated from tissues or cells differentiated from iPSCs. 3D bioprinted tissues are generated by using primary or iPSC-derived relevant cells combined with hydrogels that enable the 3D bioprinting of pre-defined, native-like tissue structures. Finally, organ-on-a-chip models attempt to capture the interaction between organs and flow aspects of vascularized tissues through the use of microchips and connected microfluidics systems. In most cases, *in vitro* 3D tissue models are produced using primary cells or iPSC-derived cells to capture more accurately the physiology of cells in the native human tissues. The application of the different techniques described above to produce 3D liver tissue models has been reviewed recently by Mazza et al. [118]. For 3D liver models, hepatocytes, endothelial, Kupffer, stellate and mesenchymal stem cells have been used, which have been isolated from organs as primary cells, progenitor cells, or derived from iPSC. In general, the data shows that the physiology of the liver function as measured by secretion of relevant proteins such as albumin, or gene expression of functional biomarkers is better recapitulated in these 3D models than in 2D cultures of hepatic cell lines or primary hepatocytes.

A goal for *in vitro* human cellular liver models is to develop disease assay platforms for compound testing. This can be achieved by using disease cells with a specific genetic mutation that induces a measurable phenotype or inducing a phenotype that it is common across multiple liver diseases with a relevant insult. One example of the latter is fibrosis, which is the final response to chronic non-resolving injury before progression to cirrhosis and liver death (see Fig. 1). Fibrosis is not a static state but is the result of a continuous complex remodeling process that involves the interaction of components of the extracellular matrix (ECM), growth factors, proteases and cytokines that may be reversible. Recent studies with rodents and human liver fibrosis models have shown that it is possible to intervene due to the extraordinary capacity of liver cells to regenerate. Examples of 3D liver fibrosis models have been published using both 3D bioprinted tissues [119] and organoids [120], in most cases, using TGFβ or MTX as fibrinogenic inducing agents, and measurements of collagen deposition, cytokine production and gene expression of fibrosis biomarkers (*e.g.* αSMA). There are also examples of organoids being developed to study specific genetic rare liver diseases. Guan et al. [121] recently reported on the generation of human hepatic organoids (HOs) that resemble human liver consisting of iPSC-derived hepatocytes and cholangiocytes, which organized into epithelia that surrounded the lumina of bile duct–like structures. The organoids were used to study how different genetic JAG1 mutations impair bile duct formation in Alagille Syndrome (ALGS), and to investigate the heterogeneity found in the clinical features associated with JAG1 mutations. The ALGS disease liver organoids were generated using ALGS liver disease patient iPSC-derived cells. The authors observed that hepatic organoids generated from patients had typical features of ALGS liver disease, which included a paucity of bile ducts, intrahepatic cholestasis, and fibrosis. They also used CRISPR-Cas9 based technology to reverse the JAG1 mutations in patient iPSC cells and the HOs recovered their capacity to form well-organized structures with bile ducts after reversion of the ALGS1 mutation. In another approach, Huch et al. have also generated liver organoids as *in vitro* disease models for ALGS, in this case, using ductal cells isolated from human liver biopsies [122]. Organoids produced from ductal cells displayed a duct-like phenotype but had to be differentiated until expression of hepatocyte markers ALB and CYP3A4 was detected. Using this liver organoid production protocol, the authors used biopsies from AAT deficiency and ALGS patients to generate disease organoids. After organoid differentiation and maturation, differentiated cells from AATD patients secreted high levels of Albumin and took up LDL similarly to that of healthy donor-derived organoid cultures; AAT protein aggregates were readily observed within the cells; there was reduced AAT protein secretion, and supernatants from differentiated mutant organoids showed reduced ability to block elastase activity. Differentiated liver organoids from AATD patients also mimicked the *in vivo* situation by showing signs of ER stress, such as phosphorylation of eIF2a and increased apoptosis. ALGS patient organoids resembled healthy cultures in their undifferentiated form but failed to upregulate biliary markers such as KRT19 and KRT7 in the differentiated form.

There is also hope that *in vitro* 3D liver tissues will be used as explants in humans to enable regenerative medicine approaches to rare liver disease, in addition to being disease-in-a-dish assay platforms. The ability of hepatic cells in the context of organoids or bioprinted tissues to regenerate a disease liver organ have already been demonstrated in animals. The company Organovo recently announced progress in its first IND-track program for its NovoTissues® treatment of alpha-1 antitrypsin deficiency (AATD). This bioprinted tissue-based therapeutic approach received orphan drug designation from the U.S. Food & Drug Administration in 2017. Organovo previously disclosed the results of its preclinical studies in the PiZ mouse model for AATD using bioprinted liver explants (Table 2) [96]. The bioprinted liver tissues were fabricated using primary human umbilical vein and liver endothelial cells, hepatic stellate cells and hepatocytes. After implantation of the liver tissue on the surface of the liver in mice expressing the mutant human form of alpha-1 antitrypsin, the protein markers of human albumin, transferrin, AAT and fibrinogen were detected in the circulation as early as 7 days, with increasing levels of human albumin detected for at least 90 days post-implantation. Serum and histopathologic evaluation of the implanted therapeutic tissue showed engraftment, retention and a high degree of disease clearing through 125 days post-implantation. These results also demonstrated the sustained presence of key human liver proteins such as albumin and AAT in the animal bloodstream. Importantly, pathologic evaluation of diseased animals receiving implanted bioprinted liver tissues suggested a significant reduction in the pathologic hallmarks of the disease in the damaged region of the liver adjacent to the tissue transplants. Organovo recently also studied the potential benefit of its NovoTissues® in an established animal model for Type 1 Tyrosinemia and showed retention and sustained functionality post-implantation. Pathologic evaluation of the diseased animals receiving implanted bioprinted liver tissues also suggested an improvement in liver health and extended survival *versus* non-treated animals. In addition to 3D bioprinted liver tissues, liver organoids have also been used for transplantation in regenerative medicine approaches. Human liver organoids generated with single donor-derived multiple cells have been shown to rescue mice from acute liver failure [123–125]. In this work, isolated endothelial cells (EC) and mesenchymal cells (MC) from a single donor umbilical cord (UC) were generated. Then EC-hiPSC endoderm cells were generated from the same donor-derived hiPSCs from the UC-ECs and simultaneously plated EC-hiPSC endoderm, UC-ECs, and UC-MCs in a 3D microwell culture system generated single donor cell-derived differentiated liver organoids (LO). These single donor cell-derived LOs (SDC-LOs) could be differentiated into functional LOs *in vitro* with enhanced hepatic capacity as compared to that of EC-hiPSC-derived hepatic-like cells. In addition, when these functional SDC-LOs were transplanted into the renal subcapsules of ALF mice, they rapidly assumed hepatic functions and improved the survival rate of ALF mice. With the goal of clinical translation of liver bud transplant therapy, the same laboratory established protocols and a platform for the scale-up production of liver organoids from endoderm, endothelial, and mesenchymal progenitor populations derived from human iPSCs, and demonstrated functionality both *in vitro* and *in vivo* [124, 125]. Another group used protocols based on the above organoids to demonstrate that paracrine interactions between hIPSc, mesenchymal stem cells, and human umbilical vein endothelial cells were needed to promote hepatocyte maturation in liver organoids, but organoid self-organization required cell-to-cell surface contact [126]. To determine whether liver organoids are capable of functioning as mature liver tissue, organoids at day-2 culture were implanted under the kidney capsule of immunodeficient mice. Three weeks after

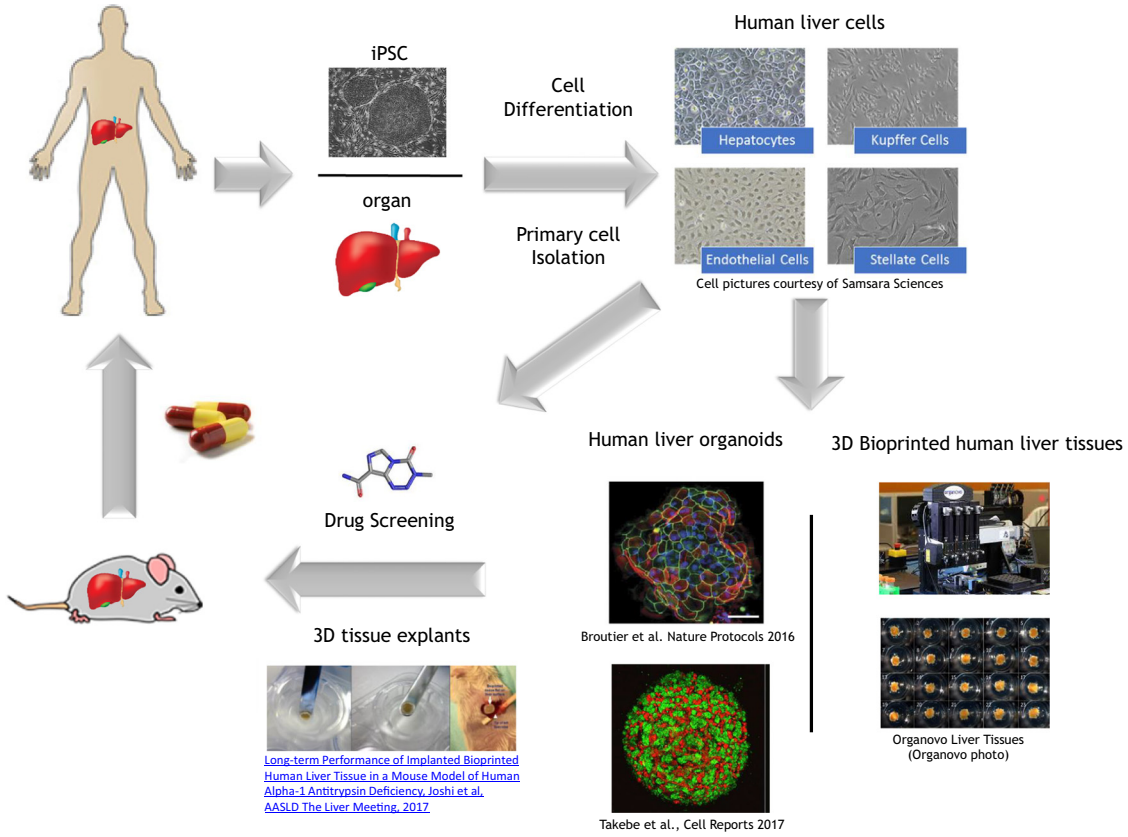

Fig. 3. Schematic for the development of *in vitro* human 3D functional liver tissue models. Human liver cells or progenitors stem cells are obtained either from isolation from human biopsies or differentiated from human iPSC. *In vitro* liver 3D tissue models include organoids or 3D bioprinted tissues, which can then be used either as disease-in-a-dish platforms for screening in drug discovery and development, or as explants for transplantation *in vivo* in regenerative medicine.

implantation, serum levels of human albumin and alpha-1 antitrypsin (AAT) were detected, further suggesting hepatic maturation of the liver organoid *in vivo*. Based also on reports that paracrine signaling between hepatocytes, endothelial cells, and stroma cells is required in both liver development and regeneration processes, another laboratory reported the fabrication of a liver tissue containing patterned human primary hepatocytes, endothelial cells, and fibroblasts in a degradable hydrogel [127]. Once ectopically implanted in mice, this liver tissue expanded > 50-fold over the course of 11 weeks in mice with injured livers. The functional characteristics of the expanded hepatic tissue grafts were measured by sustained protein synthesis, such as human albumin in serum from engrafted mice, and drug metabolism. The presence of red blood cells in the expanded tissue seed graft also suggested that vascular networks might be present in these grafts. Huch et al. [122] tested the ability of their patient cell-derived organoids to engraft as functional hepatocytes *in vivo*, by treating Balb/c nude mice with CCl4-retrorsine to induce acute liver damage, a treatment that allows engraftment of hepatocytes. They were able to initially detect KRT19-positive, ductal-like cells at 2 h and 2 days after transplantation, distributed throughout the liver parenchyma, and at later time points, they observed ALB+, KRT19$^-$ human cells as singlets/doublets or, more rarely, in larger hepatocyte foci. Finally, in addition to bioprinted and organoid liver models, there has been substantial amount of work towards the successful decellularization of rat, rabbit or pig livers as natural 3D scaffolds for liver bioengineering and transplantation [128–130]. Decellularization protocols for human livers have also been reporter recently [131–133]. This work shows that it is possible to completely decellularize a whole human liver and lobes to form an extracellular matrix scaffold with a preserved architecture, which could then be repopulated with different human hepatic cell lines and primary hepatocyte and remain viable and functional for at least 10 days. In addition, these decellularized whole human liver scaffolds appear to be biocompatible and do not appear to induce a significant immune reaction or rejection when cubic fragments of these scaffolds are implanted subcutaneously or intra-abdominally in immune competent mice. In addition, the implanted scaffold appeared to be incorporated into the host tissues with progressive host cell infiltration and arteriolar neovascularization [133]. This decellularization approach provides an interesting opportunity for the alternative use of human livers found to be unsuitable for transplantation following organ retrieval.

3D organoids and bioprinted tissues that integrate many different liver cell types and ECM can therefore, in the near future, provide a platform to elucidate the cellular mechanisms of liver disease, identify molecular targets, and screen for molecules that revert the disease process and could be applied to multiple liver diseases, in addition to providing tissue explants for transplantation in regenerative medicine (Fig. 3) [125, 134]. The techniques used for the production of 3D models are now being developed by specialized bioengineering academic laboratories or companies but the relative experimental amenability of the approach and wealth of the data produced will likely push these technologies to be implemented by the end users sooner rather than later. As explained above, the challenge will remain finding a reliable source of relevant cells, both primary and iPSC-derived, and establish agreed upon criteria to harmonize the quality of the cells from different sources, as well as those of the tissues produced with these cells. The availability of disease iPSC and the corresponding robust protocols for differentiation to disease hepatocytes and other liver cells will enable the generation of personalized disease liver models.

It is unlikely the 3D liver models will replace animal model testing any time soon, and the different models will complement each other until enough data is generated to be able to establish the predictive value of each approach. The liver function of animals and humans is

different enough that there have been cases of drugs that were toxic in animals but do not show toxicity in humans, and *vice versa*, compounds that were not toxic in animal models and then showed toxicity in humans. The hope is that human 3D *in vitro* liver tissue models will help prioritize compounds for animal testing, thus reducing costs and maximizing chances of selecting the best compounds for humans, and perhaps eventually replace animal testing when enough data supports the predictive value of these models for efficacy and toxicity data. The use of these 3D tissue disease models for efficacy studies and to make decisions during the drug discovery and development will take longer to fully come into practice because it is more complex to produce well established and validated relevant models for each disease.

3. Conclusion

The modeling of human diseases in animals has been critical for the development of therapies. In most cases though, animal models do not fully capture all aspects of the physiology of human diseases and that has partly led to a very low success rate from clinical testing to approvals. The low predictive power of animal models is particularly concerning for rare diseases. As highlighted in this review of a select group of rare liver disorders, differences in animal and human physiology, inter species background strain, and genetic predisposition all contribute to the variations and ability to fully predict outcomes in humans. Despite the challenges, advances have been made in drug development for several of the rare liver disorders leading to investigational agents currently undergoing clinical development or to the approval of new medicines. However, with only about 10% of rare diseases with an approved therapy, the need remains to continue refining existing models or develop new more relevant models. For example, larger animal species such as canine, feline or porcine models, complementary *ex-vivo* technologies such as disease relevant 3D tissue models, or investments in platform approaches for therapeutic development, for example, liver interventions that address general processes such inflammation, fibrosis and accumulation of proteins, lipid and metals that are common across the stages of liver disease, regardless of the initial insult.

Transparency document

The Transparency document associated with this article can be found, in online version.

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