Review

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Animal models for liver disease – A practical approach for translational research

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Summary

Animal models are crucial for improving our understanding of human pathogenesis, enabling researchers to identify therapeutic targets and test novel drugs. In the current review, we provide a comprehensive summary of the most widely used experimental models of chronic liver disease, starting from early stages of fatty liver disease (non-alcoholic and alcoholic) to steatohepatitis, advanced cirrhosis and end-stage primary liver cancer. We focus on aspects such as reproducibility and practicality, discussing the advantages and weaknesses of available models for researchers who are planning to perform animal studies in the near future. Additionally, we summarise current and prospective models based on human tissue bioengineering.

Keywords: Non-alcoholic fatty liver disease; NAFLD; Non-alcoholic steatohepatitis; NASH; ALD; Fibrosis; Cirrhosis; Primary liver cancer.

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Introduction

Alcohol-related (AFLD) and non-alcoholic fatty liver disease (NAFLD) are hepatic insults with similar pathogenesis and histopathology, ranging from pure fatty liver and simple steatosis through alcohol-related (ASH) and non-alcoholic (NASH) steatohepatitis, to advanced chronic liver disease (ACLD): stages that encompass fibrosis, progression to cirrhosis, and end-stage complications such as primary liver cancer.

Considering the high prevalence of liver disease worldwide,[1] and the lack of preclinical alternatives, animal models are essential to further elucidate the pathophysiology of the progression of AFLD/ASH and NAFLD/NASH to ACLD, and to define effective treatments and potential biomarkers. In this comprehensive review we describe the most widely used experimental models mimicking these liver diseases, including their pros and cons, practical advice, and recommendations.

Overall, we show that translating findings from animals to humans (and vice versa) can be challenging, but the selection of a valid model that recapitulates important features of human liver disease is crucial.

Alcohol-related liver disease

To date, none of the current animal models can reproduce all major features of human alcohol-related liver disease (ALD). Rodent models are often characterised by relatively mild hepatic damage and an impaired ability to obtain and maintain a high blood alcohol concentration (BAC), which can be explained by certain physiological features: (i) high basal metabolic rate; (ii) natural aversion to alcohol; (iii) fast catabolism of alcohol; (iv) spontaneous reduction in alcohol intake when acetaldehyde blood level increases; (v) different progression time-lines between human (over 10 years of drinking) and rodents (12 weeks of ALD model); (vi) absence of addictive behaviour and (vii) differences in the innate immune system.[2] Nevertheless, current animal models remain a very useful tool to study ALD (Fig. 1).

In this section, a summary of the key classical ALD experimental models is provided (Table 1). The appropriate selection of a model strongly depends on the scientific question being addressed. Moreover, it is critical that researchers seriously consider the impact of several factors, including rodent strain, age, gender, control groups and husbandry. All these details should also be clearly and accurately described in the methodological section of publications. Most critical factors that can lead to misinterpretation of phenotypic results are specified below and outlined in Table 2.

Alcohol in drinking water

Single bottle

Alcohol in drinking water (ADW) is the simplest model of experimental alcohol administration, originally developed in the late 70s. In this model, age and sex-matched animals have free access to a single drinking bottle containing alcohol in water and standard rodent chow diet over the course of several hours, days or weeks. The available drinking water is supplemented gradually with increasing amounts of ethanol (normally starting from 5% (v/v)), and thereafter, the highest concentration of ethanol is used throughout the study. Mice drinking alcohol usually consume

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Key point

Animal models represent a fundamental preclinical tool to further elucidate the pathophysiology of chronic liver disease, and to define effective treatments and potential biomarkers.

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concentrations up to 25.32% (v/v) and rats as high as 40% (v/v). Control animals are allowed free access to rodent chow and drinking water.[3] In most studies, ADW for 8–10 weeks is sufficient to initiate steatosis. Long-term (months) alcohol feeding has been reported to induce oxidative stress, steatosis, very mild fibrosis, increased inflammatory cell infiltrates, increased hepatic injury, and depletion of cellular antioxidant defence in mice.[4] Rats maintained on 32.40% w/v ADW for 29 weeks developed histological changes of fatty liver, inflammation, focal necrosis and perivenular fibrosis.[5] However, animal care should be considered when selecting the proposed study length, since high alcohol content can be associated with increased mortality.

Multiple bottles

However, the single bottle ADW model is not suitable for use in non-alcohol-preferring, or low alcohol-preferring animals, as their aversion to alcohol will affect their fluid intake. Hence, the model can be modified to incorporate 2 bottles — a choice between water and alcohol, or multiple bottles — a choice between water and alcohol of varying concentrations. In this variation, individually housed animals receive unlimited access to 1 bottle containing tap water and at the same time to multiple bottles containing steadily increasing concentrations of ethanol (3–10% v/v) for a few days/weeks.[5]

Because rodents are not forced to drink the alcohol to assuage hunger or thirst, this method provides a measure of "spontaneous" consumption, and the alcohol intake is relatively free of non-specific effects, such as body size, customary fluid intake, or minor motor dysfunction.[6] Moreover, self-administration of alcohol in this model appears to share a great similarity with human addiction, therefore these variations can be used to explore molecular and neurochemical pathways that contribute to alcohol abuse in behavioural and dependence studies.

Moreover, availability is clearly a factor in the consumption of alcohol in rodents, as is likely the case in humans. The alcohol intake and preference of mice and rats is profoundly influenced by the source of alcohol available. The more bottles of alcohol the animals have, the more they drink and thus they will have significantly higher plasma alcohol concentrations than mice given 1 bottle.[6]

Yet, it is a challenge to get standard laboratory rodents to voluntarily consume high amounts of ethanol without the use of initiation procedures

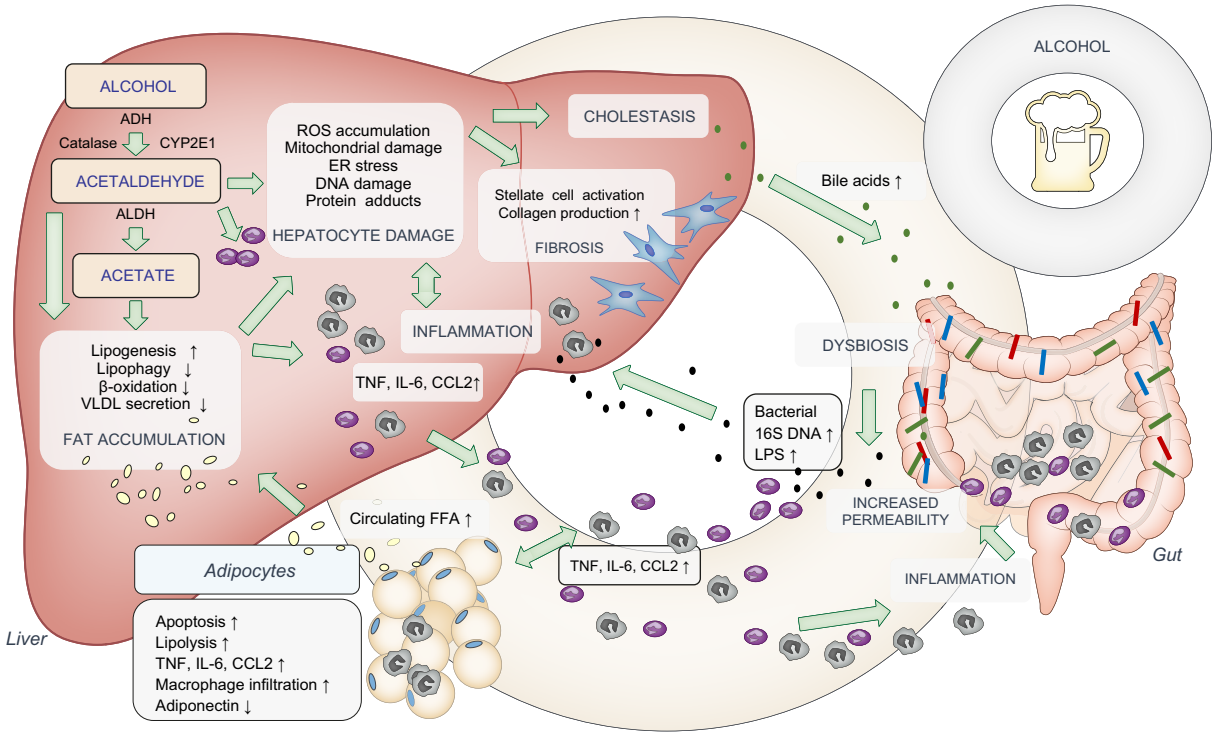

Fig. 1. Recapitulation of human ALD in animal models. A suitable animal model for the study of ALD pathogenesis should consider that ethanol is majorly metabolised into acetaldehyde by ADH, and to a lesser extent, by CYP2E1. A tertiary pathway for the oxidation of ethanol is carried out by catalase, a peroxisomal enzyme that also catalyses the removal of reactive oxygen species (*e.g.* H_2O_2). Acetaldehyde is further metabolised to acetate by the action of the ALDH. As a result, ethanol metabolism brings about gut dysbiosis, inflammation and increased permeability, which bidirectionally affect the liver in terms of significant lipid accumulation — caused by increased circulating free fatty acids, hepatic immune cell infiltration, hepatocyte damage, cholestasis and fibrosis. ADH, alcohol dehydrogenase; ALDH, acetaldehyde dehydrogenase; CCL2, C-C motif chemokine ligand 2; CYP2E1, cytochrome P450 isoenzyme 2E1; ER, endoplasmic reticulum; FFAs, free fatty acids; IL-6, interleukin 6; LPS, lipopolysaccharide; ROS, reactive oxygen species; TNF, tumour necrosis factor; VLDL, very low-density lipoprotein.

Table 1. Main features of widely used models in alcoholic liver disease (ALD) and NAFLD/NASH.

Experimental model	Type of induction	Administration route	Species	Induction time	Steatosis	Inflammation	Fibrosis	Portal hypertension	HCC	Other relevant characteristics	Used for drug discovery	Omics data
ALD/ASH												
Alcohol in drinking water[3]	Hepatotoxic substance (ethanol)	Oral (drinking water)	Rat or mouse	8–70 weeks	+	–	–	–	In combination with DEN	BAC [55–150 mg/dl]	Yes[115]	Yes[13]
Lieber-deCarli diet[10]	Hepatotoxic substance (ethanol)	Oral (liquid diet)	Rat or mouse	3–12 weeks	++	+/–	–	–	–	BAC [100– 160 mg/dl] (1/2 h after gavage)	Yes[116–118]	Yes[19,119,120]
NIAAA model[22]	Hepatotoxic substance (ethanol)	Oral (liquid diet + single or multiple ethanol binges)	Rat or mouse	10 days – 8 weeks	++	+	+	–	–	BAC [400 mg/dl]	Yes[121–123]	Yes[124,125]
LdC modifications[18]	Hepatotoxic substance (ethanol) + second hit (DEN, CCl_4, LPS or APAP)	Oral	Rat or mouse	4–10 weeks	++	++	++	–	In combination with DEN	Advanced fibrosis with LPS/CCl_4	–	–
Tsukamoto-French model[33]	Hepatotoxic substance (ethanol)	Intragastric infusion	Rat or mouse	4 weeks – 4 months	++	++	++	–	–	BAC [250–500 mg/dl]	–	Yes[126,127]
NAFLD/NASH												
Methionine- and choline-deficient diet[128]	Deficiency in methionine and choline	Oral (diet)	Rat or mouse	4–10 weeks	+++	+++	++	In rats[129]	–	Cachexia, no MS	Yes[130–132]	Yes[133,134]
Choline-deficient L-amino-defined diet[43,45]	Deficiency in choline	Oral (diet)	Rat or mouse	12–84 weeks	+++	++	++	Yes, in CDAA– HFD[53]	+	Not clear	Yes[135,136]	Yes[137,138]
High-fat diet	Diet rich in fat	Oral (diet)	Rat or mouse	16 weeks / 1 year	+++	+(after long feeding)	+(after long feeding)	–	–	+MS	Yes[139,140]	Yes[49,141]
HFD+CD[51]	Diet rich in fat + choline deficiency	Oral (diet)	Rat or mouse	24 weeks	+++	+++	++	+[53]	+	+MS	Yes[142]	–
HFD+ fructose[57]	Diet rich in fat fructose	Oral (diet)	Mouse	16–30 weeks	+++	++	++	–	–	+MS	–	–
HFD+cholesterol[60]	Diet rich in fat +cholesterol	Oral (diet)	Mouse	16–20 weeks	+++	++	++	–	–	+MS	–	–
HFD+cholesterol+ fructose[62]	Diet rich in fat +cholesterol & fructose	Oral (diet)	Mouse	12–24 weeks	+++	++	++/+++		–	+MS	Yes[143,144]	–
HFD+cholesterol+ fructose +trans-fat[64]	Diet rich in fat cholesterol fructose +trans-fats	Oral (diet)	Mouse	26–52 weeks	+++	++	++/+++	–	+	+MS	Yes[144]	Yes[145]
HFD +CCl_4[70,71]	Diet rich in fat +chemical	Oral (diet) and i.p./inhalated	Rat or mouse	12–52 weeks	+++	+++	+++	Yes in rat[71]	Yes in mouse		Yes[70]	Yes[70,71]

APAP, acetaminophen; BAC, blood alcohol concentration; CCl_4, carbon tetrachloride; CD, choline deficiency; CDAA, choline-deficient L-amino-defined; DEN, diethylnitrosamine; HCC, hepatocellular carcinoma; HFD, high-fat diet; LdC, Lieber-deCarli; LPS, lipopolysaccharide; MS, metabolic syndrome; +, low; ++, moderate; +++, high.

Table 2. Advantages and weaknesses of AFLD/ASH and NAFLD/NASH modelling.

Experimental model	Required skills	Costs	Safety for researchers	Severity	Mortality rate	Reproducibility	Weaknesses
Alcohol in drinking water	+	+	+++	+ ++ (long term)	+ ++ (long term)	++	General: It induces moderate steatosis and comparatively low elevations of serum aminotransferases, but no signs of fibrosis or inflammation. For the DID variation: nutritional effects associated with ethanol con sumption difficult to assess and control, difficult to sustain considerable BAC long term, possible dehydration.
Lieber-DeCarli diet	+	+	+++	+	+	++	It is not completely physiological since forces ethanol consumption when animals are hungry or thirsty, requires daily change of the liquid diet during the experimental period and it does not induce liver inflammation or hepatic fibrosis even after prolonged administration. Isocaloric diets should be used if including a paired control group. LdC modifications: may promote advanced liver disease but are usually accompanied by lower reproducibility and higher mortality.
NIAAA model	++	+	+++	++	++	++	The models still represent only moderate ASH. The combination of long-term chronic feeding and multiple binges of ethanol feeding is rather challenging, because of body weight loss and high mortality. In addition, housing mice in a low-temperature environment and improper technique during the oral gavage are other leading causes of mortality.
Tsukamoto-French model	+++	+++	+++	+++	+++	++	Not a real physiological model, requiring skilled surgical implantation in combination with expensive equipment. It needs extensive animal monitoring. Can be classified as severe procedure, thus have potential difficulties with local ethics committees.
Methionine- and choline-deficient diet	+	++	+++	++	++	+++	It does not replicate the NAFLD-related metabolic syndrome (decreased triglycerides and cholesterol, low leptin, absence of insulin resistance, among others). Animals become cachectic. Scarce transcriptomic similarity when compared to human NASH
Choline-deficient L-amino-defined diet	+	++	+++	+	+	+++	Very mild gain of weight and unclear increase in hepatic and peripheral insulin sensitivity.
High-fat diet	+	++	+++	+	+	++	Requires large sample size due to high inter-individual variability in steatosis, inflammation and fibrosis. Differences in species/strains, fat content in diet, duration, etc. difficult comparison between groups and protocols.
Modified HFD	+	++	+++	+	+	++	Fructose: no obvious limitations Cholesterol-modified: Cholesterol content in the diet may not be physiological Trans-fats: Prohibition to use trans-fad additives in all type of foods. Chemical: CCl_4-derived metabolites have unknown role in human NASH pathogenesis

ASH, alcohol-related steatohepatitis; BAC, blood alcohol concentration; CCl_4, carbon tetrachloride; DID, drinking in the dark; HFD, high-fat diet; LdC, Lieber-DeCarli; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; +, low; ++, moderate; +++, high.

such as sucrose fading, food and water deprivation. The repeated cycles of excessive drinking and abstinence, or the so-called intermittent drinking paradigm, also produces high ethanol consumption that can be maintained over a longer period.[7]

Drinking in the dark

The "basic" variation of drinking in the dark (DID) involves replacing the water bottle with a bottle containing 20% ethanol for 2 to 4 hours, beginning 3 hours into the dark cycle, in cages of singly housed mice. Using this procedure, mice typically consume enough ethanol to achieve psychologically relevant BAC (>1.0 mg/ml) and exhibit behavioural evidence of intoxication.[8] The DID model is also quite commonly used to mimic prenatal human binge drinking.[9] The available variations of this model make it one of the best suited chronic alcohol abuse models for a wide range of studies. It is physiological, inexpensive, without significant mortality and with very simple animal husbandry. Higher BAC can be achieved if the ethanol-containing drinking fluid is given alongside inhibitors of alcohol dehydrogenase that raise BAC by slowing its metabolism.[3,10] Alternatively, flavours can be added to make alcohol taste better.

ADW + second hit

As mentioned, despite the widespread use of the ADW model, there are important limitations in the severity of ALD progression (Table 2). Therefore, modified experimental models using secondary hepatic stressors have been widely used. For example, diethylnitrosamine (DEN) and alcohol act synergistically to induce liver injury, further promoting hepatocellular carcinoma (HCC) development and suitably mimicking human ALD.[11] A single dose of DEN given at a late stage on an 8-week, 10/20% (v/v) ADW (alternate days) regime significantly increased tumour incidence and burden, but only in male mice.[12]

Likewise, several approaches have been pursued to combine carbon tetrachloride (CCl_4) applications with alcohol. In a recent study,[13] advanced liver injury was achieved by supplementing CCl_4 exposure with ADW. Yet, the use of hepatotoxic chemicals, including CCl_4 or DEN, to study fibrosis is likely to represent the effects of the toxin more than ALD. Therefore, more physiological models that mimic the typical drinking pattern are urgently required.

BASH (both alcohol-related and non-alcoholic steatohepatitis)

In the prosperous parts of the world, lifestyles often include the overlapping of alcohol intake with high fat consumption. Consistently, moderate alcohol consumption was related to an increased risk of NAFLD progression, leading to advanced fibrosis[14] and hampering disease resolution.[15] Some animal models have already been suggested to evaluate the influence of dietary factors on chronic alcohol-induced liver injury.[16] As an example, a combination of ADW (5%) with high-fat diet (HFD) for 6 weeks leads to enhanced expression of proinflammatory genes, activation of hepatic stellate cells (HSCs), and extracellular matrix (ECM) deposition in the liver tissue, with synergistic effects on fibrosis development.[16] A complementary model that combines HFD feeding followed by gavage with a single dose of ethanol induces liver inflammation and injury through the elevation of hepatic and serum free fatty acids, leading to hepatic neutrophil infiltration.[17] This model could be used to study the effect of interactions between obesity and binge drinking on liver inflammation and injury.[17] Still the new BASH models from other laboratories are awaited with interest as a major advance in this field.

The Lieber-DeCarli diet

Although the ADW model was widely used for mild alcoholic liver injury, the need for a method which achieves significant BAC and allows reasonable and controlled nutrition led to the development of a unique liquid diet procedure over 25 years ago by Charles Lieber in collaboration with his technical assistant Leonore M. DeCarli.[3,10,18]

In order to overcome the aversive gustatory properties of alcohol, the Lieber-deCarli (LdC) diet involves incorporating ethanol into a liquid only diet. When rodents have nothing to eat or drink except the ethanol-containing liquid diet formula, their intake is sufficient to sustain a high ethanol consumption of 14 to 16 g/kg/day, which approximately resembles the intake of 3.4 L of whiskey (40% v/v) in a 75 kg human.[18] The amount of ethanol in the diet should be increased gradually, up to the final concentration of 6.4% v/v, during a primer period of approximately 5 days. This allows the animal to adapt to the ethanol-containing diet gradually, thus ensuring the effect of subsequent formal feeding. The diet is also supplemented with essential fat-soluble vitamins (A, D, E, K) and water-soluble vitamin B12, minerals and fibre.[3]

Depending on the scientific question or the rodent's species, strain and gender the duration of the LdC feeding can be modified. The earliest step of ALD may manifest as early as 3 to 4 weeks after alcohol exposure. Four weeks of LdC represents an excellent model to study the initial stages of ALD with mild steatosis, slightly elevated aminotransferases, and minor inflammation.[19,20] Stepwise feeding with the LdC up to 8 to 12 weeks induces remarkable fatty liver with mild inflammation and moderate liver damage, without fibrosis.[21] For the development of the advanced form of ALD, second hit models are recommended instead of prolonging the diet.

The NIAAA model

One of the most well-known modifications of the LdC diet is the NIAAA model developed by the group of Bin Gao.[22] According to the protocol, mice receive LdC containing 5% v/v ethanol for 10 days. A single dose of ethanol (5 g/kg body weight) is given at day 11, and 9 hours later animals are euthanized. This model can be extended to longer periods of chronic feeding (up to 8 weeks) and combined with multiple binges.

This protocol triggers higher BAC, more severe steatosis, hepatocellular damage, and hepatic neutrophil infiltration, compared with LdC feeding. However, it still represents only early ASH. Long-term chronic feeding and multiple binges of ethanol feeding induce more severe steatohepatitis; however, it is challenging to establish due to body weight loss and high mortality. The model mimics alcoholic liver injury in the form of heavy binge drinking in chronic alcohol abusers.[23]

Key point

Mostly due to animal's aversion to alcohol, current preclinical models mimicking alcoholic liver disease do not reproduce all major features of human disease; however, they are useful to understand key pathophysiological events such as steatosis and inflammation.

Other LdC variations

Besides binge drinking, other hepatotoxins including DEN,[24] CCl_4,[25,26] lipopolysaccharide (LPS)[27,28] or acetaminophen (APAP)[29] can be added during chronic LdC feeding to provide a "second hit" and increase liver damage. These studies have expanded the use of the LdC diet and provide useful insight into the effects of ethanol on the progression of severe liver injuries such as cirrhosis or HCC.

For example, the severity of fibrosis is significantly magnified by combining alcohol and CCl_4 in a dose-related manner.[25] The group of Laura Nagy established an interesting model where mice are subjected to chronic CCl_4-treatment combined with the LdC diet containing moderate concentrations of ethanol (2% v/v), leading to hepatocyte apoptosis, HSC activation and accumulation of ECM.[30]

Another synergistic model which may deserve attention is the combination of LPS with the LdC diet, which mimics leakage of bacterial products such as LPS from the intestine into the blood, leading to the release of various proinflammatory cytokines that ultimately exacerbate steatohepatitis, liver injury and fibrosis.[27,28]

Finally, we obtained a new model of advanced ALD by administering the LdC diet to transgenic mice overexpressing the c-MYC oncogene specifically in hepatocytes; these mice exhibited early ballooning degeneration, incremental collagen deposition and altered fat metabolism. Long-term feeding resulted in substantial fibrosis and expression of pre-neoplastic markers.[19,31]

The Tsukamoto-French model

To overcome the limitations of the LdC diet and reach the advanced stages of ALD, in 1984 Tsukamoto and co-workers developed a new feeding model of direct ethanol infusion through a surgically implanted intragastric cannula. In this model, a catheter is implanted into the stomach under aseptic conditions. Alcohol is added to the LdC diet and infused via the implanted catheter directly into the stomach with the aid of an infusion pump.[32,33]

This model is really valuable because the investigator has complete control of ethanol intake and diet, as well as administration rate (amount) and mode of delivery (continuous, intermittent or bolus). Moreover, the dietary factors can be manipulated and a second hit such as LPS administered enterally.[34] The animals can be maintained on the diet for several months, and blood alcohol levels of 250–500 mg/dl can be achieved and sustained. The application of this model leads to pathologic changes which resemble human ALD, including microvesicular and macrovesicular fat, megamitochondria, apoptosis, central necrosis, and mixed inflammatory infiltrate including polymorphonuclear cells and lymphocytes, central lobular and pericellular fibrosis, portal fibrosis, bridging fibrosis, but not cirrhosis or other irreversible changes.[3,35] Another interesting modification is the "hybrid" combination of the Tsukamoto-French model with *ad libitum* feeding of a western diet (WD).[36]

Non-alcoholic fatty liver disease

Animal models that mirror the pathophysiology of every stage of human NAFLD progression provide important insights into disease pathogenesis, guiding the development of much needed therapeutic options (Fig. 2). This section summarises the current and most frequently used animal models in NAFLD research, focusing on each model's major advantages and drawbacks (see also Tables 1 and 2). NAFLD researchers must have a very clear understanding of which pathological event they aim to study in order to choose the animal model that best suits their research goals.

Methionine- and choline-deficient diet

The methionine- and choline-deficient (MCD) diet has been widely used in NAFLD animal studies. MCD is high in sucrose (40%) and provides a moderate amount of fat (10%) but is deficient in methionine and choline. Methionine is an essential amino acid that cannot be synthesized *de novo* and is a crucial compound for the synthesis of cysteine, lecithin, phosphatidylcholine and many other macromolecules. Similarly, choline is a constituent of cell and mitochondrial membranes and a precursor for acetylcholine, a well-known neurotransmitter.[37,38] Due to the deficiency of both components, the synthesis of phosphatidylcholine is impaired, resulting in diminished VLDL assembly and secretion. Consequently, triglyceride (TG) clearance is reduced and lipids accumulate in the liver. Other pathological features of this model include impaired mitochondrial β-oxidation,

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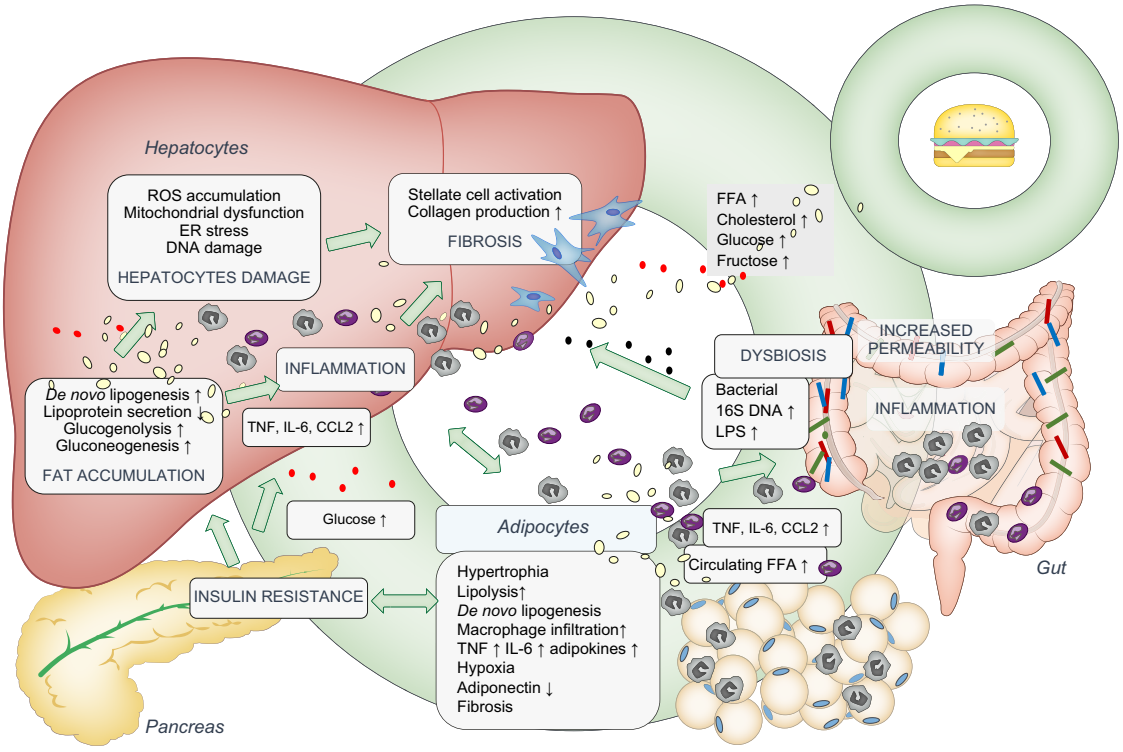

Fig. 2. Towards an ideal experimental model of NAFLD/NASH. The ideal characteristics of an animal model of NAFLD/NASH should mimic human disease with respect to its development by diet-induced obesity and insulin resistance (the most common risk factors for the disease in humans), thus triggering predominantly macrovesicular steatosis, lobular inflammation, hepatocellular ballooning and/or with Mallory-Denk bodies and, activation of hepatic stellate cells and consequently liver fibrogenesis. An important model of NAFLD should not only recapitulate the diet, systemic milieu and histological spectrum of the disease but also demonstrate activation of key cellular pathways, such as ER stress, lipotoxicity and activation of *de novo* lipogenesis. In addition, other pathogenic elements such as oxidative stress, apoptosis and fibrogenic pathways that are relevant in human disease should also be activated. Advanced fibrosis is required when studying NASH. Moreover, it has also become clear that intestinal dysbiosis plays a pivotal role in disease progression. CCL2, C-C motif chemokine ligand 2; ER, endoplasmic reticulum; FFAs, free fatty acids; IL-6, interleukin 6; LPS, lipopolysaccharide; ROS, reactive oxygen species; TNF, tumour necrosis factor.

induction of CYP2E1-derived oxidative stress, and depletion of hepatic anti-oxidants, promoting reactive oxygen species (ROS) and steatohepatitis.[39]

The MCD model replicates part of the histological phenotype typical of human NASH in a relatively short period. It usually takes 3 weeks to develop obvious steatohepatitis and 5–8 weeks for appreciable fibrosis.[40] After 10 weeks of MCD feeding, a large number of necroinflammatory foci containing lymphocytes and neutrophils can be observed in the murine liver tissue,[41] associated with markedly elevated plasma alanine aminotransferase levels.

However, it does not replicate the NAFLD-related metabolic syndrome. Mice fed the MCD are cachectic and by 8 weeks of feeding lose 40% of their weight, most likely because of a hypermetabolic state.[40] Moreover, animals have decreased plasma TG and cholesterol levels and reduced liver weight/body weight ratio. Additionally low serum levels of leptin, unchanged or increased adiponectin, an absence of overt insulin resistance, with low fasting blood sugar and peripheral insulin sensitivity, create a metabolic profile opposite to the human disease.[42] Importantly, there is also a poor concordance between differentially expressed genes in this model and human NASH.[7] Thus, the use of the MCD model is limited by the disparity in metabolic changes observed between the model and the vast majority of human patients with NAFLD.

Choline-deficient L-amino-defined diet

Like the MCD diet, the choline-deficient L-amino-defined (CDAA) diet is also deficient in choline. In contrast, the semisynthetic CDAA diet has normal or only moderately lowered levels of methionine and the proteins in the formula are comparably replaced by a mixture of L-amino acids.[43] Hence, the development of experimental NAFLD takes longer with the CDAA than the MCD diet. Steatohepatitis occurs after 12 weeks of the CDAA diet and is accompanied by mild ballooning and fibrosis, with an approximately 3-fold increase in hepatic collagen levels. Fibrosis further progresses to moderate stages after approximately 21 weeks of feeding. Moreover, CDAA-fed rodents frequently exhibit hepatic tumours associated with fibrosis; thus, they can be used to study the progression

from NAFLD to NASH and further to HCC.[40,44] Interestingly, female mice did not develop these lesions.[45]

High-fat diet

Aimed at recapitulating the pathogenesis of human NAFLD, obesogenic or the so-called HFDs are widely used to generate obesity and NAFLD in rodents. These diets are particularly rich in fat without any artificial nutrient deficiencies. In the first paper from the 1940s, Samuels *et al.*[46] reported that rats fed with a diet containing 70% energy as fat developed obesity and elevated basal and postprandial blood sugar values. From subsequent studies, it is now accepted that HFD (accounting for 45–75% of total calorie intake from fat) may be used to generate metabolic syndrome, hepatic steatosis and NASH in experimental animals.

Furthermore, the severity of HFD-induced NAFLD may depend on the: i) species — rats appear to be more sensitive than mice and require a shorter time for severe histological NAFLD manifestation[47]; ii) gender — male mice are more susceptible than females, as oestrogen (the major female sex hormone) is protective against NAFLD[48]; iii) strain of the animals, (*e.g.* Sprague-Dawley rats fed with HFD easily develop steatohepatitis, while Wistar rats are resistant[41]). In mice, the C57BL/6 strain exhibits high sensitivity to HFD. In contrast, BALB/c and C3H/HeN are significantly less prone to develop diet-induced hepatic necroinflammation.[44] In fact, irrespective of diet, C57BL/6 mice are already genetically prone to obesity, hyperinsulinemia and glucose intolerance as they age.

The importance of the genetic background has been exemplified in the diet-induced animal model of NAFLD.[49] Sanyal's group developed a novel inbred isogenic mouse strain, derived from the C57BL/6J and 129S1/SvImJ backgrounds, that upon feeding with 0.1% cholesterol HFD + fructose/sucrose-enriched drinking water results in obesity, insulin resistance and a time-dependent progression of NAFLD. Bridging fibrosis and HCC were observed in almost all mice at week 52. These mice recapitulate the key physiological, metabolic, histologic, transcriptomic and cell-signalling changes seen in humans with progressive NAFLD. However, a major drawback of the model relates to the fact that most knockout or transgenic animals are typically maintained on more commonly available backgrounds (such as C57BL/6 and B6/129), thus this model is not readily available for such studies.[44,50]

Apart from this, HFD-fed animal models present other limitations highlighted in Table 2. Therefore, they should mainly be used for the characterisation of potential drug effects on body weight, hepatic steatosis, and, to some extent, inflammation.

Key point

Animal models for NAFLD/NASH are diverse, mostly based on nutritional intervention, and their pathophysiology ranges from simple steatosis to advanced liver fibrosis. The latter is obtained when combining nutritional intervention with a second proinflammatory hit.

Modified HFD

CD diets

Among various dietary approaches, the interesting combination of HFD with choline deficiency (CD-HFD) reported by the group of Heikenwalder[51] and others[52,53] deserves closer attention. Indeed, long-term application of CD-HFD fully recapitulates chronic metabolic disorders leading to advanced NAFLD. In addition to severe steatosis, the livers of CD-HFD mice displayed all features reminiscent of human NASH including ballooned hepatocytes, infiltration of immune cells, satellitosis, Mallory-Denk bodies and glycogenated nuclei. Moreover, CD-HFD feeding induces oxidative stress and mitochondrial damage and causes mild pericellular fibrosis accompanied by activation of HSCs. At 24 weeks, mice developed signs of cirrhosis with severe perisinusoidal pan-lobular chicken wire fibrosis, regenerative nodule formation, pronounced ductular reaction and significant portal hypertension.[53] Importantly, within 24 weeks all CD-HFD-fed animals develop tumours, fully resembling human HCC.[51,53]

In order to amplify NAFLD and trigger a robust fibrotic response without significantly compromising nutrient balance, different modified HFDs with increased levels of fructose and/or cholesterol have been used.[44]

Fructose

Recent studies suggest that over-consumption of fructose, primarily in the form of soft-drinks, is tightly linked to weight gain and increases the risk of NAFLD, particularly in children and adolescents.[54] In animals, as well as in clinical studies, fructose stimulates *de novo* lipogenesis and blocks hepatic β-fatty acid oxidation leading to fat accumulation in the liver.[55] In addition to these direct effects, fructose contributes to intestinal dysbiosis, impaired intestinal barrier function and increased translocation of bacterial metabolites in NAFLD.[56]

Experimental animals fed a fructose-enriched diet are recognised as good models of metabolic syndrome. High fructose feeding for a long duration (up to 30 weeks) typically results in steatohepatitis progressing to moderate fibrosis. In the experimental study from Kohli *et al.*,[57] C57BL/6 mice were fed *ad libitum* with a high-fat high-carbohydrate diet (58% of calories from fat) and drinking water with high fructose (55% fructose) over a 16-week period; these mice developed a phenotype encompassing obesity, insulin resistance, hepatic injury, oxidative stress, macrophage infiltration, and fibrosing NASH.[41,48] Similarly, hepatocellular injury, inflammation, and hepatic fibrosis were reported in the rats fed with a fructose-enriched diet.[41,57,58]

Cholesterol

Many epidemiological studies have identified dietary cholesterol intake as a factor related to the risk and

severity of NAFLD.[59] In line with this concept, the liver phenotype of HFD-based mouse models can be aggravated by increasing cholesterol concentrations in the diet. Thus, C57BL/6 mice fed with cholesterol-containing HFD for 16–20 weeks develop more pronounced liver damage, inflammation and fibrosis than animals fed with HFD without cholesterol. Cholesterol significantly increased serum leptin, interleukin-6, liver weight and insulin resistance, thus leading to a phenotype that closely resembles the clinical features of NASH in patients with metabolic syndrome.[60] It is important to note that 2% cholesterol diets (frequently used) are not physiological, and those diets with lower cholesterol (which more closely model human cholesterol consumption) may either not induce NAFLD or do so in a time frame that is economically (in time and money) unrealistic.

In practice, to induce NASH and significant liver fibrosis within reasonable time frames (usually around 4 to 6 months) an increasing number of studies are using HFD enriched with both fructose and cholesterol. Such diets are referred to as "fast food diet" or "western diet (WD)". In humans, a "western" dietary pattern is characterised by high intakes of red/processed meat, fast food, refined grains/cereals, sugar-sweetened beverages, eggs, sweets/desserts and low intake of fruit and vegetables or dairy products. This diet induces obesity, metabolic syndrome and NAFLD. Nowadays, commercial diet companies sell many open-source formulations of WD for animal research. However, researchers must be cautious, because animal WD do not fully recapitulate many features of this human dietary pattern (*e.g.*, diverse fat sources, amounts of micronutrients). For example, the micronutrient content of these WD is formulated to promote animal health, which is totally inconsistent with the western dietary pattern in humans.[61] Therefore, these diets should be considered as powerful tools to generate disease, such as NAFLD for example, but not as a model of "western" nutrition.

Amongst other studies, an excellent WD model has been introduced by Charlton and colleagues.[62] In this study C57BL/6 mice were fed with HFD (40% of energy as fat, milk fat, 12% saturated) enriched with 0.2% cholesterol. Additionally, high-fructose corn syrup was also administered in the drinking water. By 6 months, animals exhibited all the hallmarks of advanced NAFLD most commonly observed in humans, including obesity, metabolic syndrome and steatohepatitis. Importantly, the most significant new feature of this model was the presence of hepatocellular ballooning and progressive fibrosis.

Trans-fats

Addition of dietary trans-fats has been reported to enhance the steatogenic and pro-fibrotic properties of WD in mice.[44] The underlying molecular mechanisms are not fully understood, but trans-fats likely sensitise mice to the effects of HFD by increasing insulin resistance, hepatic lipogenesis and oxidative stress.[63]

Consequently, an 'American Lifestyle-Induced Obesity Syndrome' (ALIOS) mouse model of NASH was developed by Tetri and colleagues[64] and subsequently refined by other laboratories.[65,66] In both variations of the diet, the source of the trans-fats is partially hydrogenated vegetable oil. However, the cholesterol content in the modified diet is greater, and fructose was provided in the food pellets rather than in the drinking water. C57BL/6 mice treated with these diets develop obesity, hepatic insulin resistance, hyperinsulinemia, marked steatosis, moderate lobular inflammation, and mild-stage hepatocellular ballooning within 26–30 weeks of dieting, and HCC after 52 weeks.[67] In general, the model accurately reflects patterns associated with human NAFLD and replicates features of metabolic syndrome, therefore representing an excellent preclinical model for identifying pharmacological interventions with greater likelihood of being translated into the clinic. However, the FDA has recently imposed a ban on the use of trans-fat additives in all type of foods including animal diets. This prompted the development of non-trans-fat WDs, with similar nutrient composition, capable of promoting comparable metabolic and liver histopathological changes.[68]

Chemical

Based on 'multiple-hit' theory for the development and progression of human NALFD, few animal models successfully combined obesogenic diets with CCl_4 administration.[69–71] In this setting, CCl_4 potentiates the effects of an HFD towards the fast development of NASH and fibrosis.

Kubota *et al.* were among the first to show that a combined model of HFD feeding and CCl_4 administration induced hepatic steatosis, inflammatory cell accumulation, hepatocellular ballooning, fibrosis, and increased serum aminotransferase levels after 12 weeks.[69] Two subsequent studies performed in mice and rats recapitulated those findings,[70,71] and showed that a combination of HFD with CCl_4 leads to prominent F4 fibrosis (*i.e.*, cirrhosis after 24 weeks of protocol). Interestingly, in both studies, rodents subjected to these novel protocols developed progressive NASH-like histology despite the fact that adding CCl_4 treatment to HFD feeding attenuated typical HFD-induced increases in body weight, cholesterol and insulin/glucose levels. Last, but not least, global transcriptome profiling of the liver by RNA sequencing showed that the dysregulated molecular pathways in WD/CCl_4 rodents at 12/24 weeks were comparable to those of mild/advanced human NASH.

Animal models of advanced chronic liver disease

Albeit the causes of chronic liver disease (CLD) may vary, the progression of the disease converges in

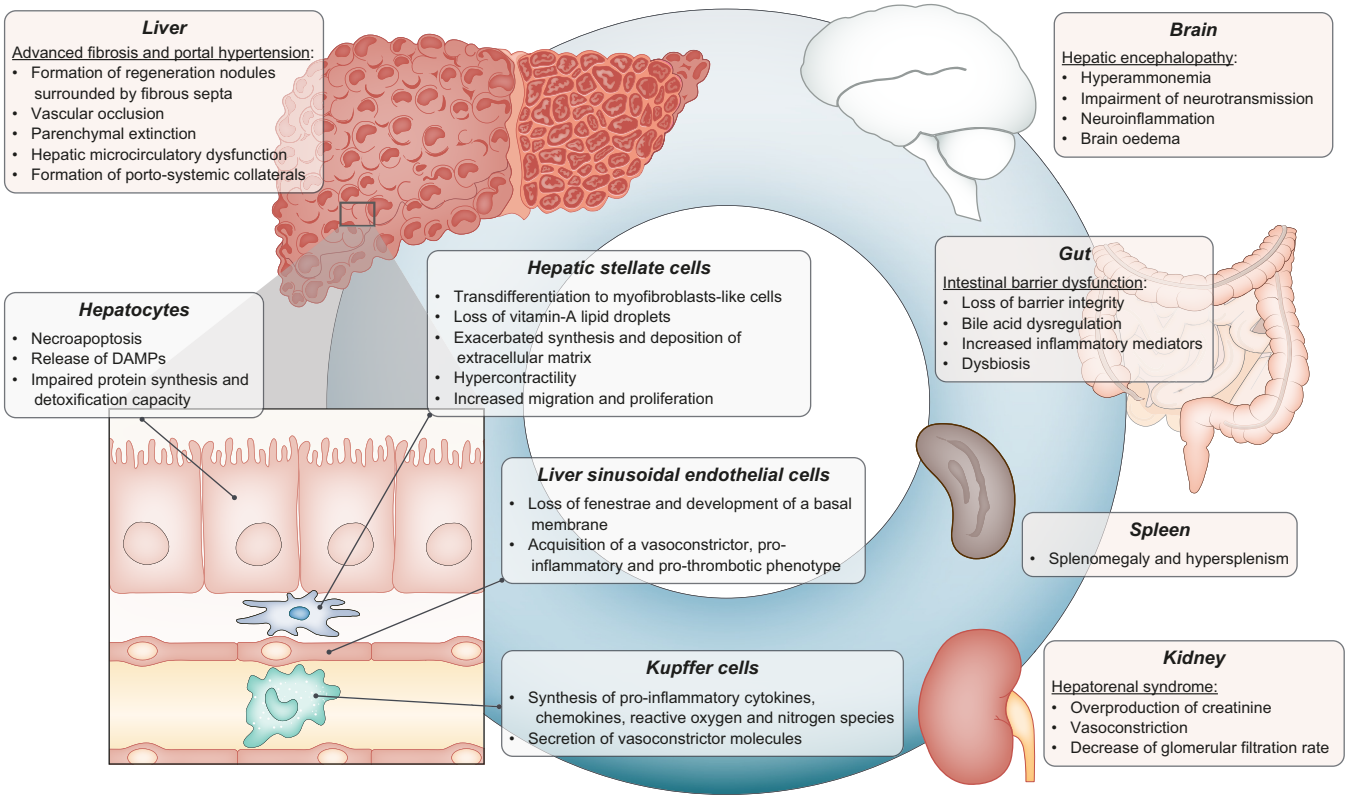

Fig. 3. Main alterations present in animal models of advanced chronic liver disease. An animal model of ACLD that closely mimics the pathophysiology of the human disease should exhibit a profound deregulation both at the systemic and cellular levels. In the liver, all cell types are affected, including hepatocyte necroptosis, hepatic stellate cell activation, liver sinusoidal endothelial cell capillarisation, and Kupffer cell shift to a proinflammatory phenotype. Altogether, these alterations cause histological distortion and vascular occlusion in the liver, leading to the development of portal hypertension, one of the most relevant syndromes associated with ACLD. At advanced stages, portal hypertension can cause severe systemic complications, such as hepatic encephalopathy, intestinal barrier dysfunction or hepatorenal syndrome, among others. ACLD, advanced chronic liver disease; DAMPs, damage-associated molecular patterns.

Key point

Animal models of cirrhosis derive from chronic exposure of rodents to hepatotoxins, resulting in advanced fibrosis, portal hypertension, and other clinical complications observed in patients. Models of acute-on-chronic liver failure (ACLF) are also described.

cirrhosis and may even progress, in some cases, to HCC. Cirrhosis, or ACLD, is characterised by an excessive deposition of ECM in the liver, leading to the formation of regenerative nodules surrounded by fibrous bands, parenchymal extinction, and vascular occlusion. One of the most relevant syndromes associated with ACLD is portal hypertension, which has serious clinical complications.[72]

Even though research efforts to find an effective treatment for ACLD have dramatically increased in recent decades, liver transplantation currently remains the only therapeutic option. While some promising candidates are emerging,[73] there is still a long path of preclinical research to undergo before they reach the bedside. For this reason, it is essential to have well-characterised animal models that closely mirror the specific aspects of ACLD (Fig. 3).

Preclinical models of ACLD that are based on the administration of hepatotoxic substances are the most widely used by researchers worldwide. However, other types of model exists (such as surgical models), as well as models of specific aetiologies of cirrhosis. We will summarise herein the most relevant and widely used animal models for ACLD research, as well as their main drawbacks and advantages. However, a more extensive list of models can be found in Table 3.

Carbon tetrachloride

CCl_4 is metabolised in the liver by cytochrome P450 enzymes and converted to a highly reactive trichloromethyl ($CCl_3^{\bullet}$) radical, ultimately leading to hepatotoxic damage, inflammation and fibrosis.[74] Although it can also be used in shorter protocols for the study of acute liver injury, chronic administration of CCl_4 has long been one of the most widely accepted models of ACLD. However, a great variety of protocols exist, making it sometimes difficult to compare results from different research groups. In general, CCl_4 is administered to rats or mice through intraperitoneal injection or inhalation. Alternatively, it can be delivered by oral gavage, although there have been mixed reports regarding mortality rates using this route.[75,76]

Intraperitoneal injection

CCl_4 is dissolved in mineral or vegetable oil and intraperitoneally injected into rats or mice commonly 2–3 times per week. Due to the toxicity

of its vapours, CCl_4 should be handled inside a fume hood. The dose must be adjusted by animal body weight, and typically ranges between 0.5–2.0 ml/kg.[77,78] The duration of the protocol may vary depending on the dose and species chosen but is generally 6–12 weeks. It is important to note, however, that most intraperitoneal mice models described only reach a stage of CLD that could be defined as fibrosis or early cirrhosis.

Inhalation

CCl_4 can equally be administered through inhalation. This model has traditionally been used in rats for the study of portal hypertension and its complications,[77,79,80] and it has more recently been adapted for use in mice.[78] In both cases, the animals must be placed in a closed chamber, inside a fume hood, connected to a source of compressed oxygen bubbling through a CCl_4 flask at a usual rate of 1 L/min. In most protocols, animals are subjected to this CCl_4 atmosphere 2/3 times per week, increasing the exposure time every week.[77] Moreover, phenobarbital can be added to drinking water (0.3 g/L) to shorten the required time to reach cirrhosis, as McLean *et al.* first described in 1969.[81] After 13–16 weeks, animals will develop ascites, marking the development of decompensated cirrhosis.

In general, CCl_4-induced ACLD constitutes a reliable model of the human pathology, mimicking all its crucial aspects both at the physiological and cellular level. CCl_4-cirrhotic animals present a profound deregulation of all the cell types present in the liver, including hepatocyte necroapoptosis, HSC activation, liver sinusoidal endothelial cell capillarisation and macrophage recruitment. It is of particular interest for the study of decompensated stages of cirrhosis since CCl_4-cirrhotic animals often present ascites, among other signs of decompensation. Nonetheless, some technical aspects must be considered when using this model (Table 4). Firstly, a suitable strain must be selected, as not all strains of mice or rats are equally susceptible to the damage produced by CCl_4. BALB/c have been described to be the most adequate among mice[82] while, in our experience, Wistar rats present higher CCl_4-susceptibility. Even among animals of the same strain, there can be a certain heterogeneity in the degree of cirrhosis obtained, which is characteristic of this model. In rats, intra-animal heterogeneity can be diminished by selecting animals for study once they have developed ascites, an unequivocal sign of cirrhosis decompensation, rather than selecting them based on the weeks of toxicant administration. Nonetheless, the number of animals per experimental group must be sufficient to compensate for said variability. Regarding sex-related differences, it has been reported that female Sprague-Dawley rats respond more severely to acute CCl_4 than males, while also presenting a greater extent of tissue

Table 3. Main features of widely used models of advanced chronic liver disease.

Experimental model	Type of induction	Admin. route	Species	Induction time	Steatosis	Inflammation	Fibrosis	Portal hypertension	HCC	Other relevant characteristics	Used for drug discovery	Omics data
CCl_4[76–78]	Hepatotoxic substance	Inhalation, i.p., or oral (gavage)	Rat or mouse	Inhalation: 13–16 weeks; i.p.: 6–12 weeks; oral: 6–12 weeks	+	+++	+++	Yes (↑ HVR, endothelial dysfunction)	With higher doses or longer induction periods, or in combination with other agents[146]	Ascites	Yes[147,148]	Yes[149,150]
TAA[87,89,91]	Hepatotoxic substance	In drinking water or i.p.	Rat or mouse	10–12 weeks	+	+++	+++	Yes (↑ HVR, endothelial dysfunction)	In combination with other agents[151]	–	Yes[87,92,152]	Yes[153,154]
cBDL[94,155]	Surgical	-	Rat or mouse	3–4 weeks	+	+++	+++	Yes (↑ HVR, endothelial dysfunction)	–	Cholestasis; enlargement of gallbladder or biliary cyst	Yes[96,156]	Yes[157,158]
DDC[159]	Hepatotoxic diet	Oral (*ad libitum*)	Mouse	4–8 weeks	-	++	++	–	–	Cholestasis; formation of intraductal porphyrin plugs; ductular reaction	Yes[160,161]	Yes[162,163]
DMN[164,165]	Hepatotoxic substance	Oral (gavage) or i.p.	Rat or mouse	4 weeks	-	++	+	Yes	Yes	Highly carcinogenic	Yes[166]	Yes[167,168]
DEN[169]	Hepatotoxic substance	Oral (gavage), i.p., or in drinking water	Rat or mouse	7–18 weeks	+	++	-/+	–	Yes	Highly carcinogenic	Yes[170,171]	Yes[172,173]
Mdr2$^{-/-}$[174,175]	Genetic	n.a.	Mouse	12–24 weeks	-	++	+	Yes	Yes	Sclerosing cholangitis; biliary fibrosis	Yes[176]	Yes[177]
ACLF[178–180]	Combination: CCl_4/TAA/cBDL with LPS/KP	CCl_4: inhalation or i.p. TAA: i.p. LPS/KP: i.p.	Rat or mouse	CCl_4: 8–16 weeks TAA: 10 weeks cBDL: 4 weeks	+	+++	+++	Yes (↑ HVR, endothelial dysfunction)	–	High mortality; renal dysfunction	Yes[179–181]	–

ACLD, advanced chronic liver disease; ACLF, acute-on-chronic liver failure; cBDL, common bile duct ligation; CCl_4, carbon tetrachloride; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DEN, diethylnitrosamine; DMN, dimethylnitrosamine; HCC, hepatocellular carcinoma; HVR, hepatic vascular resistance; KP, *Klebsiella pneumoniae*; LPS, lipopolysaccharide; TAA, thioacetamide; +, low; ++, moderate; +++, high.

Table 4. Advantages and weaknesses of advanced chronic liver disease modelling.

Experimental model	Required skills	Costs	Safety for researchers	Severity	Mortality rate	Reproducibility	Weaknesses
CCl_4	+	+++	+ (inhaled) ++ (i.p.)	+	+	++	Differences in administration route, frequency, and dose are associated with discrepancies between studies and research teams. For CCl_4 inhalation, special facilities and equipment are required. Animal tolerability to CCl_4 varies, making it difficult to have homogeneous groups. Necessity to increase the n. CCl_4 withdrawal may lead to partial fibrosis regression.
TAA	+	+++	++	+	+	++	Possible low yield of cirrhosis when TAA is administered in drinking water. High variability in fibrosis grade. Significant mortality if TAA dose is not corrected to body weight.
cBDL	++	+	+++	++	++	+++	Not adequate for studies involving pharmacological drugs eliminated through the biliary route. Extrinsic compression of portal vascular structures due to exaggerated dilatation of the remnant extrahepatic bile duct.

cBDL, common bile duct ligation; CCl_4, carbon tetrachloride; TAA, thioacetamide; +, low; ++, moderate; +++, high.

Key point

All animal models present pros and cons, which are herein summarised. Standardisation of protocols, as described in this review, is key to obtain reproducible and representative results, and therefore a higher impact in the field.

repair and cellular regeneration.[83] Lastly, CCl_4-induced ACLD exhibits a relatively rapid rate of partial regression once the toxicant is removed, therefore being of specific interest for the study of fibrosis regression.[84]

Thioacetamide

After CCl_4, thioacetamide (TAA) is the second most widely used model of hepatotoxin-induced ACLD. Its hepatotoxicity, first reported in rats in 1948,[85] stems from the generation of highly reactive metabolites in the liver that can bind to proteins and lipids causing oxidative stress and necrosis.[86] In the past decades, TAA has been extensively used both in mice and rats; it is generally administered by intraperitoneal injection or in drinking water.

Intraperitoneal injection

For its intraperitoneal delivery, TAA is dissolved in saline solution and the dosage is adjusted by body weight. There are a variety of published protocols of TAA-induced cirrhosis with differences in dose and frequency of administration. In our experience, Sprague-Dawley rats receiving TAA twice per week at a dose of 250 mg/kg will develop ACLD in 12 weeks.[87] Others have described milder guidelines for its use in mice (200 mg/kg twice per week for 8–10 weeks),[88] but many of them only reach fibrosis or an early stage of cirrhosis. Moreover, abdominal adhesions should be expected.

TAA in drinking water

Alternatively, TAA can be added to drinking water at a concentration of 300 mg/ml, for a duration of 12 weeks.[89] Some research groups have reported alternative protocols in which TAA concentration was gradually increased according to body weight gain throughout the duration of ACLD induction.[89,90]

Overall, TAA constitutes a highly reliable and reproducible model that effectively mirrors the patterns of human ACLD.[91,92] It presents a low mortality rate when dosage is correctly adjusted by body weight and requires little technical training and equipment. Moreover, the fibrosis developed with this model persists for weeks after TAA withdrawal and is more similar in nature to human fibrosis compared to other toxicants such as CCl_4, presenting more prominent regenerative nodules and a periportal and lobular distribution.[2] Also, unlike CCl_4, where genetic background plays a key role in toxicant-susceptibility, no strain-related variability has been reported for TAA. However, a certain inter-individual variability in the degree of fibrosis reached is to be expected.

Common bile duct ligation

Common bile duct ligation (cBDL) is a model of secondary biliary cirrhosis that can be performed both in rats and mice. The surgical obstruction of the common bile duct causes bile to accumulate in the liver, leading to hepatic injury, inflammation and, ultimately, fibrosis and cirrhosis.[93,94]

Briefly, the animal is anesthetised and placed in a disinfected surgical area. After an abdominal incision along the *linea alba*, the common bile duct is isolated. A double ligature is performed, with the distal ligature being at the cross section of the duodenum and the common bile duct, and the proximal ligature being as close to the junction of the hepatic ducts as possible. Finally, the bile duct is resected between the 2 ligatures and both abdominal layers, the peritoneum and abdominal skin, are closed separately with simple interrupted sutures. After 3–4 weeks, both mice and rats will exhibit ACLD.[95,96] To increase survival, it is recommended to administer vitamin K once per week until the end of the protocol.[97] Additionally, antibiotics can be administered during the first few days after surgery. However, they may not be necessary if surgery was performed under sterile conditions and they could interfere with some aspects of the disease, such as bacterial translocation.

While it requires a certain degree of surgical expertise, especially when performed in mice, cBDL constitutes a relatively simple and highly

Table 5. Overview of current and future methods in hepatic tissue bioengineering.

Liver model	System	Disease	Culture	Advantages	Weaknesses
Spheroids[182]	*In vitro*	NAFLD/NASH	PHH, IC, NPC	High-throughput drug screening, co-cultures with NPC possible without any specialised system	Cell choice can modify the output, suboptimal cell–cell interaction, access to fresh human tissue, healthy tissue not really extracted from healthy individuals, lack of complete hepatocyte polarity
Organoids[108]	*In vitro*	NAFLD/NASH, HCC/CCA	PSC	A genetically stable 3D model, long-term culture, unlimited source of cells, high-throughput drug screening and personalised medicine (gene therapy)	PSCs express foetal markers, limited cell maturation/cell function, failure to recapitulate multiple cell types of the liver
Liver-on-a-chip[109]	*In vitro*	NAFLD/NASH, ACLD	PHH, IC, NPC	Dynamic fluid flow, commercially available, single and multi-chamber design, sustained functionality for at least 4 weeks, modelling zonal liver phenotypes possible	Low throughput drug screening, access to fresh human tissue, healthy tissue not really extracted from healthy individuals
Precision-cut liver slices[111]	*Ex vivo*	ALD/ASH, NAFLD/NASH, HCC	Whole tissue	Reproducible, low cost, hepatic environment and ECM present, low amount of tissue	Access to fresh tissue, healthy tissue not really extracted from healthy individuals, functions as metabolic capacity only maintained 3 days
Decellularized liver scaffold[113]	*Ex vivo*	Under development	PHH, IC, NPC	Provides a neutral environment that mimics (patho)physiological conditions, low antigenicity, simple and safe protocol	Divergence in decellularisation and recellularisation protocols, reendothelisation is a concern, long-term engraftment

ACLD, advanced chronic liver disease; ALD, alcohol-related liver disease; ASH, alcohol-related steatohepatitis; CCA, cholangiocarcinoma; ECM, extracellular matrix; HCC, hepatocellular carcinoma; IC, immortalised cell lines; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NPC, non-parenchymal cells; PHH, primary human hepatocytes; PSCs, pluripotent stem cells.

reproducible model for the study of cholestatic disease. However, it should be noted that an exaggerated dilatation of the remnant extrahepatic bile duct could lead to extrinsic compression of portal vascular structures. Mortality in this model is moderate and mainly due to the occasional perforation of a biliary cyst, or the gallbladder in mice, and subsequent choleperitoneum. This can be avoided by performing a concomitant cholecystectomy in mice. On a similar note, the risk of biliomas can be significantly decreased by ligating all extrahepatic bile duct branches. Finally, another main drawback of this model is that it cannot be used for the evaluation of drugs that are eliminated through the biliary route.

Modelling primary liver cancer: hepatocellular carcinoma and cholangiocarcinoma

ACLD is a common risk factor for the development of liver cancer. In the last few years a number of *in vivo* models of HCC and cholangiocarcinoma (CCA) — the main primary liver cancers — have been developed. The ideal animal model of HCC/CCA must be immunocompetent and/or developed in a host with a species-matched microenvironment, with a full immune response including all immune cell subsets and chemokine signalling. Moreover, tumours must develop relatively quickly and recapitulate the genetic (*e.g.* driver mutations), anatomic, and phenotypic features of human HCC/CCA.

A variety of strategies have been used to generate primary liver cancer in rodents. These include genetic modifications such as expression of oncogenes (*e.g.* MYC, Kras/PTEN),[31,98–102] disruption of single or multiple tumour suppressor genes (*e.g.* NEMO, TAK),[103,104] or stem cell transduction.[105] Comorbid liver disease can be modelled by the injection of chemotoxic agents.[98–102] Xenograft models are based on the implantation of tumour cells in hosts that lack a fully functional immune system.[100,101,106] The future success of these models will depend on the establishment of immunocompetent humanised mice (*e.g.* pre-transplantation with human hematopoietic cells).[107]

Future perspectives

Although animal models are still the best approach to comprehensively study the pathophysiology of liver diseases and to develop new drugs, sometimes translating the findings from animals to humans can be challenging. Thus, complementary advanced *in vitro* 3D human cellular culture systems have been developed to enable researchers to study specific cellular functions and regulation, as well as drug efficacy and toxicity, in a liver-centred environment. Spheroids consisting of primary human hepatocytes or immortalised cell lines and pluripotent stem cell-derived organoids have benefited from the understanding of ECM biology. Both bioengineering systems have been applied to the understanding of NAFLD/NASH, whilst the expansion and establishment of organoids as tumouroids for the study of primary liver cancer has great potential, but has not yet been explored for precision oncology.[108] When the goal is to better recapitulate the microphysiological conditions occurring at the hepatic sinusoid, the liver-on-a-chip system adds the biophysical factors, such as shear stress and hydrodynamic pressure that normally affect liver sinusoidal endothelial

cells, and paracrinally affect other cells. These advanced *in vitro* systems may use immortalised liver cells, and importantly primary cells isolated from human livers.[109,110] Additionally, precision-cut tissue slices represent an *ex vivo* tissue culture technique by which different stages of (human) liver AFLD/ASH, NAFLD/NASH and HCC have been successfully investigated.[111,112] Last but not least, the decellularisation and repopulation of human liver represents the present and the future of hepatic tissue bioengineering[113,114] (Table 5).

Abbreviations

ACLD, advanced chronic liver disease; ACLF, acute-on-chronic liver failure; ADH, alcohol dehydrogenase; ADW, alcohol in drinking water; ALD, alcohol-related liver disease; ALDH, acetaldehyde dehydrogenase; APAP, acetaminophen; ASH, alcohol-related steatohepatitis; BAC, blood alcohol concentration; CCA, cholangiocarcinoma; CCl_4, carbon tetrachloride; CCL2, C-C motif chemokine ligand 2; CD, choline deficiency; CDAA, choline-deficient L-amino-defined; CLD, chronic liver disease; CYP2E1, cytochrome P450 isoenzyme 2E1; DAMPs, damage-associated molecular patterns; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DEN, diethylnitrosamine; DID, drinking in the dark; DMN, dimethylnitrosamine; ECM, extracellular matrix; FFAs, free fatty acids; HCC, hepatocellular carcinoma; HFD, high-fat diet; HSCs, hepatic stellate cells; HVR, hepatic vascular resistance; IC, immortalised cell lines; IL-6, interleukin 6; KP, *Klebsiella pneumoniae*; LdC, Lieber-deCarli; LPS, lipopolysaccharide; MCD, methionine- and choline-deficient; NAFLD, non-alcoholic fatty liver disease; NPC, non-parenchymal cells; PHH, primary human hepatocytes; PSC, pluripotent stem cell; ROS, reactive oxygen species; TAA, thioacetamide; TNF, tumour necrosis factor; VLDL, very low-density lipoprotein; WD, western diet.

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Conflict of interests

The authors declare no conflicts of interest that pertain to this work.

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Authors' contributions

All authors: conception of the work, literature search, writing, revision of the manuscript, and figure illustrations.

Supplementary data

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