BBA - Molecular Basis of Disease 1865 (2019) 943–953

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Animal models of NAFLD from a hepatologist's point of view☆

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ARTICLE INFO

Keywords:
Non-alcoholic fatty liver disease
Non-alcoholic steatohepatitis
Animal models
High-fat diet
Fructose
Cholesterol

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disorder closely linked to obesity, hyperlipidemia and type 2 diabetes and is increasingly recognized as a major health problem in many parts of the world. While early stages of NAFLD are characterized by a bland accumulation of fat (steatosis) in hepatocytes, the disease can progress to non-alcoholic steatohepatitis (NASH) which involves chronic liver inflammation, tissue damage and fibrosis and can ultimately lead to end-stage liver disease including cirrhosis and cancer. As no approved pharmacological treatment for NAFLD exists today, there is an urgent need to identify promising pharmacological targets and develop future therapies. For this purpose, basic and translational research in NAFLD animal models is indispensable. While a large number of diverse animal models are currently used in the field, there is an ongoing challenge to identify those models that mirror human pathology the closest to allow good translation of obtained results into further clinical development. This review is meant to provide a concise overview of the most relevant NAFLD animal models currently available and will discuss the strengths and weaknesses of these models with regard to their comparability to human disease conditions.

1. Introduction

1.1. The clinical and socioeconomic problem of NAFLD

The prevalence of obesity has reached alarming proportions in many countries with several hundred million people being affected world-wide. Obesity has become a major public health problem because of its close association with the metabolic syndrome and related organ disease. These disorders include non-alcoholic fatty liver disease (NAFLD), a chronic liver disease principally characterized by fat accumulation in the liver in the absence of excessive alcohol intake. While hepatic fat accumulation per se represents a relatively benign state (termed non-alcoholic fatty liver, NAFL), the disease can progress to non-alcoholic steatohepatitis (NASH) which is characterized by persistent liver inflammation, tissue damage and fibrosis (Fig. 1). Liver fibrosis, in turn, can progress to end-stage liver disease such as cirrhosis and cancer, and is a strong predictor of long-term mortality in NAFLD patients [1,2].

NAFLD has a rising prevalence and is estimated to affect already about one fourth of the global population [3]. In patient cohorts with type 2 diabetes or morbid obesity, the number of individuals with NAFLD can even exceed 70% and 90%, respectively [4]. With regard to its potential progression to end-stage liver disease, it is therefore not surprising that NAFLD is currently among the three most common indications for liver transplantation in the United States and is projected to become an even larger problem in the future [5]. In addition to its liver-related complications, NAFLD is independently associated with an increased risk of cardiovascular disease [6]. Recently, the direct medical costs for NAFLD patients were estimated to be more than 103 billion Dollars per year in the United States and about 35 billion Euros for Germany, France, Italy and the United Kingdom [7].

Despite its high clinical and socioeconomic burden, efficient pharmacological treatment strategies for NAFLD are currently not available. Preclinical animal models are indispensable to identify novel drug targets for the developments of future therapies. In recent years, a large number of studies using a multitude of different NAFLD animal models have been published. While all these models have their specific advantages and disadvantages, it is important to note that the degree of diversity between individual models – in terms of their specific pathophysiology – can be relatively high. This circumstance sometimes complicates the comparability of studies and can hamper transferability of the results to human disease. It is therefore an ongoing challenge in

☆ This article is part of a Special Issue entitled: Animal Models in Liver Disease edited by Peter Fickert and Martin Wagner.

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https://doi.org/10.1016/j.bbadis.2018.06.023
Received 13 April 2018; Received in revised form 22 June 2018; Accepted 25 June 2018
Available online 07 July 2018
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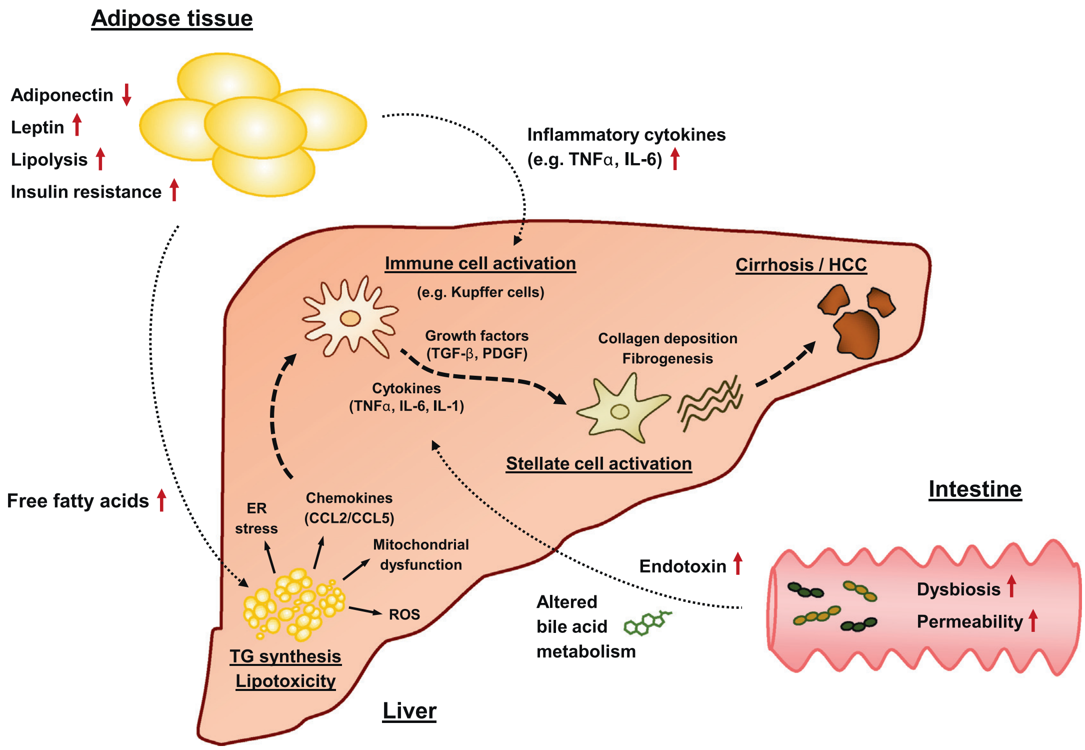

Fig. 1. Pathophysiology of NAFLD.
The pathophysiology of NAFLD is a complex multi-organ process involving metabolic and inflammatory changes in liver, adipose tissue and gut. Increased flux of fatty acids from the insulin resistant adipose tissue or increased production of fatty acids derived from de novo lipogenesis result in increased hepatic triglyceride (TG) synthesis and accumulation (i.e. liver steatosis or NAFL). Persistent liver steatosis can induce metabolic stresses including lipotoxicity, mitochondrial dysfunction and reactive oxygen species (ROS) that trigger the activation of immune cells and pro-inflammatory signaling thus resulting in the progression of NAFL to NASH (i.e. non-alcoholic steatohepatitis). NASH can also be triggered or aggravated by pro-inflammatory signals coming from the inflamed adipose tissue or by bacterial products such as endotoxin coming from the leaky gut. The metabolic and inflammatory changes associated with NASH can lead to hepatocyte apoptosis/necrosis, tissue damage and, subsequently, scar tissue formation (i.e. liver fibrosis) through activated stellate cells. In a subset of affected individuals, NASH can ultimately progress to end-stage disease including liver cirrhosis and hepatocellular carcinoma (HCC). Abbreviations: CCL, C-C motif chemokine ligand; ER, endoplasmic reticulum; IL, interleukin; PDGF, platelet-derived growth factor; TGF, transforming growth factor, TNF, tumor necrosis factor.

the field to identify and develop clinically relevant and reliable NAFLD models that allow the generation of valid, reproducible and transferable results. In this regard – and as already indicated by others [8–10] – preclinical NAFLD research and drug development could strongly benefit from a more specific consensus in the community about the minimal requirements of an appropriate NAFLD animal model. In the following sections, we will discuss – from a hepatologist's point of view – the most relevant features that define a useful NAFLD animal model. These features are summarized in Table 1, which also gives an overview about the way in which the respective parameters should be reported in experimental studies.

1.2. Features of an ideal NAFLD animal model

1.2.1. Liver phenotype

It is obvious that an ideal preclinical model should mimic the pathophysiology of the human disease as closely as possible. If applied to identify and validate potential drug targets, an ideal NAFLD model should moreover display features of the disease that are clinically most relevant (i.e. advanced stages of the disease). While the presence of bland liver steatosis (i.e. NAFL) in humans is most often an asymptomatic state, liver-related outcomes of NAFLD are primarily driven by the inflammatory changes observed in NASH and, particularly, by the presence of liver fibrosis [1]. With regard to the liver phenotype, a clinically relevant mouse model of NAFLD should therefore display the typical histopathological features of NASH (i.e. steatosis, inflammation and ballooning) together with a certain degree of liver fibrosis (Fig. 2).

These features require histopathological examination of the liver tissue by an experienced pathologist and can be staged according to various scoring systems. Among these, the scoring system introduced by Kleiner et al. [11] has been found to be applicable in mouse livers in its original or a slightly modified version [12]. According to Kleiner et al., the presence of steatosis (0–3), inflammation (0–3) and hepatocyte ballooning (0–2) is graded in a semi-quantitative manner. Disease activity is then calculated as the sum of these individual scores and expressed as the NAFLD Activity Score (NAS) (0–8). To prove the existence of bona fide NASH, at least one point is necessary for each individual category. This is supplemented by the histopathological evaluation of liver fibrosis from F0 to F4 with F0 being absence of fibrosis and F4 being liver cirrhosis (Fig. 3).

Until recently, histopathological assessment of NAFLD activity in preclinical animal studies was restricted to one single analysis at the end of the study. This contrasts with most clinical studies where the efficacy of a certain intervention is typically monitored by within-subject comparisons between baseline and end-of-treatment. The advantage of the latter procedure is that it allows for stratification of the experimental groups at baseline and reduces the risk to produce false positive or false negative results due a reduction of heterogeneity within and between the groups. To transfer this methodological advantage from clinical practice into preclinical studies, recent papers have successfully introduced methods to perform liver biopsies in living mice [9,13,14]. The use of these methods in preclinical studies is not yet widely established and seems technically relatively challenging. However, it will be interesting to see whether the use of such methods will improve the general validity of preclinical NAFLD studies and make the results more transferable to human disease in the future.

Table 1
Characteristics of an ideal NAFLD model from a hepatologist's point of view.

Features	Measurement parameters
Metabolic	
Obesity	• Baseline and final body weights • Weight gain during the course **of the** study (measured once weekly)
Hyperglycemia/insulin resistance	• (Fasting) glucose and/or insulin levels at the end of the study • *Optional*: glucose and/or insulin tolerance tests
Liver	
Hepatic fat accumulation	• (Semi-)quantitative histological evaluation using liver tissue sections (e.g. stained with H&E, Oil Red or Sudan IV) • *Optional*: direct measurement of liver triglyceride content (e.g. using Folch extraction)
Characteristics of definite NASH • Liver steatosis • Hepatocyte ballooning • Lobular inflammation	• Quantitative histological evaluation using appropriate scoring systems such as NAS [11] or SAF [91] • *Optional*: measurement of liver function/damage in blood (ALT, AST) • *Optional*: gene expression analysis in liver tissue to study activation of inflammatory signaling pathways (qPCR, Western blot)
Liver fibrosis	• Quantitative histological evaluation in liver tissue sections specifically stained for collagen e.g. with Trichrome (using the criteria of NAS or SAF) • *Optional*: morphometric measurement of fibrosis area in microscopic images of liver tissue sections stained for collagen (e.g. with Sirius Red) • *Optional*: gene expression analysis in liver tissue to study activation of pro-fibrotic signaling pathways (qPCR, Western blot)
Other	
Systemic inflammation	• Measurement of markers of systemic inflammation in serum/plasma (e.g. TNFα, IL-6 by ELISA)
Adipose tissue inflammation	• Number of "crown-like structures" present in stained adipose tissue sections as a histological measure of inflammatory activity [17,18] • *Optional*: gene expression analysis in adipose tissue (qPCR, Western blot)
Dysbiosis/intestinal inflammation/intestinal barrier dysfunction	• Measurement of circulating endotoxin (LAL assay), LPS-binding protein (ELISA) or other markers of intestinal barrier dysfunction [92] • *Optional*: gene expression analysis in intestinal samples (qPCR, Western blot) • *Optional*: analysis of gut microbiota composition (sequencing)

Irrespective of the mode of sample collection (end-of-treatment only vs. biopsy at baseline and end-of-treatment), the most central requirement of a valid NAFLD mouse model is a liver phenotype that shows a time-dependent progression from NAFL to NASH and liver fibrosis, and that these changes are thoroughly validated by histopathological analysis. As recently pointed out by others, a large proportion of studies published in the field unfortunately fail to meet this requirement [10].

1.2.2. *Obesity, insulin resistance and adipose tissue inflammation*

NAFLD is a complex multi-organ disease and its pathophysiology involves metabolic and inflammatory changes not only in the liver, but also in other organs (Fig. 1) [2,15,16]. Besides obesity, insulin resistance and type 2 diabetes are strong risk factors for the development of NAFLD in humans [4]. These metabolic complications involve a chronic low-grade inflammation of the adipose tissue which is mainly driven by the infiltration of certain immune cells including macrophages [17,18]. This results in increased secretion of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) or interleukin-6 (IL-6) from the adipose tissue and an increased delivery of these factors to the liver [19]. Ideally, NAFLD animal models should therefore display obesity, insulin resistance and a certain degree of adipose tissue inflammation.

1.2.3. *Alterations of intestinal physiology*

Besides the adipose tissue, a major extra-hepatic organ contributing to the pathophysiology of NAFLD is the gut [2,15,16]. Changes in the gut microbiome – termed dysbiosis – are frequently observed in obesity and NAFLD. Intestinal dysbiosis is closely linked to chronic alterations of the gut immune system, intestinal inflammation and an impairment of intestinal barrier function [20,21]. Increased intestinal permeability can, in turn, lead to the enhanced translocation of bacterial metabolites – such as lipopolysaccharide (LPS) – into the portal and systemic circulation. Increased LPS levels and subsequent activation of downstream inflammatory signaling pathways have been suggested to contribute to hepatic inflammation and fibrogenesis in NASH [22–24]. An ideal NAFLD model should therefore include features of intestinal inflammation and a certain degree of intestinal barrier dysfunction.

Patients affected by NAFLD show specific changes in the metabolism of bile acids (BA) – soap-like molecules produced from cholesterol in the liver and secreted into the gut lumen to aid the digestion of lipids. NAFLD-associated alterations in BA homeostasis include an increased overall BA pool size and/or alterations of the relative abundance of individual BA species [25–30]. Since gut bacteria extensively metabolize and modify BAs, changes in the intestinal microbiota present in obesity and NAFLD are thought to contribute to the alterations of BA metabolism and their down-stream signaling properties [31]. Future studies should aim to establish a better understanding of this cross-talk between BAs and gut bacteria, and should reveal how it is related to the pathophysiology of NAFLD. As the intestinal microbiome composition significantly differs between humans and mice, models using germ-free mice colonized with a human microbiota (i.e. humanized mice) may be particularly helpful for this kind of studies [32,33].

1.2.4. *Other aspects*

As another desirable feature of an ideal NAFLD model, progressive NAFLD should be observable in a genetic background broadly available and – if required – well suited for genetic loss-of-function studies. Although most knock-out mice are currently maintained on the C57BL/6 background (favoring this mouse strain for loss-of-functions studies), it is important to keep in mind that other inbred strains (such as BALB/c or certain recombinant inbred strains) are actually more prone to the development of liver fibrosis in chronic injury models [34,35] or experimental NASH (see below).

Moreover, the diets (if applicable) used to induce NAFLD should not be too artificial in terms of excessively high amounts of specific nutrients or complete absence of essential factors that are normally present in human diet. Finally, the reproducibility and the inter-individual variability of a specific model as well as the temporal resources required to induce advanced disease stages (i.e. NASH/fibrosis) are also important aspects to consider.

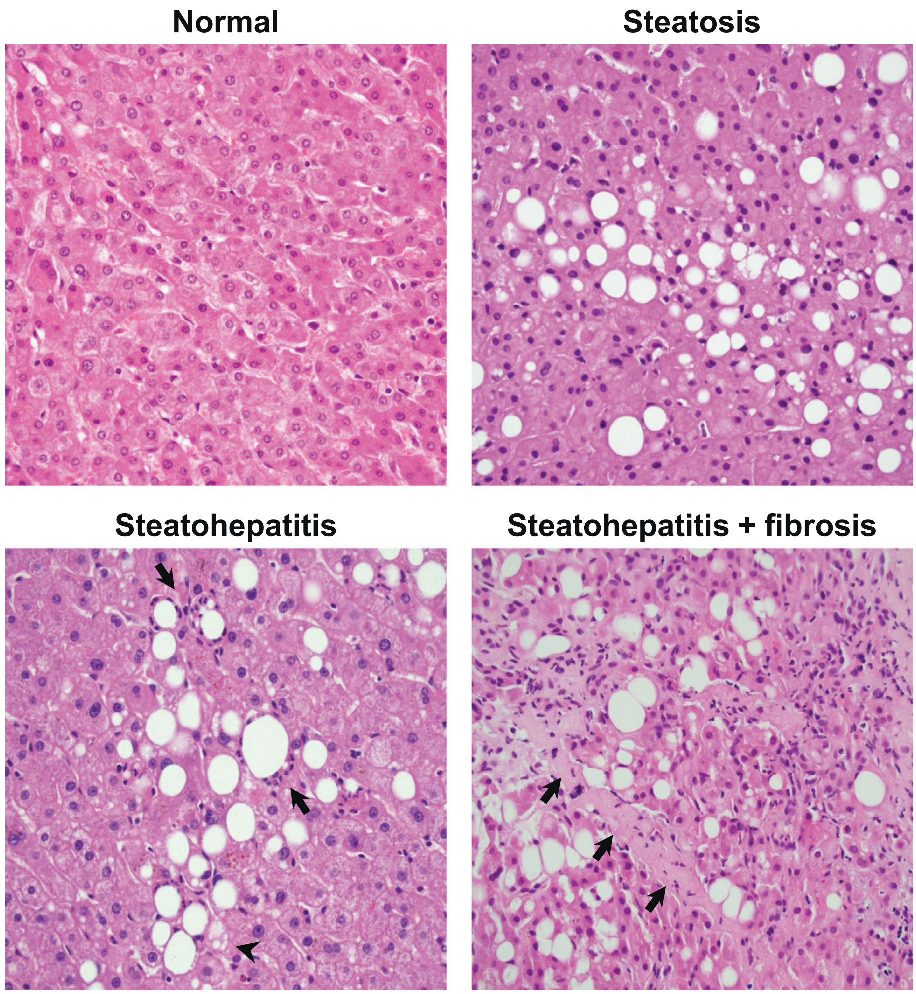

Fig. 2. Histopathology of human NAFLD.
Hematoxylin and eosin (H&E) staining of human liver samples showing normal tissue (upper left panel), macro-vesicular liver steatosis (NAFL; upper right panel), steatohepatitis (NASH; lower left panel) and steatohepatitis with liver fibrosis (lower right panel). Arrows in lower left panel indicate inflammatory infiltration, while arrow head indicates hepatocyte ballooning. Arrows in lower right panel indicate the presence of scar tissue associated with liver fibrosis. All specimens are presented in 400× magnification.

Based on the multitude of these requirements and the fact that many aspects of NAFLD pathophysiology in humans are still incompletely understood, an ideal preclinical model does probably not yet exist. Thus, a very important first step in preclinical NAFLD research is the selection of the appropriate animal model best suited to address the specific research question. Due to the sheer number, it is not possible to present a comprehensive overview of all different NAFLD models currently applied in the field. Therefore, this review is meant to summarize the general properties of the most commonly used NAFLD mouse models including a discussion of their strengths and weaknesses with respect to the pathophysiology of the human disease. Our discussion will be led from a hepatologist's point of view and will therefore have a specific focus on the liver phenotype of the selected models.

2. Genetic models of NAFLD

2.1. Leptin-deficient mice (ob/ob)

The leptin-deficient mouse model (*ob/ob*) is a longtime and frequently used model in general metabolic and NAFLD research (Table 2). Leptin is a satiety hormone predominantly expressed in the adipose tissue. *Ob/ob* mice do not produce functional leptin due to a point mutation in the *ob* gene and, consequently, these mice are hyperphagic and develop obesity, hyperlipidemia and insulin resistance on a normal chow diet [36]. Although this is accompanied by the spontaneous development of bland liver steatosis, *ob/ob* mice do not progress to NASH unless challenged by additional metabolic stimuli such as exposure to small doses of LPS or feeding with specialized diets (i.e. high-fat diets (HFD) or methionine and choline-deficient (MCD) diets; see also below). Moreover, in addition to its role as a satiety hormone, leptin seems to be directly involved in hepatic fibrogenesis [37] and therefore only few studies have so far reported the development of NAFLD-associated liver fibrosis in *ob/ob* mice [14,38]. As an additional drawback of the *ob/ob* mouse model, leptin deficiency is commonly absent in human NAFLD patients who rather have normal or elevated serum leptin levels [39,40].

2.2. Leptin receptor-deficient mice (db/db)

Leptin receptor-deficient mice (*db/db*) are physiologically similar to the *ob/ob* model since they carry a spontaneous mutation in the *db* gene encoding the leptin receptor (according to new nomenclature renamed *lepr*). This leads to defective leptin receptor signaling and renders *db/db* mice resistant to most physiological effects of this hormone. Similar to *ob/ob* animals, *db/db* mice are hyperphagic, obese and insulin resistant, and spontaneously develop liver steatosis under normal dietary conditions [41]. They also require additional stimuli to show disease progression to advanced NASH, however, *db/db* mice seem to be more

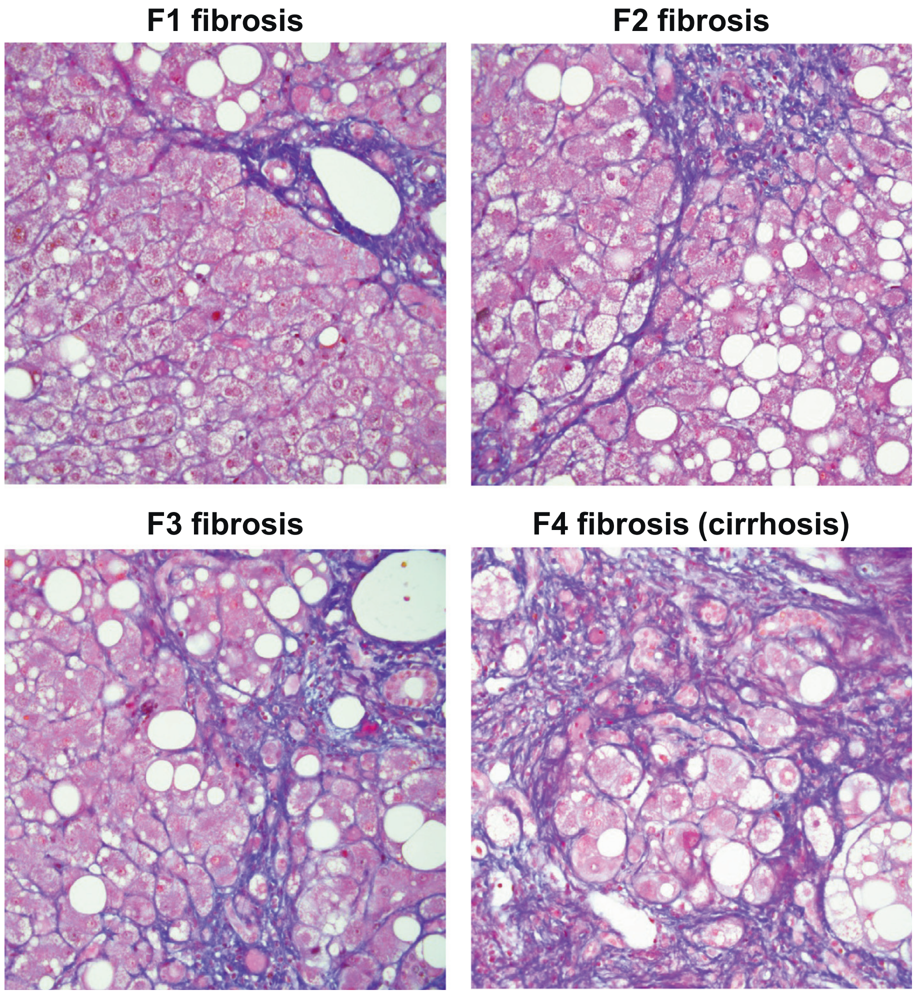

Fig. 3. Stages of liver fibrosis in NASH.
Ladewig trichrome staining of human liver samples showing F1 fibrosis (i.e. periportal fibrosis; upper left panel), F2 fibrosis (i.e. periportal/perisinusiodal fibrosis; upper right panel), F3 fibrosis (i.e. bridging fibrosis; lower left panel) and F4 fibrosis (i.e. cirrhosis; lower right panel). All specimens were scored according to Kleiner et al. [11] and are depicted in 400× magnification.

susceptible to liver fibrosis than their *ob/ob* counterparts [42].

2.3. Low-density lipoprotein receptor-deficient mice (Ldlr-/-) and apolipoprotein E-deficient mice (Apoe-/-)

The low-density lipoprotein receptor (LDLR) is a cell surface receptor expressed on multiple tissues and cell types that mediates the endocytosis of cholesterol-rich LDL particles. This pathway is defective in LDLR-deficient mice (*Ldlr-/-*) leading to an impairment of cholesterol clearance from the circulation and, consequently, severe hypercholesterolemia. These mice have been widely used as an atherosclerosis model in the past. However, more recent studies suggested that *Ldlr-/-* mice are also a useful model to study NAFLD including liver inflammation and fibrosis when fed with appropriate diets (i.e. high-fat/high-cholesterol diets; see also below) [43].

Apolipoprotein E (ApoE) is a key component of lipoprotein particles transporting high amounts of lipids and cholesterol. Like loss of LDLR, the absence of ApoE in *Apoe-/-* mice results in impaired clearance of lipoproteins from the blood and, consequently, leads to severe hyperlipidemia and hypercholesterolemia. Next to the development of atherosclerosis, feeding of *Apoe-/-* mice with a high-fat/high-cholesterol diet for seven weeks resulted in hepatic steatosis associated with increased markers of liver inflammation and histologically visible collagen deposition [44]. Hypercholesterolemia is an important risk factor for the development of NAFLD in humans and cholesterol is increasingly recognized as a direct pathophysiological mediator for the inflammatory changes observed in NASH [45]. Thus, *Ldlr-/-* and *Apoe-/-* mice can be regarded as a physiologically relevant model to study the direct contribution of cholesterol to NASH development.

2.4. Other genetic NAFLD models

In addition to the models outlined above, a number of other genetic NAFLD mouse models exist. For more detailed information on these models – which also include genetic models for NASH-related HCC – the reader is referred to other recent reviews [46,47].

3. Nutrient-deficient NAFLD models

3.1. Methionine and choline-deficient (MCD) diets

Several currently available animal models are based on diets deficient for certain essential nutrients to induce a liver phenotype resembling human NAFLD. Among these, the methionine and choline-deficient (MCD) diet has been widely used in the past as a useful model to study the onset and progression of NAFLD in genetically unaltered animals. As a major advantage, MCD diet rapidly induces liver steatosis and inflammation which is followed by significant tissue injury and

Table 2
Genetic animal models of NAFLD.

Model	Description	Metabolic and liver phenotypes					Duration of feeding	Limitations
		Obesity	Hyperglycemia/insulin resistance	Steatosis	NASH	Fibrosis		
ob/ob	Spontaneous mutation in the leptin-coding *ob* gene	Yes	Yes	Yes	Yes (in combination with specialized diets [14,38])		Variable	Development of NASH and liver fibrosis require additional metabolic insults (specialized diets) Leptin-deficiency not commonly observed in human NAFLD patients
db/db	Spontaneous mutation in the leptin receptor-coding *db* gene	Yes	Yes	Yes	Yes (in combination with specialized diets e.g. [42])		Variable	Development of NASH and liver fibrosis require additional metabolic insults (specialized diets) Leptin receptor-deficiency not commonly observed in human NAFLD patients
Ldlr-/-	Targeted mutation of the *Ldlr* gene encoding the low-density lipoprotein receptor; usually used in combination with high-fat/high-cholesterol feeding	Mild (less pronounced than in BL6 WT [93])	Less pronounced than in BL6 WT [93]	Yes	Yes	Yes	Usually 3–5 months [43,94,95]	Obesity and hyperglycemia/insulin resistance less pronounced than in WT mice
Apoe-/-	Targeted mutation of the *Apoe* gene encoding apolipoprotein E; usually used in combination with high-fat/high-cholesterol diets	Mild (less pronounced than in BL6 WT and *Ldlr-/-* [93])	Mixed results [44,93]	Yes	Yes	Yes	7 weeks [44]	Obesity and (potentially) hyperglycemia/insulin resistance less pronounced than in WT mice

fibrosis within only a few weeks of feeding (typically after two to eight weeks).

Although the exact pathophysiology of this model is not fully understood, methionine and choline are important methyl group donors. Sufficient methylation capacity in hepatocytes is crucial for the maintenance of many physiological functions including lipid metabolism [48]. Based on this, the rapid onset of NASH in MCD-fed mice seems to be partly due to an impairment of hepatocyte lipid excretion via very low-density lipoprotein (VLDL) particles and a concomitant increase in oxidative stress [49–52]. With regard to the metabolic phenotype apart from the liver, MCD diet feeding in mice results in pronounced weight loss, lowered blood glucose levels and increased insulin sensitivity [53,54]. As these metabolic changes are opposite to those observed in the vast majority of human NAFLD patients, this represents a major drawback of the MCD model.

3.2. Choline-deficient (CD) diets

In contrast to MCD diets, choline-deficient (CD) diets have normal or only moderately lowered levels of methionine preventing a pronounced weight loss. From a clinical perspective, recent studies suggest that low choline intake in humans can promote the development and progression of NAFLD [55–58]. Similar to methionine and choline, folate is a third important methyl group donor in hepatocyte metabolism [59] and certain clinical studies found an association of folate deficiency with the presence and severity of NAFLD [60–62]. Moreover, it was recently suggested that rapid changes in intestinal folate metabolism could contribute to the beneficial effects of an intervention with low-carbohydrate diet on liver steatosis in NAFLD patients [63]. From this point of view, NAFLD animal models based on choline deficiency (and potentially also folate deficiency [64]) seem to be attractive physiological models for hepatic methyl donor deficiency that are clinically relevant to the human disease.

CD diets supplemented with varying levels of methionine, L-amino acids and/or increased levels of fat (i.e. choline-deficient high-fat diets) have been published which induce experimental NAFLD in rodents. This has, for example, led to the development of models showing relatively rapid onset of NASH with increased serum transaminases and significant liver fibrosis (within six to twelve weeks of feeding) [65,66] as well as the onset of NASH-driven hepatocellular carcinoma within a reasonable time frame (twelve months) [67]. As a general experimental advantage of CD over MCD diets, the sufficient supply with methionine in these diets limits the marked weight loss typically observed with MCD feeding. However, with few exceptions [67] most CD models lack the development of significant obesity and do not show alterations of glucose tolerance. Moreover, CD diets do usually not promote insulin resistance and therefore (like the MCD diets) have only limited use for the validation of drug targets with a known or anticipated involvement in insulin function [9].

4. NAFLD models based on obesogenic high-fat diets

In recent years, an increasing number of studies made use of obesogenic diets to validate potential drug targets for the treatment of NAFLD in the absence of artificial nutrient deficiencies. These models are typically based on diets particularly rich in fat, but they additionally contain – to variable levels – increased amounts of sugar and/or cholesterol. Collectively, these diets are therefore referred to as “high-fat diets” (HFD). Compared to other models outlined above, HFD-based models are relatively time consuming and often require slightly larger sample sizes due to a higher inter-individual variability. However, the use of these obesogenic diets led to preclinical models mimicking the full spectrum of the metabolic and histological features of human NAFLD including (but not limited to) obesity, insulin resistance and, most importantly, liver inflammation and fibrosis. In addition, the use of wild-type animals with broadly available genetic background facilitates the validation of potential drug targets by genetic loss-of-function experiments in these models. In some HFD-based models, even the spontaneous emergence of NASH-driven hepatocellular carcinoma (HCC) has been reported [68,69] – a characteristic previously restricted to genetic models or the application of artificial chemical carcinogens.

4.1. General aspects of HFDs

HFDs are quite different with regard to their macronutrient compositions and, unfortunately, no widely accepted standard for NAFLD research exists to date. This high degree of variability most likely originates from the use of variable amounts of sugar and fat (typically 40–60% of the calories are derived from fat) and different sources of fat (e.g. use of vegetable oils derived from coconut or soybean vs. animal fats derived from lard or milk). In addition to this, HFDs can significantly vary with regard to cholesterol contents (typically 0–2%) and the type of sugar that is added to the diet and/or the drinking water (e.g. sucrose or fructose).

A subset of "traditional" HFDs with fixed compositions (such as D12451, D12492, TD88137 or Surwit diet) has been used for more than 20 years in metabolic research. These diets generally contain only moderate levels of cholesterol (0–0.2%) and sucrose as the major source of sugar. Feeding these diets leads to the rapid onset of obesity-associated glucose intolerance/insulin resistance and pronounced liver steatosis. As a major drawback, however, most of these traditional HFDs do not induce marked liver inflammation and fibrosis unless fed for very long time periods (> one year) and are therefore suboptimal models to investigate advanced stages of NASH [8,9,70]. To overcome this limitation, several attempts have been made to use modified HFDs which increased levels of cholesterol and/or fructose.

4.2. The role of cholesterol in HFD-based NAFLD models

Hypercholesterolemia is a known risk factor for the development of NAFLD and cholesterol has been suggested to directly contribute to the pathophysiology of NASH [45]. In line with this concept, the liver phenotype of HFD-based mouse models can be aggravated by increasing amounts of dietary cholesterol. Among others, this is exemplified by a study in wild-type C57BL/6 mice demonstrating that a 16-week feeding regimen with a HFD containing 0.2% cholesterol led to more pronounced liver damage and fibrosis than the same HFD without cholesterol [71]. This finding was recently extended by Henkel et al. demonstrating that the addition of 0.75% of cholesterol to a plant oil-based HFD dramatically increased NASH activity and liver fibrosis on the histopathological level and, moreover, led to a marked induction of pro-inflammatory and pro-fibrotic pathways after 20 weeks of feeding [72].

4.3. The role of fructose in HFD-based NAFLD models

Next to cholesterol, increased dietary intake of fructose is an important risk factor for the development of NAFLD in humans and correlates with increased disease severity including the degree of liver fibrosis [73–75]. Mechanistically, increased intake of fructose is thought to enhance the development and progression of fatty liver disease via metabolic effects in liver and gut [76]. In the liver, fructose is rapidly metabolized to fructose-1-phosphate by the enzymatic activity of fructokinase C. In contrast to glucose metabolism by hexokinase or glucokinase, this reaction is not subject to negative feedback inhibition. Therefore, high fructose metabolism by fructokinase C can lead to intracellular ATP depletion and, subsequently, to enhanced nucleotide turnover resulting in uric acid generation. Uric acid, in turn, is thought to have several unfavorable metabolic effects in the liver including increased triglyceride accumulation and inflammasome activation [77]. In line with this concept, metabolic studies in humans demonstrated that increased fructose consumption leads to activation of hepatic de novo lipogenesis and suggested that this effect is specific for fructose and is not seen for glucose [78,79]. In addition to these direct effects on hepatic de novo lipogenesis, an increasing number of studies suggest a crucial contribution of fructose to intestinal dysbiosis, impaired intestinal barrier function and increased translocation of bacterial metabolites (such as LPS) in NAFLD [80,81].

The profound impact of fructose on liver and gut homeostasis is now widely used to induce NAFLD in animal models and to accelerate disease progression to NASH and liver fibrosis in experimental settings. Among other studies, such a model has been introduced by Kohli et al. using a medium chain fatty acid-based HFD in combination with ad libitum administration of drinking water enriched with fructose and sucrose. Feeding of this diet for 16 weeks resulted in obesity associated with insulin resistance and histopathologically detectable liver inflammation and fibrosis. In contrast, animals fed with the same HFD but receiving normal drinking water (without fructose and sucrose) developed less severe inflammation and no detectable liver fibrosis [82]. In practice, an increasing number of studies are presently using HFD enriched with both, high levels of fructose and cholesterol, to induce NASH and significant liver fibrosis within reasonable time frames (usually around four to six months). A part of these studies is summarized in Table 3.

4.4. The role of the genetic background in HFD-based NAFLD models

Besides macronutrient composition of the diet and the duration of feeding, the genetic background of the animals is a further variable that determines disease severity in preclinical NAFLD models. This is exemplified in a recent study by Asgharpour et al. using a novel isogenic mouse strain derived from the C57BL/6J and 129S1/SvImJ backgrounds [69]. The researchers fed mice from this strain – which has approximately 60% of its genome originating from C57BL/6J and 40% from 129S1/SvImJ – with a HFD containing 0.1% cholesterol in combination with fructose/sucrose-enriched drinking water. This resulted in obesity, insulin resistance and a time-dependent progression of NAFLD from liver steatosis after eight weeks to NASH with mild fibrosis (mostly stage 1) after 16–24 weeks and severe bridging fibrosis after 52 weeks. Progression of NASH in this model was also accompanied by the formation of liver tumors from week 32 onwards. Importantly, NAFLD activity and liver fibrosis in the isogenic B6/129 mice were more pronounced than in either of the two parental strains and C57BL/6J mice did not develop liver tumors. As a potential limitation of this model, the isogenic B6/129 background is not readily available for studies in knock-out or transgenic animals since these are typically maintained on more commonly available backgrounds such as C57BL/6.

4.5. NASH-HCC models based on the combination of HFD feeding and the application of toxins

As outlined above, some of the existing HFD-based NAFLD models can result in the spontaneous emergence of NASH-driven HCC after a sufficiently long period of feeding (i.e. approximately twelve months) [67–69]. Since temporal resources are often limited in practice, it is interesting to note that the combined use of HFD feeding with the application of certain toxins/carcinogens can significantly accelerate the development of experimental liver cancer [83].

In this regard, one of most widely used NASH-HCC models is typically referred to as "STAM mice". In this model, neonatal animals are exposed to one single low-dose treatment with streptozotocin (STZ) two days after birth [84]. STZ is an anti-neoplastic agent particularly toxic to insulin-producing β-cells in pancreatic islets. Treatment with STZ therefore leads to islet injury and islet inflammation resulting in partially impaired insulin secretion and a mild diabetic condition. This first metabolic insult is followed by the induction of HFD feeding at week four after birth. The main advantage of this model is its rapid

Table 3
Animal models of NAFLD based on high-fat diets.

Model	Description	Metabolic and liver phenotypes						Duration of feeding	Limitations
		Obesity	Hyperglycemia/ insulin resistance	Steatosis	NASH	Fibrosis	HCC[a]		
CD-HFD	Choline-deficient high-fat diet (CD-HFD)	Yes	Yes	Yes	Yes	Yes	Yes (25%)	NASH: 6 months Fibrosis/HCC: 12 months [67]	Incidence of liver tumors relatively low Progression to fibrosis requires relatively long feeding period
HFHC	"High-fat, high-carbohydrate" (HFHC) High-fat/high-sucrose diet (coconut oil) +Fructose/sucrose in drinking water	Yes	Yes	Yes	Yes	Yes	n.d.	16 weeks [82]	Liver tumors not reported in this model
ALIOS	"American Lifestyle-Induced Obesity Syndrome" (ALIOS) High-fat/high-sucrose diet (vegetable oil) +Fructose/sucrose in drinking water	Yes	Yes	Yes	Yes	Yes	Yes (40%)	NASH (no fibrosis): 16 weeks [96] Fibrosis/HCC: 12 months [b] [68]	Incidence of liver tumors relatively low Progression to fibrosis requires relatively long feeding period Contribution of the genetic background not entirely clear since [68] was performed in C57BL/6 × 129 mice
AMLN	"Amylin liver NASH model" (AMLN) High-fat/high fructose diet (vegetable oil) 22% Fructose (in diet) 2% Cholesterol	Yes	Yes	Yes	Yes	Yes	n.d.	30 weeks [13]	Liver tumors not reported in this model
"Fast Food Diet"	High-fat/high-sucrose diet (milk fat) 0.2% Cholesterol +High-fructose corn syrup in drinking water	Yes	Yes	Yes	Yes	Yes	n.d.	6 months [97,98]	Liver tumors not reported in this model
DIAMOND	"Diet-induced animal model of non-alcoholic fatty liver disease" (DIAMOND)[b] High-fat/high-sucrose diet (milk fat) 0.1% Cholesterol +Fructose/sucrose in drinking water	Yes	Yes	Yes	Yes	Yes	Yes (89%)	NASH/mild fibrosis: 4–6 months Fibrosis/HCC: 12 months [69]	Requirement of unique genetic background (isogenic C57BL/6J x 129S1/SvImJ)
STAM	Perinatal injection with streptozotocin leading to β-cell injury followed by high-fat diet feeding	No	Yes	Yes	Yes	Yes	Yes (100% in males)	NASH/fibrosis: 4 weeks HCC: 12–16 weeks [84]	Lack of obesity Diabetes-like phenotype artificially induced by streptozotocin

[a] Spontaneous development of hepatocellular carcinoma (HCC; without chemical carcinogens); numbers in brackets indicate proportion of animals with tumors.
[b] Study was performed in a genetic background other than C57BL/6.

progression of NAFLD starting with the emergence of bland liver steatosis at week six (i.e. after two weeks of HFD). Afterwards, the histopathological changes of NASH (inflammation, ballooning) and liver fibrosis are observable from week eight (i.e. after four weeks of HFD) onwards. Increased hepatocyte proliferation indicative of tumor initiation is detectable at week 16 and, finally, liver tumors are macroscopically visible with a very high incidence (100% in males) at week 20. In contrast to other NASH-HCC models which report similar incidences for both sexes [67], liver tumors develop only in male STAM mice [84]. As a potential drawback of STAM mice, this model is based on the artificial application of STZ. Although perinatal STZ treatment at low doses in rodents induces a phenotype resembling type 2 diabetes with hyperglycemia and insulin resistance in adulthood [85–87], STAM mice do not show overt obesity but have rather a slightly reduced body weight compared to age-matched controls [84]. This contrast the situation typically found in patients with NAFLD where insulin resistance normally develops in the context of overweight and obesity.

4.6. Non-murine NAFLD models

Next to the mouse, other model organisms exist and are well-suited to study the pathophysiology of NAFLD and validate potential treatment options. Similar to mice, certain other species including rats, rabbits and pigs (such as the Ossabaw miniature swine) develop a liver phenotype resembling human NASH (including liver fibrosis) when challenged with appropriate diets containing increased amounts of fat and/or cholesterol and/or sugar [88–90]. One advantage of rabbits and pigs is their longer pre-pubertal stage which allows mimicking the physiological situation of pediatric NAFLD more precisely than it would be possible with mice or rats. As another specific advantage of the pig model, the general anatomy and certain aspects of lipid/cholesterol metabolism of this species are more similar to the human condition as compared to rodent models. As the major drawbacks of these non-murine species, genetic approaches are less well established and the housing of larger animals is generally more challenging with regard to costs and logistics.

5. Conclusion

The prevalence of NAFLD has reached alarming proportions in many parts of the world and the projected socioeconomic impact of the disease within the next decades will be significant. Since no approved pharmacological treatment for NAFLD is presently available, there is an urgent need to identify and validate promising drug targets in preclinical studies. These preclinical studies require the use of animal models that mimic the pathophysiology of the human disease as closely as possible. In addition to this important and most central requirement, certain other aspects such as the use of broadly available genetic backgrounds and reasonable timeframes are relevant features of a useful NAFLD model.

Despite considerable advances in recent years, the ideal animal model comprising all desirable features and suited for all possible types of studies does not yet exist. For the research community, this circumstance results in the necessity to identify the minimal requirements of valid NAFLD models and to define common guidelines for their use to address specific research questions. This will be imperative to allow the generation of reliable results, make valid scientific conclusions and foster comparability of studies performed in different laboratories and countries in the future. From the general clinical perspective, a useful animal model needs to mimic the most important metabolic changes observed in human NAFLD patients – namely obesity, insulin resistance/hyperglycemia and hyperlipidemia. As another most central feature – particularly from the hepatologist's point of view – a relevant NAFLD animal model should show progression to advanced disease stages (i.e. NASH and liver fibrosis) and this phenotype needs to be thoroughly monitored by histopathological examination and quantitatively analyzed by appropriate scoring systems.

Funding information

This study was supported by grants of the Interdisciplinary Center for Clinical Research, Würzburg to AG and HMH (A-242 and Z-4/135) and of the Else Kröner-Fresenius-Stiftung to AG (2014_A67).

Potential conflict of interests

DJ received travel grants from Alexion and Falk. HMH consulted for GlaxoSmithKline and received travel grants from Falk. SK declares no potential conflict of interests. AG advises for AbbVie, Alexion, BMS, Gilead, Intercept, Novartis, and Sequana, is on the speakers' bureau for AbbVie, Alexion, BMS, Falk, Gilead, Intercept, Novartis, and Sequana, and received research grants from Intercept and Novartis.

Transparency document

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

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