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Mouse models of nonalcoholic steatohepatitis in preclinical drug development

Henrik H. Hansen, Michael Feigh, Sanne S. Veidal, Kristoffer T. Rigbolt, Niels Vrang and Keld Fosgerau

Gubra Aps, Hørsholm Kongevej 11b, Hørsholm DK-2970, Denmark

Nonalcoholic fatty liver disease (NAFLD) has become the most common cause of chronic liver disease in the Western world. NAFLD is a complex spectrum of liver diseases ranging from benign hepatic steatosis to its more aggressive necroinflammatory manifestation, nonalcoholic steatohepatitis (NASH). NASH pathogenesis is multifactorial and risk factors are almost identical to those of the metabolic syndrome. This has prompted substantial efforts to identify novel drug therapies for correcting underlying metabolic deficits, and to prevent or alleviate hepatic fibrosis in NASH. Available mouse models of NASH address different aspects of the disease, have varying clinical translatability, and, therefore, also show different utility in drug discovery.

Introduction

The prevalence of NAFLD is rapidly increasing worldwide and it is now the most common liver disorder in the Western world [1]. Obesity, type 2 diabetes (T2D), hyperlipidemia, and hypertension are highly prevalent in individuals with NAFLD and, therefore, NAFLD risk factors are almost identical to the constituents of the metabolic syndrome [2,3]. NAFLD is a complex spectrum of liver diseases ranging from benign, usually asymptomatic, steatosis to the more aggressive necroinflammatory form, nonalcoholic steatohepatitis (NASH). NASH is characterized by varying degrees of steatosis, cytoskeletal damage (hepatocellular ballooning), and lobular inflammation with or without fibrosis [4]. Although not all patients with NAFLD develop liver-related complications, patients with NASH are at increased risk of developing hepatic fibrosis, which can progress to cirrhosis, hepatocellular carcinoma (HCC), and end-stage liver disease [5,6]. As a consequence, NASH is currently the second indication for orthotopic liver transplantation, and it is projected that NASH will become the leading indication for liver transplantation within developing countries by 2020 [7]. To date, no evidence-based drug therapy has been approved for NASH management and, because therapeutic advances have been slow, NASH is classified as a medical condition with high unmet therapeutic need.

To facilitate the development of novel diagnostic and therapeutic interventions in NASH, a plethora of animal models have been used to identify molecular targets that are involved in the onset and progression of NASH. In view of recent advances in the understanding of the pathogenesis of NASH and progress in the clinical development of anti-NASH compounds, here we discuss the advantages and limitations of current *in vivo* mouse models of NASH.

NASH pathogenesis

Current NAFLD treatment focuses on reducing metabolic risk factors, with lifestyle intervention being the mainstay therapy; however, this approach is often inefficient because of long periods of dieting and weight cycling [8]. Recently, several breakthroughs have been made in the understanding of NASH pathogenesis, which is now known to be multifactorial, implicating several pathways in disease onset and progression. The pathogenesis of NASH was originally interpreted with a 'dual-hit' hypothesis, where steatosis ('first hit'), resulting from increased lipolysis and lipogenesis (accentuated by insulin resistance), predisposes to the initiation of NASH through downstream ('second hit') proinflammatory mediators [9]. Today, more complex 'multiple-hit'

Corresponding author: Hansen, H.H. (hbh@gubra.dk)

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hypotheses have been proposed with the aim to explain how fatty acids and their metabolites promote NASH through multiple sequential or parallel cytotoxic pathways. In general, most recent hypotheses involve fatty acid-mediated lipotoxicity, which exhausts hepatocyte adaptive and regenerative responses, enabling accumulating oxidative stress to trigger hepatocyte necroinflammation, scar tissue formation (fibrosis), and disruption of hepatic cytoarchitecture, which can ultimately progress to cirrhosis and HCC [10,11]. A recent meta-analysis study of microarray data sets from rodent activated hepatic stellate cells (HSCs, principal collagen-producing cells) underlined the complexity in fibrogenesis signaling pathways and suggested several novel candidate genes potentially serving as biomarkers or therapeutic targets for fibrotic NASH [12]. NASH-specific pathways and druggable targets are also likely to be expanded in detail by 'omics' approaches (gut metagenomics, plasma metabolomics, and liver transcriptomics), which are increasingly applied in NASH research [13–15].

There is evidence for concurrent immune imbalances in NASH. Although the immune signaling pathways involved are incompletely understood, activation of hepatic resident Kupffer cells (specialized macrophages) and neutrophils, in addition to the recruitment of other innate immune cells, is an important effector of parenchymal inflammation in NASH [16]. Recent research on the potential role of the adaptive immune system in NASH has focused on proinflammatory T cells, including T helper (Th)-17 cells, which are the primary producers of the IL-17 family of proinflammatory cytokines [17]. Given that IL-17 receptors are ubiquitously expressed in the liver (including by hepatocytes, Kupffer cells, and HSCs), dysregulated IL-17 secretion could lead to the mobilization of several deleterious cell signaling pathways [18,19]. These cell types also express other receptor families that have been implicated in NASH immunopathology, including Toll-like receptors (TLRs [20]) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs [21]). NLRs have received special attention because they are recognized as inflammasome sensory molecules. Metabolic inflammation triggered by the inflammasome (multiprotein complexes that assemble upon the sensing of danger signals and initiate the release of potent proinflammatory cytokines and chemokines) is suggested to link the metabolic syndrome and NAFLD [22], and could have an important role in the transition to fibrotic NASH [23].

Gut microbial imbalances, bacterial translocation, and maladaptive host responses ('gut dysbiosis') are emerging as important contributing factors in the pathogenesis of obesity-related disorders, including NASH. The gut microbiota also has a critical role in bile acid metabolism, and might thereby indirectly modulate farnesoid X receptor (FXR) function, which is an important therapeutic target for NASH (see below). Gut dysbiosis causes gut dysmotility and inflammation. Importantly, dysbiosis can also lead to increased gut permeability to dietary factors and bacterial immunogens, thereby increasing hepatic exposure to injurious stimuli that promote hepatic inflammation and fibrogenesis. Compositional changes in the gut microbiome, reduced intestinal barrier function, translocated bacterial proinflammatory products, and associated inflammasome activation have been reported in NAFLD, and multiple studies in mouse models of NASH have supported these findings [24]. However, most of the current evidence in this field comes from animal experiments, and further human studies are needed to determine whether gut dysbiosis translate into NASH pathology, and whether gut microbiome alterations precede and precipitate NASH, or simply reflect secondary adaptive responses to the dysmetabolic features of the disease.

Clinical development of anti-NASH drug therapies

The current understanding of NASH pathogenesis has led to broad efforts to target several features of the disease, alone or in combination, even in the absence of liver-guided therapies. Therefore, drug development in NASH is a rapidly changing field. A considerable number of single modality therapies are in various stages of clinical development, and it is expected that combination therapies will also soon be targeted. Most investigational new drugs have a hepatic metabolic target, engineered to reduce hepatic fat accumulation, inflammation, insulin resistance, or mitochondrial dysfunction. In addition, several emerging medical therapies are directly interfering with fibrosis pathways aiming to decrease hepatic fibrosis progression [25]. It is advantageous that drug therapies in NASH also induce weight loss, because successful weight management (≥5–10% weight loss) *per se* improves liver histology in NASH [26]. Current drug targets under clinical investigation are summarized in Fig. 1.

Given that there are no benchmark standard endpoints that can be followed in lieu of histology, liver histology remains the main outcome variable for clinical trials. Liver biopsy is applied to confirm (or exclude) the diagnosis and stage of NASH, which also provides a rational basis for evaluation of treatment efficacy upon completion of the trial [4]. Several histological scoring systems have been developed for monitoring histopathological changes in NASH, including the NAFLD activity score (NAS) and steatosis–activity–fibrosis (SAF) systems [27,28]. The NAS system is widely used in clinical trials and grades the severity of macrovesicular and/or microvesicular steatosis, hepatocellular ballooning, and lobular inflammation on liver biopsies. Fibrosis is not included in NAS because it is a sign of the disease stage rather than of the grade of injury; hence, a separate semiquantitative scoring system is utilized for fibrosis stage monitoring [27]. The disease scoring and staging systems are semiquantitative and only consider changes in hepatic tissue architecture, which could narrow the window of treatment efficacy. Consequently, there is an increasing consensus that quantitative histology is required to fully conclude on treatment outcome [29].

The extent of liver fibrosis, rather than of NASH, is the major driver for cardiovascular co-morbidity, malignancy, and mortality in NASH [30]. Therefore, antifibrotic therapeutics have gained considerable focus in NASH drug discovery. Current antifibrotic strategies include reducing the primary disease, improving hepatocyte integrity, suppressing hepatic inflammation, downregulating HSC activation, or promoting extracellular matrix degradation (reviewed in [23]). Several of these strategies are approached by emerging immunotherapies for NASH and other fibrotic liver diseases [31]. Although there are currently no universal regulatory approval pathways for drug development in NASH, there is an emerging consensus that NASH resolution with halted progression or improvement of liver fibrosis stage are tangible primary endpoints in most clinical trials [32,33]. From a regulatory perspective,

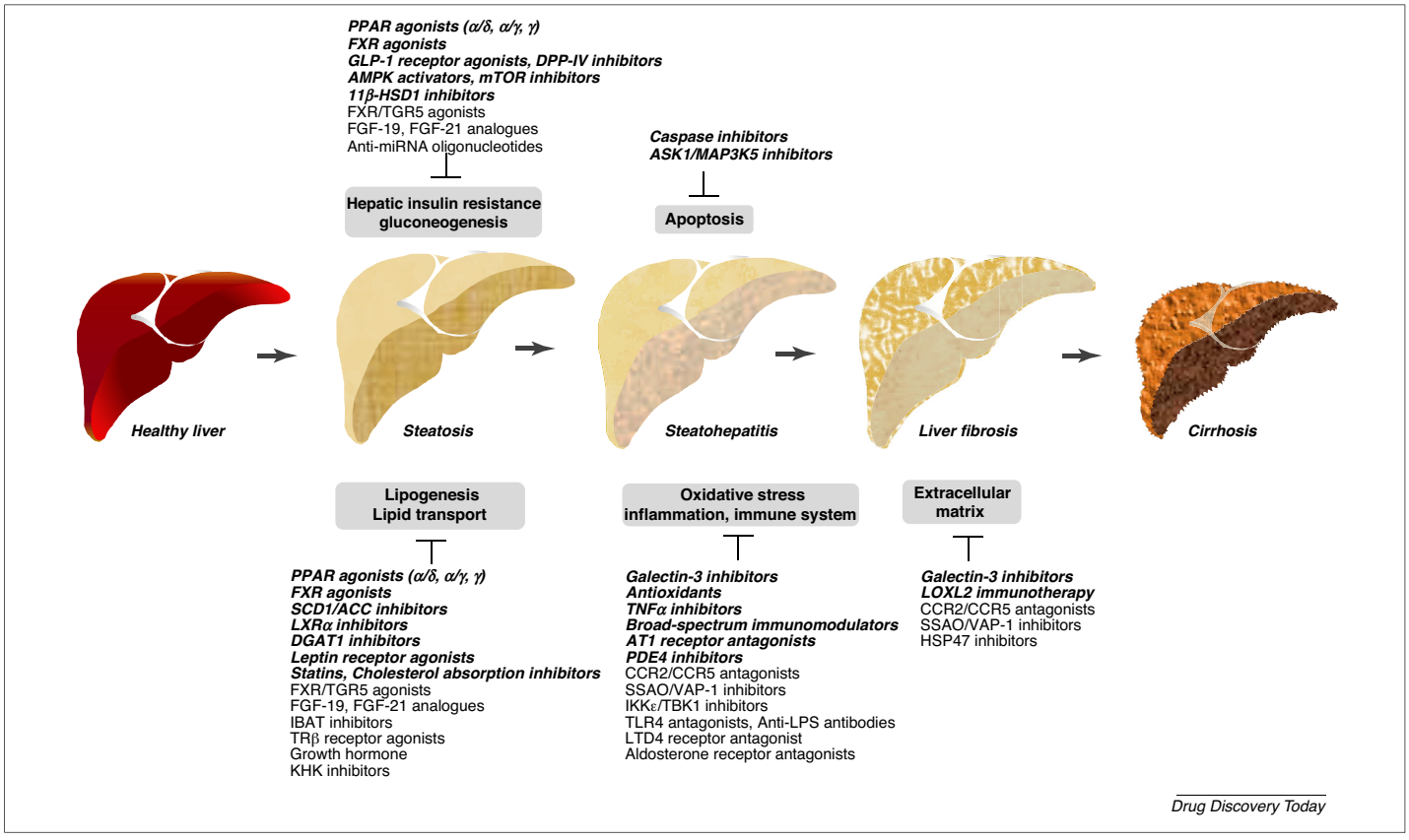

FIGURE 1

Hepatic drug classes in current (recruitment/active phase) or recently concluded clinical trials for NASH (source: ClinicalTrials.gov). Drug classes representing drugs with completed Phase II trials are indicated in bold italics. For abbreviations, see Table 1.

current pivotal clinical trials for precirrhotic NASH will likely need to demonstrate a decreased rate of progression to cirrhosis, which will require long-term extension trials [32].

In summary, an ideal drug candidate for NASH should reduce key clinical endpoints (i.e., steatosis, hepatic inflammation, and liver cell injury) and have antifibrotic effects, while also correcting underlying metabolic derangements, such as hepatic insulin resistance and obesity (Fig. 1). In this regard, most advanced clinical trials in NASH have indicated improved NASH with no worsening (but not reversal) of hepatic fibrosis on liver biopsies, but not all compounds have shown additional beneficial effects on insulin resistance and body weight [34–36].

Animal models of NASH

In drug discovery, an applicable animal model of NASH should enable the assessment of test compound pharmacodynamics with an emphasis on the key metabolic, biochemical, and histological parameters mentioned above. Considering the array of rodent models of NASH reported over the past decade, the models are essentially distinguished by their ability to mimic the etiology and/or natural history (obesogenic dietary models) or histopathology (nutrient-deficient dietary models or chemically induced models). Also, genetic models (monogenetic or polygenetic) are widely used in NASH research. Consequently, available animal models of NASH have different utility and clinical translatability.

The human NAS system (see above) is largely reproducible in NAFLD mouse models [37] and, therefore, has been increasingly applied in the preclinical assessment of liver histological responses to test compounds. In general, the NAS system is well suited for this purpose, although there is not a complete overlap in NASH pathology between humans and rodents. For example, distinct hepatocyte ballooning is often absent or marginal in rodent NAFLD/NASH models [37] and, therefore, composite NAS in experimental NASH models is largely determined by the grade of hepatic steatosis and inflammation. This also indicates that currently available mouse models of NASH are not optimal for evaluating drug effects on hepatocyte degeneration and, thus, measures should also include apoptotic markers.

Murine models constitute the bulk of research in preclinical NASH pathology, and a subset of mouse models exhibits good clinical translatability. Thus, here we discuss selected mouse models to demonstrate the diversity of the attempts to establish *in vivo* models that recapitulate the etiology, natural history, histopathology, and disease progression. We focus on murine NASH models used for test of pharmacological agents, as listed in Tables 2–4.

Obesogenic dietary models

The primary driver of NAFLD is overnutrition and a sedentary lifestyle leading to increased weight and, ultimately, obesity. The strong association between NAFLD and obesity has spurred the development of various diet-induced obesity (DIO) models aimed at mimicking the etiology and natural history of NASH. However, there are significant mouse interstrain differences in the susceptibility to NASH when fed an obesogenic and/or atherogenic diet.

C57BL/6 mice exhibit high sensitivity to obesogenic diets and, therefore, are the most common mouse strain used in experimental NASH (Table 2). For example, C57BL/6 mice are significantly more prone to develop diet-induced hepatic necroinflammation and fibrosis compared with BALB/c and C3H/HeN mice [38,39].

The diet formulas are varied to induce different degrees of adiposity (40–70% fat calories, i.e. high-fat diet) and dyslipidemia (0.1–2.0% cholesterol, i.e. atherogenic diet). A major limitation with this approach is that animals fed these diets, regardless of the dieting periods used (≥20–30 weeks), typically develop dyslipidemia, fatty liver, and mild NASH without appreciable fibrosis. Thus, such dietary models of NASH can only be used for the characterization of potential drug effects on body weight, hepatic steatosis, and (to some degree) inflammatory markers [40,41]. Therefore, different attempts have been made to add dietary factors that would amplify NASH and trigger a robust fibrotic response without significantly compromising the nutrient balance. For example, composite diets are often supplemented with fructose or sucrose ('Western diets') to promote hepatic insulin resistance with more pronounced weight gain and dyslipidemia. Even though these diets elicit more marked steatohepatitis and inflammation, only inconspicuous and mild-stage (perisinusoidal) fibrosis has been reported with these diet modifications [42]. Thus, standard Western diet formulas are suboptimal for preclinical NASH research, and only a few compounds have so far been reported to be profiled in these DIO mouse models of NASH [43–45].

To circumvent this limitation, a different concept has recently been introduced with the use of a dietary lipid composition that more closely reflects a prototypic fast-food diet. Accordingly, an 'American Lifestyle-Induced Obesity Syndrome' (ALIOS) mouse model of NASH was developed by Tetri and colleagues [46], and subsequently refined for NASH research by Amylin Pharmaceuticals and other research laboratories [47–49] (now termed 'Amylin Liver NASH model', abbreviated AMLN). Affected C57BL/6 mice on a AMLN diet ('AMLN mice') develop marked steatosis, moderate lobular inflammation, and mild-stage hepatocellular ballooning within 26–30 weeks of dieting. Notably, the addition of cholesterol (2%) and *trans*-fatty acids (45% of total fat amount) to the diet are critical factors for steatohepatitis to progress to mild–moderate fibrosis in AMLN mice [47–49].

In addition, an inbred isogenic C57BL/6J x 129S1/SvImJ mouse strain (termed DIAMOND) with age-dependent onset of NASH and fibrosis has been developed [50]. DIAMOND mice are kept on a prototypical Western diet (with 0.1% cholesterol), but affected mice nevertheless developed robust NASH and mild fibrosis at week 16–22. Bridging fibrosis (stage 3) was observed in almost all mice at week 52. Moreover, a major proportion of DIAMOND mice also showed HCC development at week 32–52. In comparison, the parent strains fed the same high-fat diet exhibited either similar (C57BL/6J) or slightly reduced NAS (129S1/SvImJ, because of a lower steatosis grade). Fibrosis was also lower (129S1/SvImJ) or almost absent (C57BL/6J), and both parent strains showed no histological evidence of HCC development. Although no pharmacological intervention has so far been reported in DIAMOND mice, this model could be applicable for the determination of drug treatment efficacy in NASH with or without co-morbid hepatocellular malignancy.

As also seen in the clinic, the NASH phenotype varies in rodent models of the disease, (i.e., have unpredictable onset, occurs at varying rates, and shows different severity). Accordingly, available data on Western diet-based NASH models indicate that mouse cohorts represent all stages of NAFLD for any dieting period ≥20 weeks, and a significant proportion of up to 30% of the animals fail to develop steatohepatitis and fibrosis [38,47,51]. This poses a challenge when designing preclinical NASH studies sensitive enough to consistently detect treatment effects. For example, the heterogeneity in the disease stage potentially limits the conclusiveness in pharmacological studies because of unintentional large variability in control group histopathology or responsiveness in treatment groups (e.g., compounds with anticipated fibrosis-preventive effects will only be efficient in nonfibrotic animals). Given the lack of diagnostic circulating biomarkers for NASH staging, such inherent problematics are usually not considered in preclinical studies, and increasing group sizes might not be sufficient to prevent false positive or negative study outcomes. With reference to standard clinical practice, biopsy-confirmation procedures have therefore recently been applied to AMLN mice for staging of baseline liver pathology to equalize NASH severity in the experimental groups [47,48] and allow for within-subject comparisons during the course of drug treatment [43,52,53].

Nutrient-deficient dietary models

To account for the insufficient hepatic fibrosis response to most hypercaloric diets, nutrient-deficient diets have been applied with the aim to provide an additional 'second hit' on hepatic metabolism. Nutrient-deficient diets are either low or devoid of certain essential nutrients, such as methionine (an essential amino acid and important methyl donor) and/or choline [precursor for *de novo* phosphatidylcholine synthesis and hepatocyte export of triglycerides via very-low-density lipoprotein (VLDL) packaging]. In addition, nutrient-deficient diets can be made even less lipotrope by replacing dietary proteins with equivalent amounts of L-amino acids. This approach has resulted in various diet types, such as choline-deficient (CD), methionine-deficient (MD), methionine-choline deficient (MCD), and semisynthetic (choline-deficient, L-amino acid-defined (CDAA); moderately low in methionine)) diets, which are commonly used in preclinical NASH research. The diets can vary in fat content (usually 20% fat kcal), and sucrose levels are typically high (45–60% carbohydrate kcal). The main advantage to using nutrient-deficient diets is the induction of NASH histological features, including mild to moderate fibrosis, within a shorter feeding period than with obesogenic diets.

These nonphysiological dietary manipulations promote NASH with different severity. For example, MD or CD feeding alone results in steatohepatitis, but only the MD diet is able to induce mild hepatocellular injury [54]. The NASH-inducing properties of the CD diet can be enhanced by a higher dietary fat content (60% fat kcal, CDHF model), which was recently reported to promote steatosis, inflammation, and moderate pericellular fibrosis after 8 weeks of dieting [55]. In comparison, MCD mice develop hepatic macrovesicular steatosis and infiltration of inflammatory cells after 1–3 weeks of dieting and robust perisinusoidal fibrosis occurred from week 5–7; thus, these mice have been frequently used to study the short-term effects of pharmacological treatments (Table 2). The degree of hepatic fibrosis in MCD mice appears to

vary across research laboratories, which could be the result of different mouse strains used, diet composition, and housing conditions. The CDAA diet is a variant of the MCD diet, because it contains reduced dietary methionine levels. Mice on CDAA develop macrovesicular steatosis and unspecific lobular inflammation starting from week 3, with an onset of fibrogenesis at week 6–9. This progresses to mild–moderate fibrosis stage around week 21 and HCC develops with a high incidence at week 44 [56].

A disadvantage with the MCD and CDAA diets is the induction of hypophagia and hypercatabolism resulting in significant body-weight loss with a proportional loss of liver mass. By contrast, CD mice display normal body weight [54,55]. The lack of obesogenic effects of the nutrient-deficient diets prevents any insulin-resistant phenotype, which could be a disadvantage if the mode of action of the test compound involves improvement of insulin function. The hypercatabolic state is particular evident for MCD-fed mice, where body-weight loss of 20–40% can occur during the feeding period [57]. In general, the catabolic profile limits the clinical translatability, which should be considered when interpreting data on nutrient-deficient diet models. Attempts have been made to reduce the catabolic impact of the nutrient-deficient diets by introducing less methionine deficiency. Although such diet modifications result in less pronounced weight loss or are weight neutral [56,58], compounds inducing weight loss are generally not feasible to test in nutrient-deficient dietary models of NASH. Hence, the principal hypercatabolic phenotype limits the utility of nutrient-deficient NASH models to only evaluate drugs directly targeting the liver for probing efficacy on hepatic injury and regeneration. Therefore, to consider aspects of the metabolic syndrome, drug effects in MCD and CDAA models should be confirmed in DIO NASH models.

Chemically induced models

Chemically induced parenchymal liver damage and fibrosis is specifically used for studying mechanisms of hepatic fibrosis progression and regression. Fibrosis in these models eventually progresses to liver cirrhosis and HCC with a very high incidence. Typical liver-targeted chemotoxins used are carbon tetrachloride (CCl_4), thioacetamide (TAA), and streptozotocin (STZ). The hepatotoxic mechanisms of CCl_4 and TAA are not fully understood, but involve hepatocyte uptake and conversion of CCl_4 and TAA to reactive metabolites causing oxidative necroinflammation and excessive activation and proliferation of collagen-producing HSCs [59]. By contrast, STZ is particularly toxic to pancreatic β cells, leading to progressive loss of insulin production, but STZ can also have hyperglycemia-independent direct hepatotoxic effects [60]. The fibrosis induction period varies among chemotoxin models but is short (1–8 weeks), depending on the relevant dosing regimen and fibrosis severity in the experimental setting. Whereas CCl_4 and TTA (with or without high-fat dieting) are used in adult mice, STZ is administered to neonatal mice (STAM model) [61]. STAM mice develop manifest NASH at 8 weeks, which progresses to fibrosis at 12 weeks, and eventually develop HCC at a rate of nearly 100% in males [61]. A recent lipidomics study revealed distinct changes in the hepatic lipid profile at different stages of NASH progression in STAM mice [62]. The STAM model has been used to study anti-NASH effects of several compounds (Table 3).

Similar to nutrient-deficient diets, hepatic chemotoxins cause weight loss in mice and, thus, do not mirror the etiology and natural history of NASH. As a result, chemotoxin-induced fibrosis models are mainly used in initial in vivo proof-of-concept studies on antifibrotic therapies (Table 3).

Genetic models

The large variety of mono- and polygenetic mouse models available for NAFLD research has been reviewed elsewhere [63]. Here, we highlight genetic models that most closely replicate the disease spectrum of the metabolic syndrome (Table 4). A major advantage with these genetic models is a generally more severe disease phenotype and development of diet-induced NASH within a shorter timeframe, compared with corresponding wild-type DIO mouse models.

ob/ob mice

Given that leptin deficiency is reported to protect against liver fibrosis, it has been interpreted that hyperphagic and obese *ob/ob* mice (homozygous for a spontaneous Lep^{ob} point mutation in the gene encoding leptin) can be used to study treatment effects on steatosis, but are less applicable for testing antifibrotics [64]. Therefore, secondary hepatotoxic insults, such as MCD diet, CCl_4, or TAA, have been applied with the aim to trigger fibrosis during the progression of steatohepatitis in *ob/ob* mice, but none of these combinations have provided an improved model of NASH. In contrast, *ob/ob* mice are consistently fibrosis prone when cholesterol (2%) and *trans*-fatty acids (45% of total fat amount) are added to the high-caloric diet (i.e., AMLN diet) [43,48,65]. *ob/ob* mice on AMLN diet (termed '*ob/ob* AMLN mice') develop steatohepatitis and fibrosis within a shorter timeframe (≤12 weeks) compared with wild-type C57BL/6 mice (AMLN mice) fed the same diet (≥26 weeks, see also 'Obesogenic diet models' above) [43,48,66]. Liver biopsy-confirmed histology was recently reported applied to *ob/ob* AMLN mice, which unequivocally confirmed marked steatohepatitis and consistent development of liver fibrosis with all severity grades represented in the cohort [48]. Compared with AMLN mice, *ob/ob* AMLN mice display a more severe NASH phenotype, reflected by higher liver triglyceride and cholesterol levels, higher liver hydroxyproline content, increased fibrosis stage, and the presence of bridging fibrosis [48]. The more marked hepatopathology in *ob/ob* AMLN mice makes this model well suited for testing the anti-NASH efficacy of various compound classes [43,53,65,67] (Table 4).

db/db mice

The *db/db* mouse is homozygous for a spontaneous diabetic mutation in the gene encoding the leptin receptor ($Lepr^{db}$). In general, the liver histology is rather similar, but less pronounced compared with that of *ob/ob* mice [68]. Depending on the diet formulation and feeding period, *db/db* mice develop micro- and macrovesicular hepatic steatosis as well as moderate necroinflammation. As for *ob/ob* mice, *db/db* mice maintained on a high-caloric diet do not present consistent histological evidence of fibrosis, and attempts to provide a suitable 'second hit' (e.g., MCD diet or diethylnitrosamine) have resulted in combination models that have been used for the characterization of antifibrotics [69–71]. However, because *db/db* mice do not display the whole spectrum of human NASH histopathology, secondary nonphysiological stimuli are necessary to induce fibrosis. Moreover, *db/db* mice are reported to show

TABLE 1

Drug classes in current (recruitment/active phase) or recently concluded clinical trials for NASH[a]

Drug class	Abbreviation	Compounds	Refs (to clinical data)
Acetyl-CoA carboxylase inhibitors	ACC inhibitors	GS-0976/NDI-010976	[87]
Aldosterone receptor antagonists		MT-3995	
AMP-activated protein kinase activators	AMPK activators	Metformin, NS-0200	[88]
Anti-lipopolysaccharide antibodies	Anti-LPS antibodies	IMM124E	
Anti-microRNA oligonucleotides		RG-125/AZD4076	
Antioxidants		Vitamin E, cysteamine	[89,90]
Apoptosis signal-regulating kinase-1/ mitogen-activated protein kinase-5 inhibitors	ASK1/MAP3K5 inhibitors	Selonsertib/GS-4997	[91]
Angiotensin II receptor type 1 antagonists	AT1 receptor antagonists	Losartan	[92,93]
11-beta-hydroxysteroid dehydrogenase inhibitors	11β-HSD1 inhibitors	RO5093151	[94]
Broad-spectrum immunomodulators		Pentoxifylline	[95]
Caspase inhibitors		Emricasan/IDN-6556, GS-9450/LB84451	[96,97]
Cholesterol absorption inhibitors		Ezetimibe	[98]
CC-chemokine receptor 2/5 inhibitors	CCR2/CCR5 inhibitors	Cenicriviroc	[99,100]
Diacylglycerol acyltransferase-1 inhibitors	DGAT1 inhibitors	Pradigastat/LCQ908	
Dipeptidyl peptidase-IV inhibitors	DPP-IV inhibitors	Sitagliptin, vildagliptin	[101]
Fibroblast growth factor-19 agonists	FGF-19 agonists	NGM282	
Fibroblast growth factor-21 agonists	FGF-21 agonists	BMS-986036, PF-05231023	
Farnesoid X receptor agonists	FXR agonists	Obeticholic acid/INT-747, GS-9674/Px104, LJN452, EDP-305	[34]
Farnesoid X receptor/transmembrane G protein-coupled receptor-5 dual agonists	FXR/TGR5 agonists	INT-767	
Galectin-3 inhibitors		GR-MD-02	[102]
Glucagon-like peptide-1 analogues	GLP-1 analogues	Liraglutide, semaglutide, exenatide	[36,103]
Growth hormone receptor agonists		Growth hormone	
Heat shock protein 47 inhibitors	HSP47 inhibitors	ND-L02-s0201	
Ileal bile acid transporter inhibitors	IBAT inhibitors	Volixibat/SHP626	
IkappaB kinase-epsilon/TANK-binding kinase-1 dual inhibitors	IKKε/TBK1 dual inhibitors	Amlexanox	
Ketohexokinase inhibitors	KHK inhibitors	PF-06835919	
Leptin receptor agonists		Leptin, metreleptin	[104]
Lysyl oxidase like 2 enzyme antibodies	LOXL2 antibodies	Simtuzumab/GS-6624	
Leukotriene D4 receptor antagonists	LTD4 antagonists	Tipelukast/MN-001	
Liver X receptor-α receptor antagonists	LXRα antagonists	Oltipraz	[105]
Mechanistic target of rapamycin protein inhibitors	mTOR inhibitors	MSDC-0602K	
Phosphodiesterase cyclic nucleotide type 4 inhibitors	PDE4 inhibitors	ASP9831, Roflumilast	[106]
Peroxisome proliferator-activator receptor agonists	PPAR agonists	Elafibranor/GFT-505 pioglitazone, rosiglitazone, fenofibrate, saroglitazar/ ZYH1, IVA337	[35,89,93,107]
Stearoyl CoA desaturase-1 inhibitors	SCD1 inhibitors	Aramchol	[108]
Sodium-glucose transporter-2 inhibitors	SGLT-2 inhibitors	Ipragliflozin, dapagliflozin, empagliflozin	[88,109]
Semicarbazide-sensitive amine oxidase/vascular adhesion protein-1 inhibitors	SSAO/VAP-1 inhibitors	PXS4728A	
Statins		Rosuvastatin, atorvastatin, pitavastatin	[110]
Thyroid β receptor agonists	TRb receptor agonists	VK2809, MGL-3196	
Toll-like receptor 4 antagonists	TLR4 antagonists	Nalmafene/JKB-121	
Tumor necrosis factor-alpha inhibitors	TNFα inhibitors	VLX103	

[a] Source: ClinicalTrials.gov; see Fig. 1 in the main text for graphical overview of drug targets.

TABLE 2

Obesogenic and nutrient-deficient dietary models of NASH in C57BL/6 mice[a]

Dietary model	Obesity	Dyslipidemia	Liver enzymes	Hepatomegaly	NASH	Fibrosis	HCC	Compounds tested in model	Induction period (weeks)	Comments on model	Refs
HF-HC	+	+	+	+	+	Very mild	–	Ezetimibe, sildenafil, leucine, metformin	6–42	Few mice show slight fibrosis	[40,41]
HF-FRUC	+	+	+	+	+	Mild–moderate	–	Liraglutide, BAR502	14-–18	Trans-fat in diet	[44,111]
HF-HC-FRUC	+	+	+	+	+	Mild	–	AC3174, elafibranor, obeticholic acid, liraglutide, YH25724, ipragliflozin, APD668	20–30	Biopsy-confirmed NASH and fibrosis; *trans*-fat in diet	[43,52,53, 66,112]
HF-SUCR	+	+	+	?	+	?	–	Fexaramine, G49, atglistatin	10	Combined with partial hepatectomy	[45]
HF-HC-SUCR	+	+	?	?	+	?		Obeticholic acid	24	Very mild NASH	[51]
MCD	–	–	+	+	+	Moderate	–	Wy-14,643, pentoxifylline, G49, YH25724, rosiglitazone, bezafibrate, GW501516, sitagliptin, MCC950, olaparib, WAY-362450	5–8	Weight loss, may be combined with partial hepatectomy	[45,52, 113,128]
CDAA	–	+	+	+	+	Mild–moderate	+	rFGF-1	3–6	Weight loss	[114]

[a] Abbreviations: FRUC, high fructose diet; HC, high-cholesterol diet; HCC, hepatocellular carcinoma; HF, high-fat diet; MCD, methionine- and choline-deficient diet; SUCR, high-sucrose diet; +, induction; –, no induction; ?, not determined/not reported.

TABLE 3

Chemotoxin-induced hepatic fibrosis models in mice[a]

Chemotoxin model	Obesity	Dyslipidemia	Liver enzymes	Hepatomegaly	NASH	Fibrosis	HCC	Compounds tested in model	Induction period (weeks)	Comments on model	Refs
CCl_4	–	+	+	+	+	Marked	+	Sorafenib, BAR502, cilostazol, brivanib, obeticholic acid	0.5–8	Dose-dependent fibrosis; weight loss; HCC after ≥12 weeks	[44,115,129]
TAA	–	?	+	+	+	Marked	+	Sorafenib, brivanib	4–8	Dose-dependent fibrosis; weight loss; HCC after ≥40 weeks	[115,116]
STZ + HF	–	+	+	+	+	Marked	+	Empagliflozin, linagliptin, telmisartan, cenicriviroc, ezetimibe, rosuvastatin, fenofibrate	2–16	Neonatal STZ model; early-onset diabetes; weight loss; HCC after ≥16 weeks on diet	[116–118]

[a] Abbreviations: CCl_4, carbon tetrachloride; HCC, hepatocellular carcinoma; HF, high-fat diet; STZ, streptozotocin; TAA, thioacetamide; +, induction; –, no induction;?, not determined/not reported.

TABLE 4

Monogenetic models of NASH[a]

Genetic model	Diet	Obesity	Dyslipidemia	Liver enzymes	Hepatomegaly	NASH	Fibrosis	HCC	Compounds tested in model	NASH induction period (weeks)	Comments on model	Refs
ob/ob	Chow	+	+	?	+	+	–	–	rFGF1	8–12	Moderate NASH	[114]
	HF-HC-FRUC	+	+	+	+	+	Moderate	–	AC3174, elafibranor, obeticholic acid, INT-767, liraglutide, SR9238	6–12	Biopsy-confirmed NASH and fibrosis; *trans*-fat in diet; fibrosis onset ≤12 weeks	[43,53, 65,67]
	MCD	–	+	+	+	+	Mild	–	LY2405319	10	Weight loss; fibrosis onset ≥8 weeks	[119]
db/db	MCD	–	+	+	+	+	Mild	–	Exendin-4, elafibranor (GFT505)	4–8	Fasting hyperglycemia; fibrosis onset 7–14 weeks	[69,70]
	Chow + DEN	+	+	?	?	+	Marked	+	Pitavastatin, metformin	14–36	Fasting hyperglycemia; fibrosis onset 16–20 weeks	[71]
foz/foz	HF-HC	+	+	+	+	+	Moderate	+	Obeticholic acid, Wy 14,643, ezetimibe, atorvastatin, SR141716A	16–28	Early-onset of fasting hyperglycemia; fibrosis onset ≥16 weeks	[51,74]
ApoE$^{-/-}$	HF-HC	+	+	+	+	+	Moderate	?	Simvastatin	7	Fibrosis onset ≥4–5 months of age	[77,120]
LDLr$^{-/-}$	HF-HC	+	+	+	+	+	Moderate	–	Rosiglitazone	12	Fibrosis onset ≥12 months of age	[78,121]
FLS	Chow	–	+	+	+	+	Mild	(–)	Fenofibrate, ezetimibe	13–20	Inbred strain; normoglycemia; fibrosis onset ≥24 weeks	[76,122, 123]
FLS-ob/ob	Chow	+	+	+	+	+	Moderate	+	Sitagliptin, aliskiren, ambrisentan, irbesartan	20–24	Inbred strain; spontaneous NASH; glucosuria; fibrosis onset ≥24 weeks	[76,1 24–127]

pontaneous reversal of steatohepatitis [68]. Therefore, *db/db* mice might have limited utility in NASH drug discovery.

foz/foz mice

foz/foz ('fat Aussie') mice carry an 11-base pair truncating mutation in the Alström gene product (*Alms1*), and were genetically engineered by researchers at the Australian National University Medical School, Canberra Hospital [72]. The exact function of the ALMS1 protein is unknown, but might include a role in the intracellular transport of lipid cargo. The rare human homolog causes the Alström syndrome, a childhood obesity syndrome complicated by T2D, premature cardiovascular disease, and cirrhosis. Similarly, *foz/foz* mice are hyperphagic and display essential characteristics of the metabolic syndrome, including obesity, fasting hyperglycemia, insulin resistance, dyslipidemia, and hypertension. The attractiveness of using *foz/foz* mice (on a high-fat atherogenic diet) in NASH research is the spontaneous development of significant fatty liver (extreme hepatic triglyceride accumulation), steatohepatitis (severe steatosis, moderate hepatocyte ballooning, and reproducible necroinflammation) with appreciable pericellular fibrosis after 24 weeks of dieting [73,74]. Thus, *foz/foz* and *ob/ob* mice have several NASH phenotypic commonalities, but *foz/foz* mice exhibit a different lipid deposition profile. Therefore, *foz/foz* mice are becoming increasingly relevant in experimental NASH pharmacology research (Table 4).

Fatty liver Shionogi mice

Polygenetic fatty liver Shionogi (FLS) lean mice were originally bred by Shionogi & Co. (Shiga, Japan), and develop spontaneous insulin resistance, hypertriglyceridemia, and steatohepatitis under normal environmental conditions [75]. Hepatic fibrosis is modest in FLS mice [38,76], and only lipid-lowering compounds have so far been tested in this model (Table 4). To provide a more robust fibrotic NASH model, a mixed genetic variant of the *ob/ob* mouse model was recently developed at Tottori University (Yonago, Japan) by backcross mating of *ob/ob* mice with FLS mice. The resulting phenotype of FLS-*ob/ob* mice combines the characteristics of both genetic models, and the mice therefore develop obesity, diabetes, severe hepatic steatosis, necroinflammation, age-dependent progression of pericellular fibrosis, and (to some degree) spontaneous tumorigenesis [76]. FLS-ob/ob mice have been increasingly used in the characterization of potential anti-NASH compounds (Table 4).

Genetic models of impaired lipoprotein function

Assembly, secretion, and transport of VLDL represents a major route for intrahepatic disposition of triglycerides. High serum levels of VLDL and LDL subclasses are linked to hepatic accumulation of cholesterol and lipids, which are considered contributing factors for hepatocellular injury in NASH. Several genetic mouse models of impaired lipoprotein function are applicable for NASH research, including Apolipoprotein E ($ApoE^{-/-}$) and LDL-receptor ($LDL^{-/-}$)-deficient mice. $ApoE^{-/-}$ mice fed a high-fat/cholesterol (1.25%) diet show slightly increased levels of fasting glucose, but the major phenotypic characteristic is marked dyslipidemia, including hypertriglyceridemia, increased serum VLDL levels, and hepatic cholesterol accumulation [77]. In contrast to chow-fed $ApoE^{-/-}$ mice, $ApoE^{-/-}$ mice maintained on the high-fat/cholesterol diet develop marked hepatic steatosis, inflammation, hepatocyte ballooning, HSC activation, and appreciable collagen deposition [77]. $LDL^{-/-}$ mice fed a high-fat/high-carbohydrate/low cholesterol (0.2%) diet develop a NASH phenotypic profile similar to $ApoE^{-/-}$ mice, although at an older age [78]. The major advantage of using these models in NASH research is the more marked dyslipidemic profile, compared with DIO NASH models in wild-type mice. Modifications of these genetic models have been used to accelerate NASH and fibrosis progression, such as by introducing nutrient-deficient diets. On a related note, a transgenic mouse model of human-like lipoprotein metabolism (APOE*3-Leiden.CETP mice) has recently been applied in preclinical NASH research [79,80].

Surgery-based models: bile duct ligation

Bile acids are ligands for the FXR, Takeda G-protein-coupled receptor 5 (TGR5, also termed GPBAR1 and GPCR19), and pregnane X receptors (PXR), which are involved in diverse metabolic functions, including regulation of glucose and lipid homeostasis, and energy expenditure, as well as prevention of intestinal bacterial overgrowth [81]. However, accumulation of bile acids is detrimental to liver function. Hepatic accumulation of bile acids promotes acute oxidative stress, necroinflammation, and apoptosis, leading to fibrosis that eventually progresses to cirrhosis and end-stage liver failure [82]. It was recently reported that patients with NAFLD show alterations in bile acids homeostasis [83], and FXR/TGR5 receptor function has been subject to intense research in NASH pathology and represent an important antifibrotic drug target [82] (Fig. 1, Table 1). Surgical manipulation of bile acid circulation has been introduced as method for fast-onset and robust induction of experimental hepatic fibrosis. For example, common bile duct ligation (BDL) is a model of obstructive cholestasis (extrahepatic biliary obstruction) in which impaired bile flow leads to hepatic accumulation of bile acids and cholestatic liver injury. BDL mice are an emerging tool in preclinical NASH research [84,85]. In addition, a range of nonsurgical models of biliary fibrosis in mice is available, including diet-induced cholestatic liver injury, chemically induced cholangitis, as well as genetic models [86].

Concluding remarks

The ideal model of NASH should faithfully replicate the multifactorial disease mechanisms, while also being reproducible and efficient. Regardless of the approaches currently used to mimic NASH in mice, none of the present models fulfill all requirements for an ideal model. Therefore, selection of the relevant NASH model must be based on prior knowledge of the individual drug target, and it is recommended that at least two individual NASH models should be used for the preclinical characterization of anti-NASH drugs. Given the marked interest in the clinical development of drugs with antifibrotic efficacy, obese NASH mouse models with consistent histology-proven fibrosis have relatively good clinical translatability and, thus, are highly applicable for preclinical drug testing in NASH.

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