Review

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New targets for NAFLD

Lucia Parlati,[1,2] Marion Régnier,[3] Hervé Guillou,[4] Catherine Postic[1,*]

Summary

Non-alcoholic fatty liver disease (NAFLD) is a growing cause of chronic liver disease worldwide. It is characterised by steatosis, liver inflammation, hepatocellular injury and progressive fibrosis. Several preclinical models (dietary and genetic animal models) of NAFLD have deepened our understanding of its aetiology and pathophysiology. Despite the progress made, there are currently no effective treatments for NAFLD. In this review, we will provide an update on the known molecular pathways involved in the pathophysiology of NAFLD and on ongoing studies of new therapeutic targets.

Keywords: NAFLD; NASH; SREBP-1c; ChREBP; FXR; LXR; animal models; PPARα; NEFA; glucotoxicity; lipotoxicity

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Introduction: NAFLD epidemiology and comorbidities

Non-alcoholic fatty liver disease (NAFLD) is a growing cause of chronic liver disease worldwide. It is the most common cause of liver disease and it will be the leading cause of liver transplantation in 2020.[1] The prevalence of NAFLD has increased over time in line with the increase of type 2 diabetes and obesity. Epidemiological studies on NAFLD are often conflicting because of the different diagnostic methods used to define NAFLD (biopsy, ultrasound, elevation of liver function tests, controlled attenuation parameter [CAP], fibroscan or MRI). Indeed, liver biopsy is the gold standard for the diagnosis of NAFLD, but because of its invasiveness, it is not routinely performed. Moreover, the epidemiology of NAFLD varies significantly in different countries. In western countries, NAFLD incidence is about 28/1,000 person/years (PY) in the general population.[2–4] In Asia the incidence varies from 19/1,000 PY to 86/1,000 PY.[5,6] Furthermore, as already mentioned, the incidence of NAFLD varies according to the diagnostic method used, from 26-80/1,000 PY in studies based on elevated liver function tests or ultrasound to 34/1,000 in those using magnetic resonance spectrometry.[7,8] In France, a recently published study from the French Constance Cohort (n = 102,344) reported a prevalence of steatosis of 16.7% (evaluated by Fatty liver index, that is based on BMI, waist circumference, gamma glutamyltransferase [GGT] and triglycerides) and of advanced fibrosis of 2.6% (evaluated by the Forns Index, that is based on platelet count, age, total cholesterol and GGT). In particular, advanced fibrosis was more prevalent among diabetic patients (7.6% *vs.* 2.5% in obese patients).[9]

NAFLD is characterised by excessive fat deposition in hepatocytes in the absence of risky alcohol consumption[10] and by the presence of features of the metabolic syndrome, such as insulin resistance, type 2 diabetes and obesity.[11] NAFLD is a composite disease which groups 2 main entities: simple steatosis and non-alcoholic steatohepatitis (NASH).[12] The common feature of these 2 entities is the accumulation of fat in hepatocytes (>5% of total liver weight) but NASH is characterised by a more severe liver injury including ballooning of hepatocytes, inflammatory cell infiltration and progressive fibrosis. The pathogenesis of metabolic liver disease involves nutritional overload, genetic factors and environmental factors. Steatosis is the result of caloric overload and an accumulation of mostly triglycerides, but also sphingolipids and phospholipids in hepatocytes. Free fatty acids (FFAs) that are esterified to form hepatic triglycerides are derived principally from adipose tissue lipolysis but also from dietary fat and *de novo* lipogenesis: 59% of FFAs come from adipose tissue lipolysis, 26% from *de novo* lipogenesis and 15% from chylomicrons (diet).[13–15] Indeed, it has been demonstrated that in NAFLD there is an increase in hepatic *de novo* lipogenesis[13] and in the secretion of very low-density lipoprotein (VLDL) that could be a compensatory mechanism for the increase in fatty acid influx to the liver.[15,16]

The pathological accumulation of lipids in the liver and adipose tissue results in lipotoxicity and insulin resistance, which leads to the worsening of steatosis (Fig. 1). Alongside its role in lipid storage, adipose tissue acts as an endocrine organ that secrets hormones and cytokines, called adipokines, such as adiponectin and leptin.[17] Adiponectin has several metabolic functions, such as regulation of fatty acid oxidation, inhibition of lipid accumulation in the liver and the adipose tissue[18] and the maintenance of glucose homeostasis, including hepatic insulin sensitivity.[19] Patients with NAFLD have lower serum adiponectin level, which

[1]*Université de Paris, Institut Cochin, CNRS, INSERM, F-75014 Paris, France;* [2]*Hôpital Cochin, 24 rue du Faubourg Saint Jacques, 75014 Paris, France;* [3]*UCLouvain, Université catholique de Louvain, Walloon Excellence in Life Sciences and BIOtechnology, Louvain Drug Research Institute, Metabolism and Nutrition Research Group, Brussels, Belgium;* [4]*Toxalim, Université de Toulouse, INRA, ENVT, INP-Purpan, UPS, Toulouse 31027, France*

* Corresponding author. Address: Institut Cochin - 24 rue du Faubourg Saint-Jacques, France.
E-mail address: catherine.postic@inserm.fr (C. Postic).

contributes to impairment of fatty acid metabolism and promotes a chronic inflammatory state in the liver.[20]

NAFLD is a progressive liver disease; it is estimated that 5% of patients with simple steatosis and 20% of patients with NASH progress to cirrhosis.[12] The process of fibrosis progression is not completely understood and it can vary considerably from one patient to another. Several risk factors for fibrosis progression have been identified: age, hypertension, obesity, type 2 diabetes, low aspartate aminotransferase/alanine aminotransferase ratio, presence of hepatic comorbidities.[21,22] It has been reported that patients with simple steatosis may progress by 1 fibrosis stage over approximately 14 years, while patients with NASH may progress by 1 fibrosis stage over about 7 years. Unfortunately, serum liver function tests are not a reliable method of follow-up since a significant proportion (40-60%) of patients with biopsy-proven NASH can have persistent normal serum transaminases; thus, better biomarkers are needed to improve clinical follow-up of high-risk patients. Among the histological characteristics of NASH, fibrosis is the most directly related to survival. Fibrosis stage is important to monitor the clinical risk of progression to cirrhosis and long-term liver outcomes and mortality.[23] Except for hepatocellular carcinoma (HCC) that can develop in a non-cirrhotic liver, all liver complications of NASH occur in the presence of cirrhosis (portal hypertension, ascites, variceal bleeding, hepato-renal syndrome, hepatic encephalopathy, hepato-pulmonary syndrome).[24] Moreover, patients with NASH are at risk of cardiovascular mortality due to its strict relationship with metabolic risk factors such as arterial hypertension, dyslipidaemia, type 2 diabetes and obesity.[25]

The only therapeutic options currently available are the control of risk factors (diabetes, hypertension and dyslipidaemia) and weight loss. In patients with histologically proven NASH and with pooled liver biopsies before and after weight loss, improvement of all the histological features of NASH (steatosis, inflammation, ballooning and fibrosis) was observed in those that achieved ≥5% weight loss, while the greatest fibrosis resolution occurred in those with ≥10% weight loss.[26] Thus, bariatric surgery effectively improves NASH, by reversing steatosis, reducing hepatocyte apoptosis, and reversing hepatic fibrosis, probably also through the modulation of bile acid signalling.[27,28] Consequently, the mechanisms underlying the ability of bile acids to improve NASH after bariatric surgery are being actively investigated.

It has been demonstrated that treatment with pioglitazone, vitamin E, and obeticholic acid could improve liver necroinflammation in NASH; however, none of these options has received FDA approval for the treatment of NASH.[29,30] Several other pharmacologic agents targeting steatosis, inflammation, or fibrosis pathways are in clinical trials for the treatment of NASH; more than 50 open phase II and III clinical trials are currently registered at www.clinicaltrials.gov.[31]

Preclinical models of NAFLD/NASH

To date, no molecule studied for the treatment of NASH has shown convincing results in terms of improvement or resolution of liver disease. An effective method of improving the probability of therapeutic success is to develop robust preclinical models that are applicable to humans. Obviously, a preclinical model will never be identical to the human pathology; however, it should mimic a diet similar to humans' diet, leading to the onset of obesity and insulin resistance, associated with systemic inflammation and an increase in serum inflammatory cytokines, such as interleukin (IL)-6 and tumour necrosis factor (TNF) α, and a reduction in serum adiponectin. Furthermore, animal models should develop similar histological changes as human NASH: steatosis, lobular inflammation, hepatocellular ballooning and fibrosis. Finally, once extensive fibrosis is established, they should develop HCC. In mouse models, which are widely used for preclinical research, high-fat diet (and high-sucrose diet) feeding which leads to obesity, glucose intolerance and steatosis does not progress to NASH on most genetic backgrounds.[32] Herein, we discuss the advantages and limitations of some of the preclinical models widely used to investigate NAFLD/NASH (Table 1).

Key points

- Non-alcoholic fatty liver disease (NAFLD) is a growing cause of chronic liver disease worldwide; it is the most common cause of liver disease and it will be the leading cause of liver transplantation in 2020.
- The pathogenesis of metabolic liver disease involves nutritional overload, genetic and environmental factors.
- NAFLD represents the hepatic manifestation of a multi-organ disease (the metabolic syndrome) which involves dyslipidaemia, obesity, high blood pressure, impaired sensitivity to carbohydrates and type 2 diabetes.
- Several animal models involving specific diets or genetic manipulations have been used to better understand the aetiopathogenesis of NAFLD.
- The aetiopathogenesis of NAFLD is based on a complex interaction between glucose and lipid metabolism in the liver.
- Currently, no drugs have proven efficacy for the treatment of NAFLD; only weight loss has shown efficacy in improving the histological characteristics of NAFLD.
- The ideal drug candidate for NAFLD should improve steatosis, hepatic inflammation and fibrosis, while ameliorating glucose metabolism, insulin resistance and obesity.

Animal models of diet-induced NAFLD

Methionine and choline-deficient diet

The methionine- and choline-deficient (MCD) diet was, until recently, one of the most commonly used dietary models; it induces severe NASH within 2–4 weeks, with steatosis, inflammation and fibrosis. This diet, which is enriched in sucrose (43 kcal%) is deficient in methionine and choline. Methionine is an essential amino acid that is involved in several processes including: i) methylation of DNA, RNA, proteins and lipids though its conversion to S-adenosylmethionine; ii) regulation of oxidation though its conversion to cysteine, the limiting reagent for glutathione synthesis.[33] Methionine deficiency is therefore associated with a reduction in S-adenosylmethionine and decreased synthesis of folate, tRNA and creatine. Moreover, if associated with a shortage of other lipotropic factors such as vitamin B12 and choline, deficiency in methionine is predictive of liver steatosis.[33] Choline is essential for different cellular processes such as the synthesis of phospholipids required for cell membrane assembly, cholinergic neurotransmission, methyl metabolism, transmembrane signalling and transport and cholesterol metabolism.[34] Choline deficiency is associated with steatosis since it is necessary for making the phosphatidylcholine portion of VLDL particles. In the absence of choline, VLDL particles are not secreted, lipoperoxidation is increased in hepatocytes,[35] which in turn causes a rise in intracellular free radicals associated with DNA damage and carcinogenesis, and

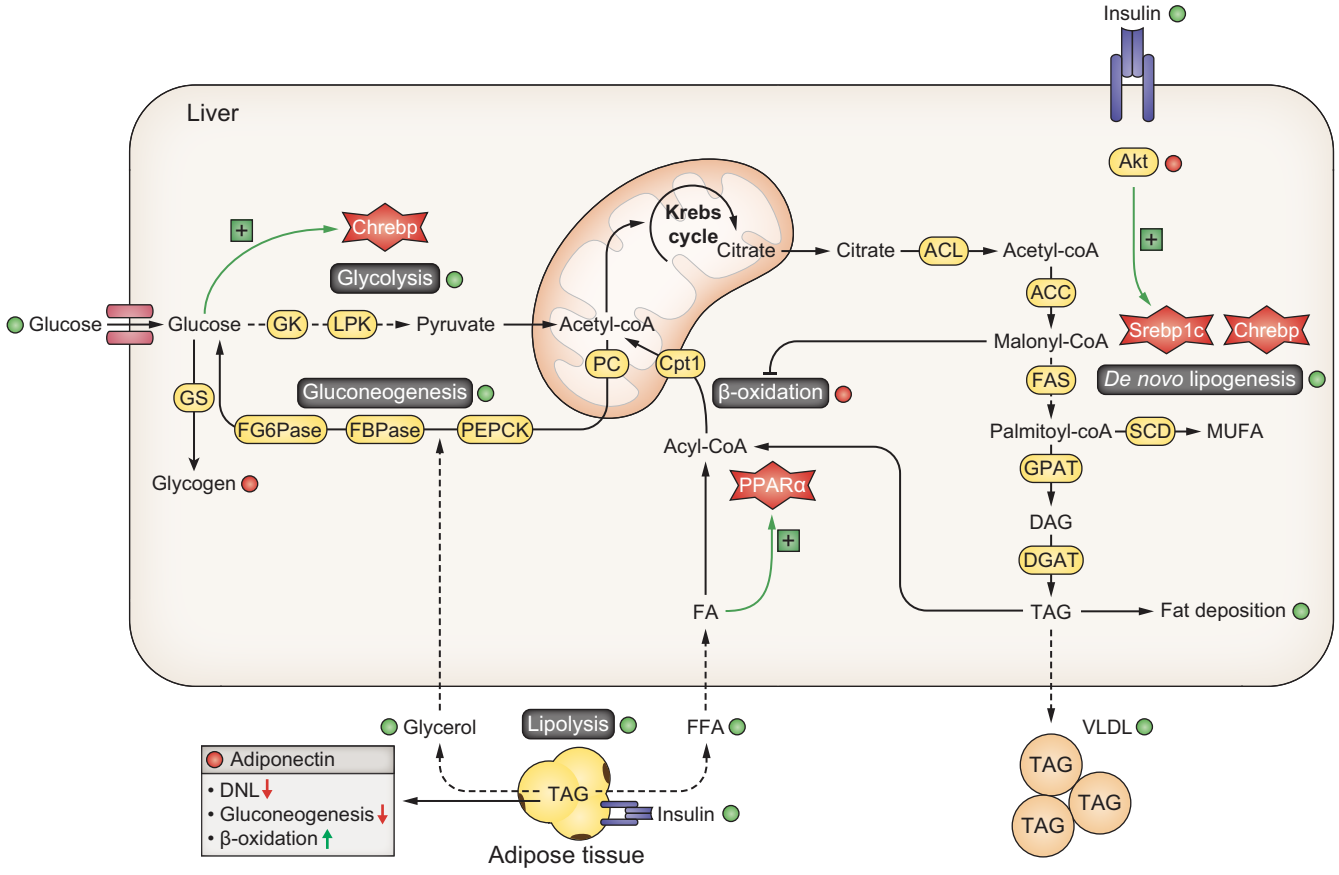

Fig. 1. Insulin resistance and NAFLD. During insulin resistance, glucose delivery increases and activates ChREBP that leads to an increase in glycolysis and *de novo* lipogenesis. Defective hepatic insulin signalling impairs the ability of insulin to inhibit gluconeogenesis while still increasing SREBP1-c-mediated *de novo* lipogenesis. Liver PPARα activity and β-oxidation are decreased in NAFLD, thereby participating in the accumulation of triglycerides in the liver. Peripheral insulin resistance leads to an incomplete suppression of adipose tissue lipolysis increasing the liberation of free fatty acids and glycerol that reach the liver to increase gluconeogenesis. During insulin resistance and NAFLD, the levels of adipose tissue-producing adiponectin decreased; thus, decreasing β-oxidation and increasing *de novo* lipogenesis and gluconeogenesis. Metabolic pathways that are increased or decreased during NAFLD/insulin resistance are labelled with green or red dots, respectively. ACC, acetyl-CoA carboxylase; ACL, ATP-citrate lyase; ChREBP, carbohydrate responsive element–binding protein; Cpt, carnitine palmitoyltransferase; DAG, diacylglycerol; DPAT, diacylglycerol phosphate acyltransferase; DNL, *de novo* lipogenesis; FA, fatty acid; FAS, fatty acid synthase; FBPase, fructose 1,6-bisphophatase; FFA, free fatty acid; GK, glucokinase; GPAT, glycerol-3-phosphate acyltransferase; GS, glycogen synthase; LPK, L-type pyruvate kinase; MUFA, monounsaturated fatty acid; NAFLD, non-alcoholic fatty liver disease; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; PPARα, peroxisome proliferator-activated receptor α; SCD1, stearoyl-CoA desaturase 1; SREBP-1c, sterol regulatory element–binding protein-1c; TAG, triacylglycerol; VLDL, very low-density lipoprotein.

apoptosis becomes dysfunctional.[36] MCD diet models replicate the histological models of NASH in humans, however this model shows no alteration in glucose tolerance and fasting glycaemia and, more importantly, weight loss occurs while NASH is progressing. In this regard, this model strongly differs from human NASH. Moreover, there is little correlation between gene expression profiles in MCD diet-induced NASH and human NASH.[37]

Choline-deficient, L-amino acid-defined, high-fat diet

An improvement of the MCD diet was more recently proposed. A mouse model of NASH with fibrosis was established by optimising the methionine content in the context of high-fat diet (HFD) feeding. Mice were fed a choline-deficient, L-amino acid-defined, high-fat diet (CDA HFD) consisting of 60% kcal from fat and 0.1% methionine by weight. Interestingly, in contrast to MCD diet feeding, C57BL6/J mice fed on CDA HFD diet maintain or even gain weight.[38] Plasma levels of alanine aminotransferase increased from the first week, when hepatic steatosis was also observed. By the sixth week, C57BL/6J mice had developed enlarged fatty livers with fibrosis, as assessed by Masson's trichrome staining and by hydroxyproline assay. Therefore, this improved CDA HFD model, in which mice develop rapidly progressive liver fibrosis, is potentially useful for improving our understanding of human NASH and for the development of efficient therapies for this condition.[38]

Western-like diet

A Western diet (WD) is a diet high in saturated fat, trans-fat, cholesterol and sugar.[39] The WD mouse model was designed to induce hepatic steatosis, NASH, and hepatic fibrosis in a pathophysiological manner similar to that observed in humans. Unlike the MCD diet model, the composition of the WD can vary greatly depending on the studies published and therefore, hepatic

Table 1. Dietary animal models of NASH.

Model name	Characteristics
Methionine and choline-deficient diet	Pros: Histological NASH by 2-4 weeks Cons: Weight loss and absence of insulin resistance
Choline-deficient L-amino-defined diet	Pros: Histological NASH and hepatocarcinogenesis Cons: Weight stability and absence of insulin resistance
Cholesterol and cholate	Pros: Histological NASH by 6-24 weeks, dyslipidaemia, lipoperoxidation and oxidative stress but the major Cons: Absence of insulin resistance, weight loss of 9% and low serum triglycerides
High cholesterol diet	Pros: Insulin resistance, increase in free fatty acids, triglycerides and serum aminotransferase levels Cons: Small weight gain and not pronounced liver histological changes, level of dietary cholesterol not applicable to humans
High-fat diet	Pros: Insulin resistance, histological NASH by 16 weeks. Cons: Phenotype is not severe
High-fructose -fat and -cholesterol diet	Pros: Insulin resistance, histological NASH, liver and fat inflammation. Cons: Not progression to liver advanced fibrosis and carcinogenesis
The streptozocin high-fat diet model	Pros: Histological NASH by 20 weeks, progressive liver fibrosis ad HCC. Cons: Beta cells loss function induced by chemical agent and not by insulin resistance et systemic inflammation, transcriptomics profoundly different from humans
Diet-induced animal model of NAFLD (DIAMOND)	Pros: Obesity, insulin resistance, dyslipidaemia, liver inflammation by 16 weeks, cirrhosis by 36 weeks and HCC, activation of the unfolded protein response, oxidative stress, apoptosis, fibrogenic process, serum adiponectin reduction and adipose tissue inflammation, concordance in transcriptomic analysis and histological features of HCC similar in mice and human NASH
Western-like diet	Pros: Induce hepatic steatosis, NASH and fibrosis Cons: Diet composition is not comparable to human diet
Diet- and chemical-induced murine NASH	Pros: Induce hepatic steatosis, insulin resistance, NASH and HCC. Same liver transcriptomic dysregulation of human NASH Cons: Small weight gain

HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis.

changes seen in mice fed a WD are variable depending on the content of the diet.[40] Aside from the administration of a HFD, researchers have explored the impact of adding cholesterol or high-fructose corn syrup on liver injury. High-fructose corn syrup is considered more lipogenic than sucrose, thereby leading to increased development of NAFLD. Kholi *et al.* reported that non-genetically modified mice maintained on a high-fat high-carbohydrate diet (high fat and water with 55% fructose and 45% sucrose (wt/vol)) not only develop obesity but also increased hepatic reactive oxygen species and a NASH-like phenotype with significant fibrosis.[41] For these reasons, studies have analysed the impact of a WD, with high fat, high cholesterol and added high-fructose corn syrup on NASH in mice.[42] Therefore, these models are highly variable depending on the composition of the diet and are often not reflective of a human WD.

The diet-induced animal model of NAFLD (DIAMOND)

This model is obtained by using an isogenic strain derived from the cross of 2 common mouse strains, 129S1/SvImJ and C57BL/6J. When fed a chow diet, these mice exhibit normal body weight and energy homeostasis. Interestingly, when fed a high-fat high-carbohydrate diet (WD, 42% kcal from fat and 0.1% cholesterol and *ad libitum* administration of glucose/fructose in drinking water), they develop obesity, insulin resistance, dyslipidaemia, liver inflammation (within 16 weeks), progressive liver fibrosis and cirrhosis (within 36 weeks), and HCC when advanced fibrosis is established. This model also mirrors other key pathways activated in human NASH: the unfolded protein response, oxidative stress, apoptosis, fibrogenesis, serum adiponectin reduction and adipose tissue inflammation. Moreover, transcriptomic analysis shows a concordance between preclinical and clinical models and histological features of HCC are similar in mice and human NASH.[43]

Diet- and chemical-induced murine NASH

This model is obtained by feeding C57BL/6J mice with a WD and by weekly injection of carbon tetrachloride (CCl_4) for 12 and 24 weeks. CCl_4 injection exacerbates histological features of NASH, fibrosis and HCC development. These mice, in fact, developed F3 fibrosis in 12 weeks and HCC in 24 weeks. However, CCl_4 reduced food intake and weight gain was attenuated in these mice compared to mice on a WD without CCl_4, but this did not impact on insulin resistance, which was present in all mice on a WD (regardless of CCl_4 injection). Furthermore, whole liver transcriptomic analysis indicated that dysregulated molecular pathways in WD/CCl_4 mice and immunological features were similar to those of human NASH. The reproducibility of this model and its similarities with human NASH make it suitable for the study of disease pathogenesis and to test new treatments.[44]

Another diet and chemical model of NASH is the streptozocin HFD model that is obtained by giving streptozocin (200 μg) to C57BL/6J mice fed an HFD. These mice develop NASH, fibrosis and HCC in about 20 weeks.[45] However, in this model, pancreatic β cell loss is caused by streptozocin and not systemic inflammation and insulin resistance. Moreover, there is little correlation between gene expression profiles in this mouse model and in human NASH.[46]

Genetic models of NAFLD/NASH

Genetic and environmental factors such as the diet strongly influence the pathogenesis of NAFLD. The great variation in hepatic lipid accumulation as well as the genetic architecture of NAFLD have been studied in more than 100 inbred mouse strains.[32] Moreover, several genetic mouse models are used to mimic

NAFLD/NASH, such as *ob/ob* mice (mutation in the leptin gene), *db/db* mice (mutation in the leptin receptor gene) or *foz/foz* mice (mutation in *Alms1* gene, essential for primary ciliary function). In humans, there are genetic polymorphisms that are strongly associated with susceptibility to NAFLD/NASH, such as mutations in genes encoding for lipid metabolism, glucose metabolism, hypertension, or inflammation. The fact that these mice have a gene modification makes them by definition different from humans. However, the study of specific pathways could help us to understand the aetiology of metabolic liver disease. Often, a specific diet must be used alongside a genetic model to reproduce the histological and metabolic modifications of human NASH.

Leptin-deficient mice (ob/ob mice)

Leptin is a hormone predominantly secreted by adipose cells and enterocytes in the small intestine that is involved in regulation of energy balance by inhibiting hunger. Obesity is associated with decreased sensitivity to leptin, thus resulting in an inability to detect satiety despite high energy stores and high levels of leptin. Leptin-deficient mice develop obesity, insulin resistance and steatosis. Liver histology shows lipotoxicity and lipo-apoptosis, but rarely progression to cirrhosis since *ob/ob* mice are resistant to hepatic fibrosis. However, when these mice are treated with lipopolysaccharide, or fed with a high-fat and MCD diet, they develop liver histological features of NASH without progressive liver fibrosis.[47] This model is therefore scarcely applicable to the study of NASH since: i) there is no progression towards extensive liver fibrosis; ii) clinical studies have shown that serum leptin levels in men with NAFLD and NASH can be normal or elevated.[48]

db/db mice

db/db mice have a loss of function of the leptin receptor. These mice are obese and exhibit insulin resistance with a fatty liver phenotype. Unlike *ob/ob* mice, *db/db* mice develop extensive liver fibrosis if fed a MCD diet, but the pathophysiological mechanism is not completely understood.[49] Recent studies reported that iron overload in *db/db mice* induces major features of NAFLD by promoting an increase in hepatic oxidative stress and impaired hepatic mitochondrial β-oxidation and fibrinogenesis.[50]

foz/foz mice

foz/foz mice are mutated or deficient in ALMS1 (Alstrom syndrome protein 1), a ubiquitous protein involved in primary cilia function, intracellular transport and appetite regulation. *foz/foz* mice are obese, insulin resistant and exhibit liver steatosis. When challenged with a HFD, *foz/foz* mice develop NASH, owing to an impairment of metabolic homeostasis.[51]

Targeting regulators of fatty acid synthesis and β oxidation

As mentioned in the introduction, different sources of fatty acids contribute to the development of fatty liver. Under conditions of insulin resistance, there is an uncontrolled release of fatty acids by the white adipose tissue, owing to the diminished antilipolytic action of insulin.[52] Therefore, peripheral fats stored in adipose tissue flow to the liver by way of plasma non-esterified fatty acids (NEFAs). FFAs coming from adipose tissue stimulate gluconeogenesis via acetyl-CoA pyruvate carboxylase. Dietary fatty acids are also taken up by the liver through the uptake of intestinally derived chylomicrons. In addition, the combination of elevated plasma glucose (hyperglycaemia) and insulin concentrations (hyperinsulinaemia) promote *de novo* fatty acid synthesis (lipogenesis) and impair β-oxidation, thereby contributing to the development of hepatic steatosis. After the esterification step (conversion of FAs into triglycerides), triglycerides can then be stored as lipid droplets within hepatocytes or secreted into the blood as VLDL (Fig. 1).

Lipogenesis is defined as *de novo* fatty acid synthesis from non-lipid precursors. This pathway is activated by high carbohydrate availability and converts excess carbohydrate into lipids. In the postprandial state, glucose is converted into pyruvate through glycolysis and pyruvate is imported into the mitochondria to join the tricarboxylic acid cycle (TCA) cycle. Citrate formed in the TCA cycle is transported into the cytosol where it is converted to acetyl-CoA by ATP-citrate lyase. *De novo* fatty acid synthesis begins with ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC)-1. Malonyl-CoA which serves as a 2-carbon donor is added to the acetyl-CoA primer by fatty acid synthase (FAS), a multifunctional enzyme complex. Palmitic acid (16:0), a saturated fatty acid (SFA), is the predominant fatty acid generated through lipogenesis. After elongation by fatty acyl-CoA elongase family members, palmitic acid can be transformed to long-chain fatty acids (over 16 carbon chain). Palmitic acid can also be desaturated by stearoyl-CoA desaturase-1 (SCD1) to palmitoleic acid or elongated to form stearic acid (C18:0). SCD1 catalyses the conversion of stearoyl-CoA to oleoyl-CoA, which is a major metabolite in triglyceride synthesis (Fig. 1).[53] It has recently been shown that fructose- and sucrose- but not glucose-sweetened beverages promote hepatic *de novo* lipogenesis. In a double-blind randomised trial, 94 healthy men were assigned to daily consumption of sugar-sweetened beverages (SSBs) containing moderate amounts of fructose, sucrose (fructose-glucose disaccharide) or glucose (80 g/day) in addition to their usual diet or SSB abstinence (control group) for 7 weeks. Regular consumption of both fructose- and sucrose-sweetened beverages in moderate doses – associated with stable caloric intake – increases hepatic fatty acid synthesis even in a basal state. Interestingly, this effect was not observed after glucose consumption.[54] Another study reported that rat liver perfused with fructose showed increased secretion of VLDL-triglycerides and enhanced incorporation of FFAs from the perfusate into VLDL-lipids. Neither of these processes was affected by infusion of glucose.[55]

β-oxidation of fatty acids occurs within the mitochondrial matrix. Short and medium-chain fatty acids (chain lengths up to 12 carbons) can freely enter the mitochondrial matrix. In contrast, long-chain fatty acids must be transported into the mitochondria by the carnitine shuttle, which is a rate-limiting step. β-oxidation involves the sequential removal of 2-carbon segments in the form of acetyl-CoA and production of shortened acyl-CoA, with concurrent reduction of 1 FAD and 1 NAD^+. The electrons carried by $NADH^+$, H^+ and $FADH_2$ immediately enter the electron transfer chain in oxidative phosphorylation, whereas acetyl-CoA enters the TCA cycle.

Addressing the mechanisms that cause lipotoxicity may be of interest to identify suitable strategies to prevent or at least retard the development of NASH and/or later syndromes. Several classes of fatty acids are associated with lipotoxicity. SFA accumulation in hepatocytes leads to liver injury through different lipotoxicity processes: i) activation of damage-associated molecular pattern receptors such as Toll-like receptor 4 (TLR4), which triggers NF-kB and the subsequent production of

inflammatory cytokines and stress kinases[53]; ii) the death receptor TRAIL-R2 (TNF-related apoptosis-inducing ligand receptor 2)[56,57] which triggers caspase 3,6,7 and subsequently promotes apoptosis. Intrahepatic SFAs can also activate endoplasmic reticulum stress and c-Jun N-terminal kinase (JNK) directly. JNK is the major mediator of SFA-induced lipotoxicity since it inactivates insulin receptor substrate-1, impairs mitochondrial respiration and increases the production of reactive oxygen species.[57] Moreover, JNK inhibits the expression of fibroblast growth factor-21 (FGF21) (a key hepatokine with beneficial properties) by suppressing peroxisome proliferator-activated receptor α (PPAR)α activity; it reduces peroxisome β-oxidation and increases the activity of pro-apoptotic protein p53.[56,57] In addition to hepatocytes, the lipotoxic effects of SFAs were reported on hepatic stellate cells (HSCs). In HSCs, the activation of TLR4 by SFAs leads to the production of pro-inflammatory chemokines that activate JNK in Kupffer cells, the resident macrophages, with subsequent activation, chemotaxis, and secretion of transforming growth factor-β and tissue inhibitor of metalloproteinase.[58]

A better understanding of the interplay between *de novo* lipogenesis and β-oxidation is of great interest in the NAFLD/NASH field. Herein, we focus on key molecular drivers of *de novo* lipogenesis and β oxidation, namely carbohydrate responsive element–binding protein (ChREBP), sterol regulatory element binding protein 1c (SREBP1c) and PPARα, respectively. We will describe a series of mouse models in which their expression has been genetically modified in the liver and discuss the consequences on hepatic steatosis, insulin resistance and NASH.

ChREBP: A carbohydrate sensor of *de novo* lipogenesis

The transcription factor ChREBP has emerged over the past years as a major regulator of lipogenesis in response to carbohydrates. ChREBP is most abundant in active sites of *de novo* lipogenesis where it has been studied as a master regulator of lipid metabolism.[59] Following a carbohydrate-enriched meal, ChREBP is upregulated at the transcriptional, translational and post-translational levels. After its translocation to the nucleus, ChREBP undergoes several post-translational modifications dependent on glucose metabolism, such as O-GlcNAcylation or acetylation, both of which are important for its transcriptional activity.[60] Global ChREBP-deficient ($ChREBP^{KO}$) mice show reduced hepatic glycolytic and lipogenic gene expression and triglyceride synthesis under high-carbohydrate feeding.[61] Interestingly, $ChREBP^{KO}$ mice fed a HFD develop severe liver injury due to endoplasmic reticulum stress.[62] In this model, hepatocyte apoptosis was linked to increase cholesterol biosynthesis, since the inhibition of this pathway via SREBP2 or hydroxymethylglutaryl-CoA synthase (the rate-limiting enzyme controlling the mevalonate pathway – the metabolic pathway that produces cholesterol) and other isoprenoids prevented $ChREBP^{KO}$ mice from HFD-induced liver injury. ChREBP loss of function was also associated with sugar intolerance and malabsorption, resulting in dysregulation of sucrose and fructose metabolism in mice.[63] ChREBP inhibition in obese and insulin-resistant ob/ob mice, through RNAi leads to reversal of hepatic steatosis, providing a direct proof for the involvement of ChREBP in the pathogenesis of NAFLD.[64] When fed a chow diet, mice overexpressing ChREBP show normal glucose metabolism without insulin resistance, in spite of the increased expression of genes regulating lipogenesis and fatty acid esterification that promote liver steatosis. Even if fed an HFD, mice overexpressing ChREBP exhibit liver steatosis without insulin resistance or impairment of glucose metabolism. In clinical models, ChREBP expression was decreased in the presence of insulin resistance and increased in the presence of more than 50% liver steatosis.[65] These results suggest that ChREBP dissociates the binomial hepatic steatosis from insulin resistance, and its overexpression could be effective in improving both glucose and lipid metabolism. Indeed, the reciprocal relation between hepatic steatosis and insulin sensitivity has greatly evolved in the past decade. Initially, as mentioned, the lipotoxic model prevailed where accumulation of deleterious lipid species (ceramides, diacylglycerols) was held responsible for impaired insulin signalling.[65] However, more recent studies suggesting dissociation between hepatic steatosis and insulin resistance gave way to another view where the metabolic consequences of hepatic steatosis would instead depend on the specific nature of generated lipid species and/or subcellular localisation. Lipidomic analysis in the livers of ChREBP-overexpressing mice revealed a decrease in SFA concentrations in contrast to enrichment in monounsaturated fatty acids (MUFAs). In mouse hepatocytes, ChREBP overexpression induced the expression of *Scd1*, the enzyme responsible for the conversion of SFAs into MUFAs. SFA impairment of phosphorylation of Akt, a critical insulin-signalling node, was rescued by the elevation of SCD1 levels upon ChREBP overexpression, whereas reduction of SCD1 activity attenuated the beneficial effect of ChREBP on Akt phosphorylation. ChREBP expression in liver biopsies from patients with NASH was increased when steatosis was greater than 50% and decreased in the presence of severe insulin resistance. Together, our results demonstrate that increased ChREBP can dissociate hepatic steatosis from insulin resistance, with beneficial effects on both glucose and lipid metabolism.[65] More recently, Bricambert *et al.* identified a coactivator of ChREBP: the histone demethylase plant homeodomain finger 2 (Phf2). Using specific deletion of the H3K9me2 methyl-marks on the promoter of the genes regulated by ChREBP, they demonstrated that Phf2 eases the incorporation of precursors into MUFAs, thus promoting the development of liver steatosis without insulin resistance and inflammation. Phf2 activation also protected the liver from oxidative stress and fibrosis in diet-induced obesity models, through the induction of the transcription factor Nrf2 (NF-E2-related factor 2), which redirects glucose metabolism to the pentose phosphate pathway and glutathione biosynthesis. In summary, H3K9me2 demethylation at specific gene promoters by Phf2 protects against steatohepatitis.[66]

SREBP1-c, an insulin-sensitive regulator of *de novo* lipogenesis

In addition to its activation by carbohydrates, *de novo* lipogenesis is also regulated by insulin through the SREBP1-c[67] transcription factor. Insulin directly activates SREBP1-c by increasing its gene expression and by promoting its proteolytic processing.[67,68] Consequently, the absence of the insulin receptor in hepatocytes leads to decreased SREBP1-c expression and activity, impacting on triglyceride accumulation.[69] Like ChREBP, SREBP1-c is induced in hepatic steatosis and its hyperactivation leads to liver triglyceride accumulation.[70,71] Moreover, hepatocyte-specific deletion of SCAP (SREBF chaperone),[71] the protein that escorts SREBP1-c into the nucleus, fully blocks SREBP1-c activity and prevents hepatic steatosis in genetic and nutritional models of obesity.[71] Dissecting the role of SREBP1-c in the context of insulin resistance is of interest since lipogenesis remains active

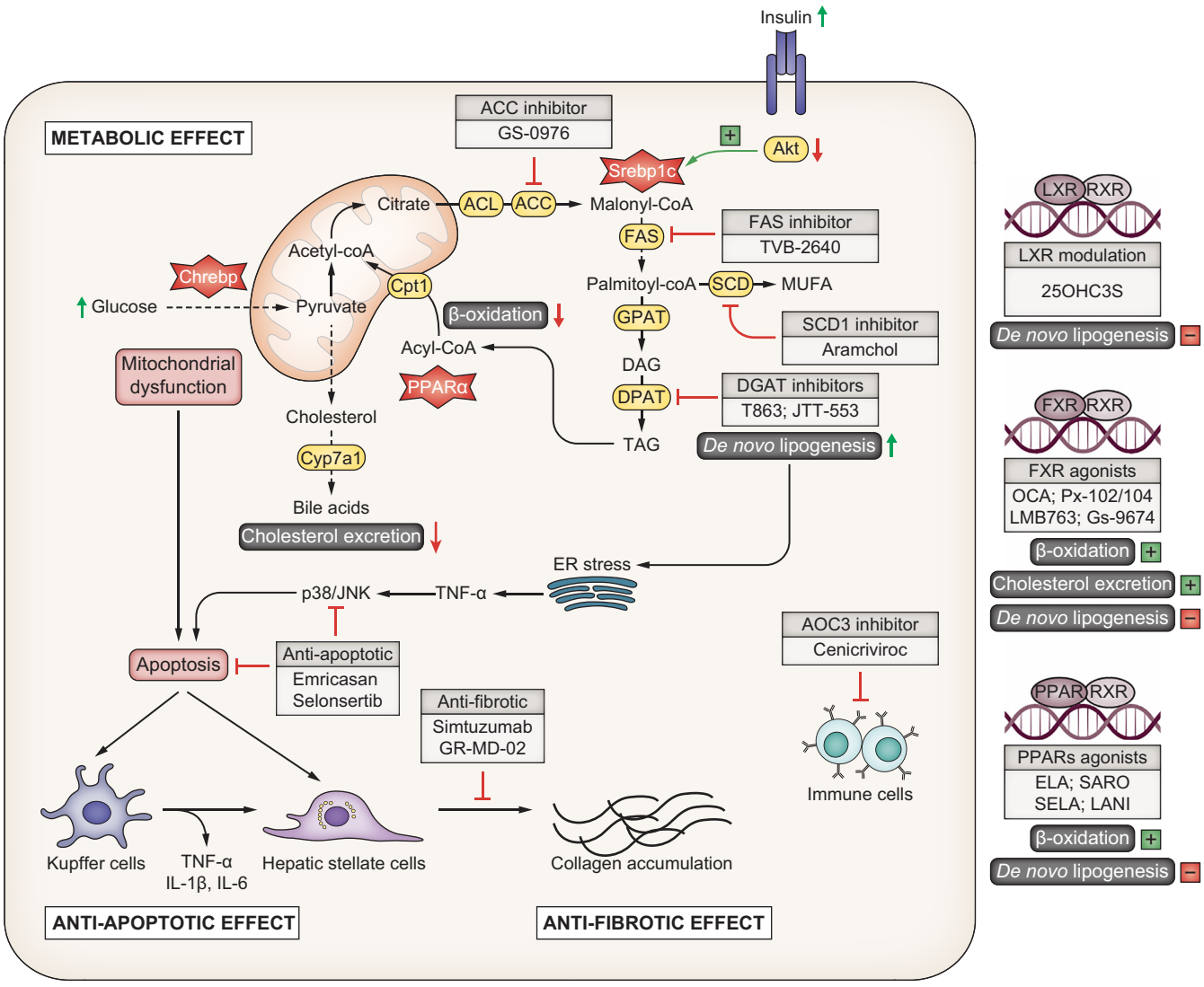

Fig. 2. Therapeutic targets in NAFLD and NASH. Therapeutic approaches in NAFLD target either the metabolism to decrease liver fat deposition or apoptosis and fibrosis to limit the progression of NAFLD. Metabolic treatments mostly target *de novo* lipogenesis, β-oxidation and bile acid metabolism. They can act indirectly by inhibiting enzymes that control *de novo* lipogenesis (inhibitors of FAS, ACC, SCD, DGAT) or be direct analogues (or antagonists) for nuclear receptors involved in *de novo* lipogenesis, β-oxidation and bile acid metabolism. Complications of NAFLD (inflammation, apoptosis and fibrosis) are targeted by different molecules. They have the ability to limit the activation of Kupffer cells and stellate cells by different mechanisms, thereby preventing collagen deposition and fibrosis. ACC, acetyl-CoA carboxylase; ACL, ATP-citrate lyase; AOC3, amine oxidase copper containing 3; ChREBP, carbohydrate responsive element–binding protein; Cpt, carnitine palmitoyltransferase; CYP7A1, cytochrome P450 7A1; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DPAT, diacylglycerol phosphate acyltransferase; ER, endoplasmic reticulum; FAS, fatty acid synthase; FXR, farnesoid X receptor; GPAT, glycerol-3-phosphate acyltransferase; IL-, interleukin-; JNK, c-Jun N-terminal kinase; LXR, liver X receptor; MUFA, monounsaturated fatty acid; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; SCD1, stearoyl-CoA desaturase 1; TAG, triacylglycerol; TNFα, tumour necrosis factor-α.

despite the inability of insulin to suppress hepatic glucose production. The so-called "selective hepatic insulin resistance" may explain why triglycerides still accumulate during insulin resistance, thereby resulting in both hyperglycaemia and hepatic steatosis. One recent study proposes a mechanism by which insulin signalling bifurcates and differentially regulates liver glucose production and SREBP1-c-mediated lipogenesis during insulin resistance. These authors suggest that peripheral insulin resistance causes liver glucose production (through adipose tissue lipolysis) while autonomous insulin signalling remains active and is still able to stimulate SREBP1-c activity and lipogenesis.[72-74] These results raise the question of how insulin regulates SREBP1-c during pathological insulin resistance and what is the contribution of SREBP1-c and ChREBP to the development of hepatic steatosis during insulin resistance.

PPARα: a lipid metabolism regulator in the liver

PPARα is a transcription factor of the nuclear receptor family. It is highly expressed in hepatocytes where it plays a crucial role in controlling lipid transport and metabolism, especially through activation of mitochondrial and peroxisomal fatty acid β-oxidation pathways.[75] PPARα is particularly active during fasting, as it controls fatty acid catabolism and ketogenesis,[76-78] as well as the endocrine hormone FGF21.[79,80] Several lines of evidence from

Table 2. Overview of the current pharmacological agents for the treatment of NAFLD/NASH.

Drug	Mechanism/target	Clinical improvements					Current phase
		IR	Steatosis	NASH	Apoptosis	Fibrosis	
Therapies targeting insulin resistance and *de novo* lipogenesis							
Obeticholic acid	FXR agonist		●	●		●	III
Cilofexor (GS-9674)	FXR agonist		●				II
Tropifexor	Non-steroidal FXR agonist		●	●			II
Firsocostat (GS-0976)	ACC inhibitor		●	●		●	II
Cilofexor + firsocostat	FXR agonist + ACC inhibitor	●	●	●		●	II
TVB-2640	FASn inhibitor		●				II
Aramchol	SCD1 inhibitor		●	●			III
Pradigastat	DGAT1 inhibitor		●				II
Elafibranor	PPARα/β agonist			●			III[1]
Seladelpar (MBX-8025)	PPARδ agonist	●					II[2]
Saroglitazar	PPARα/γ agonist			●			II
25-hydroxycholesterol3-sulfate	LXR inhibitor		●	●		●	I
Pegbelfermin (BMS-986036)	FGF21 analogue		●				II
NGM-282	FGF19 analogue		●	●		●	II
Ursodeoxycholic acid (UDCA)	Bile acid		●	●			II
Therapies targeting apoptosis							
Emricasan	Caspase inhibitor			●	●	●	II[3]
Selonsertib	ASK1 inhibitor		●	●		●	III[4]
Simtuzumab	Antibody against LOXL2		●	●		●	II
Selonsertib + simtuzumab			●	●		●	II
Therapies targeting fibrosis							
Cenicriviroc	CCR2/5 antagonist	●				●	III[5]
Belapectin (GR-MD-02)	Galactin-3 inhibitor			●		●	II

ACC, acetyl-coA carboxylase; ASK1, apoptosis signal-regulating kinase 1; CCR2/5, C-C chemokine receptor type 2/5; DGAT1, diacylglycerol acyltransferase 1; FASn, fatty acid synthase; FGF21, fibroblast growth factor 21; FXR, farnesoid X receptor; IR, insulin resistance; LOXL2, lysyl oxidase-like 2; LXR, liver X receptor; PPAR, peroxisome proliferative activated receptor; SCD1, steroyl-coA desaturase 1.

[1] Phase III has been stopped because elafibranor failed to reach the primary endpoint

[2] Phase IIb trial had to be halted after the appearance of liver cell damage and signs of inflammation in some participants.

[3] Phase II trials of emricasan failed to reach their primary endpoints.

[4] Phase III trial of selonsertib failed to reach its primary endpoint.

[5] Phase III trial of cenicriviroc was terminated early due to a lack of efficacy.

studies in mice also suggest that PPARα may protect against steatosis and inflammation.[77,81-84] PPARα is highly activated upon binding of ligands. It has been shown to be activated by fatty acids and eicosanoids[85-88] as well as other lipids such as phospholipids[89] and endocannabinoids.[90,91]

Fibrates are pharmacological ligands of PPARa known as lipid-lowering agents; they are currently used in humans to treat dyslipidaemia and hypertriglyceridaemia.[92] They have demonstrated many hepatic benefits in preclinical studies (improvement of hepatic steatosis, inflammation and fibrosis),[93,94] but these results were not replicated in humans, thereby limiting their use to the treatment of dyslipidaemia rather than NAFLD and NASH.

Previous studies reported a relationship between lower expression of PPARα and progression of fibrosis in human liver tissue, thus justifying the study of fibrates and PPAR-targeted treatments in the context of liver diseases.[95] Moreover, PPARα has anti-inflammatory properties as it enhances FGF21 activity and reduces NF-kB activity.[96] These findings indicate that PPARα represents an interesting target in NAFLD since it regulates key metabolic pathways in the liver and correlates negatively with liver diseases in humans. However, while PPARα-targeted treatments have shown efficacy for NAFLD in preclinical studies, their effects in humans remain controversial; hence, further studies are required before they can be considered as candidates for the treatment of NAFLD.

New metabolic/pharmacological targets

As mentioned, there are currently no drugs that can cure or treat NAFLD; thus, pharmacological research in the field of NAFLD is extremely active with different pathways being targeted: insulin resistance and gluconeogenesis, lipid transport and lipogenesis, apoptosis, oxidative stress and inflammation, extracellular matrix deposition and fibrosis (Fig. 2). The most important histological determinant in NASH is fibrosis since it has been demonstrated that it is the major driver of cardiovascular comorbidity, malignancy and mortality in NASH.[97] Thereafter, antifibrotic therapeutics are a central focus in NASH drug research.

In summary, an ideal drug candidate for NAFLD should improve steatosis, hepatic inflammation and fibrosis, while ameliorating glucose metabolism, insulin resistance and obesity. The molecules currently being studied for NASH are innumerable and the purpose of this review is not to describe all of them but to give preliminary results on the main classes (Table 2).

Targeting insulin resistance and *de novo* lipogenesis

FXR agonists

The farnesoid X receptor (FXR) is a crucial molecular actor in hepatic homeostasis. The activation of FXR reduces lipotoxicity (by inactivating the *de novo* lipogenesis mediated by SREBP-1c), increases mitochondrial β-oxidation and increases cholesterol

excretion, thus resulting in reduced insulin resistance, inflammation and fibrosis. The first clinical study published in 2015, the FLINT trial, reported that treatment for 72 weeks with obeticholic acid, a semisynthetic bile acid FXR agonist, improved necro-inflammation without worsening fibrosis in 46% of patients with histologically proven NASH and led to NASH resolution in 22% of treated patients. The FLINT study was the first to demonstrate that FXR activity appears to be a potent target, not only for improving lipid metabolism, but also for some histological alterations in NASH and laid the foundation for a long-term phase III trial.[98] In a recently published multicentre, randomised, placebo-controlled study, 931 patients with intermediate hepatic fibrosis (F2-F3) were randomised to placebo, 10 mg and 25 mg of obeticholic acid for 18 months (interim analysis). The primary endpoint of fibrosis improvement was achieved by 12% of the placebo group, 18% of the study group treated with 10 mg of obeticholic acid (p = 0.045) and 23% of patients treated with 25 mg of obeticholic acid (p = 0.0002), respectively. The secondary endpoint of NASH resolution was not met and the impact on insulin resistance was not evaluated in this trial. The most common adverse events were pruritus, a transient increase in total cholesterol and LDL-cholesterol, and a decrease in HDL-cholesterol. Long-term clinical outcomes are being assessed in a long-term phase III trial.[99] There are other non-steroidal FXR agonists (Px-102, Px-104, LMB763, Gs-9674) that are being studied in phase I–IIa randomised controlled trials (RCTs) in patients with NASH. In the FLIGHT-FXR phase II study, tropifexor treatment efficiently decreased steatosis and reduced circulating alanine aminotransferase and GGT levels (NCT02855164).[100] Other FXR agonists currently investigated include cilofexor (NCT03449446), EDP-305 (NCT03421431), EYP 001 (NCT03812029) and nidufexor (NCT02913105). The ATLAS trial, a phase II, randomised, double-blind, placebo-controlled study (NCT03449446), evaluated the safety and efficacy of monotherapy and dual combination regimens of cilofexor 30 mg, firsocostat 20 mg (an ACC inhibitor) and selonsertib 18 mg in patients with advanced fibrosis, including those with NASH-related cirrhosis. In this study, 392 patients were enrolled (56% had compensated cirrhosis) and a numerically higher percentage of patients in the combination therapy group (cilofexor and firsocostat) achieved a ≥1 stage improvement in fibrosis without worsening of NASH after 48 weeks of treatment compared with the placebo group (20.9% *vs.* 10.5%, p = 0.17), respectively.[101] The ATLAS trial exemplifies efforts to use FXR agonists (cilofexor) at a lower dose, with an ACC inhibitor (firsocostat) in order to reduce side effects related to FXR agonism. Norursodeoxycholic acid, a bile acid derivative like other FXR agonists led to a dose-dependent reduction in serum transaminases in a double-blind randomised, placebo-controlled phase II trial without liver histology(NCT03872921).[102]

Acetyl-coenzyme A carboxylase inhibition

ACC promotes the adenosine triphosphatase-dependent carboxylation of acetyl-CoA to malonyl-CoA, the first rate-limiting step in *de novo* lipogenesis.[103] The ACC-1 isoenzyme has a cytosolic localisation and is expressed in hepatocytes and adipocytes, while the ACC-2 isoenzyme is expressed on the mitochondrial surface of oxidative tissues such as the liver, heart, and skeletal muscle.[103] The inhibition of ACC decreases *de novo* lipogenesis and increases FA β-oxidation,[103] indeed mice with constitutively active ACC exhibit hepatic insulin resistance and increased *de novo* lipogenesis, developing NASH with progressive liver fibrosis. This phenotype is prevented by the genetic ablation of ACC or its pharmacological inhibition.[104] GS-0976, an oral treatment currently being evaluated in a phase IIa RCT, is an inhibitor of hepatic ACC-1 and ACC-2, which leads to a reduction of steatosis, suppression of *de novo* lipogenesis and a reduction of serum fibrosis markers in non-cirrhotic patients with NASH. Nausea, abdominal pain, diarrhoea, headache and the asymptomatic increase of serum triglycerides were the most common adverse events associated with GS-0976, which did not impact on insulin resistance.[105]

Firsocostat, an ACC inhibitor, led to a 29% reduction of liver fat content in 126 patients with NASH when given at a dose of 20 mg daily for 12 weeks in a phase II trial (NCT02856555).[106]

FASn inhibition

FASn is one of the enzymes involved in *de novo* lipogenesis in the liver. In a phase II randomised, placebo-controlled trial, 99 patients with NASH were given 25 mg or 50 mg of TVB 2640 (FASn inhibitor) or placebo per day. Liver fat was measured by MRI.

Patients on the lower and higher dose showed a 9.6% and 25% reduction of liver fat content, respectively, compared to a 4.5% increase in the placebo group.[107] Safety monitoring revealed that this drug was well tolerated, without an increase in plasma triglycerides. Alopecia occurred in 2 patients (reversed after stopping the drug), but otherwise no changes were observed in fasting glucose, insulin, ketones, and renal function. A larger phase IIb clinical trial in patients with NASH and stage 2–3 fibrosis is expected to start in the first half of 2021.

SCD1 inhibition

Aramchol is an inhibitor of SCD1, one of the key enzymes of *de novo* lipogenesis. In a phase IIa randomised, double-blind, placebo-controlled trial of 60 patients with a histological diagnosis of NAFLD, aramchol (300 mg/day for 12 weeks) reduced liver fat (measured with MR spectroscopy) by 12.6% (*vs.* an increase of 6.4% in the placebo group).[108] These results led to a phase IIb study (ARREST) where patients were randomised to 400 or 600 mg/day of aramchol or placebo. The 400 mg group showed a significant reduction of liver fat content (p = 0.045) and a trend was observed in the 600 mg group (p = 0.066) compared with placebo. Resolution of NASH without worsening of fibrosis was more frequent in the 600 mg group than in the placebo group (16.7% *vs.* 5%, odds ratio = 4.74; p = 0.0514).[109] However, while no improvement in insulin resistance was observed, glycated haemoglobin was reduced. No severe side effects were observed in this study. Currently a phase III study is ongoing (NCT04104321).

Diacylglycerol acyltransferase 1 and 2 inhibition

Diacylglycerol acyltransferase (DGAT) is the enzyme that catalyses the last reaction in triglyceride synthesis and diglyceride esterification by long-chain acyl-CoA esters. There are 2 isoforms: DGAT-1 is expressed in enterocytes of the small intestine, where it reassembles triglycerides from dietary FAs to form chylomicrons,[103] while DGAT-2 is present in the liver, skin and adipose tissue, where it synthesises triglycerides from *de novo* FFAs and diglycerides.[110] DGAT-1^{KO} mice are viable and show a

reduction of tissue triglycerides, while DGAT-2^{KO} mice are not viable since they develop severe serum lipid reduction and have impaired skin barrier function.[111] It has been reported that dietary SFA absorption is increased in patients with NASH,[112] which has led investigators to evaluate selective DGAT-1 inhibitors for the treatment of NASH, obesity and hyperlipidaemia. The first molecule studied in humans, pradigastat, decreased liver steatosis (assessed by MRI) in about 24 weeks but it was associated with diarrhoea and steatorrhea in almost 90% of patients.[113] Abdominal pain, diarrhoea and headache were the most common adverse events, while no improvement in insulin resistance was reported with DGAT-1 inhibitors.

More recent data show that selective hepatic DGAT-2 inhibition decreases lipidic hepatic accumulation in HFD-fed animal models, moreover, it improves insulin resistance though the downregulation of SREBP-1c-mediated *de novo* lipogenesis and it reduces NF-kB-mediated inflammation and fibrogenesis.[114-116]

PPAR agonists

PPARα, β/δ and γ isotypes are involved in glucose and lipid metabolism as well as inflammation and fibrosis, which makes them pharmacological targets in the field of NAFLD and NASH.[117,118] While there are substantial preclinical links between PPAR and NAFLD, the most promising results of clinical trials came from pan-agonist targeting of at least 2 PPAR isotypes. Experimental models show an improvement of NASH and a reduction of liver fibrosis after PPARα and β/δ stimulation.[119] In a phase IIb RCT, elafibranor, a PPARα and PPAR β/δ agonist, ameliorated serum lipid profile and insulin resistance and improved NASH without worsening fibrosis.[120] This has not yet been confirmed in phase III but the data are not fully released.[121]

Saroglitazar, a predominant PPARα and moderate PPARγ agonist, and lanifibranor, a pan-PPAR agonist improved diet-induced NASH by upregulating β-oxidation and FA desaturation.[122,123] Saroglitazar was also reported to improve liver enzymes, liver fat content, insulin resistance and atherogenic dyslipidaemia in participants with NAFLD/NASH.[124] While promising, larger trials over longer durations, with more extensive analysis of efficacy and safety, are required.

LXRα modulation

Liver X receptor (LXRα) is an important regulator of FFA and cholesterol metabolism through the activation of SREBP-1c. LXRα promotes *de novo* lipogenesis and reduces LDL catabolism, favouring hepatic steatosis. LXRα activation also induces intestinal excretion of cholesterol and increases its conversion into bile acids by stimulating the expression of cytochrome P450 7A1, the rate-limiting enzyme in bile acid synthesis. A recent study reported that LXRα inhibition by 25-hydroxycholesterol3-sulfate had anti-inflammatory, anti-steatotic and antifibrotic effects in mouse models of NASH.[125] The effects of this class of molecules on insulin resistance are still debated.[126] In a clinical trial, 25-hydroxycholesterol-3-sulfate was proven to be safe in a phase Ib study in patients with NASH (clinicaltrials.gov numbers ACTRN12615001355561).

FGF21 agonist

FGF21 is produced by the liver, adipose tissue and pancreas. FGF21 exerts several beneficial metabolic effects: it increases energy expenditure, reduces sugar intake, stimulates β-oxidation, increases the production of adiponectin and improves insulin resistance.[80] Pegbelfermin, a pegylated FGF21 analogue, administered for 16 weeks to patients with NASH, decreased hepatic steatosis (evaluated by MRI) in a phase II study (NCT02413372).[127] The efficacy and safety of pegbelfermin, which has to be injected subcutaneously, are currently being investigated in phase IIb clinical trials: FALCON 1 (NCT03486899) in patients with NASH with bridging fibrosis; and FALCON 2 (NCT03486912) in those with NASH and compensated cirrhosis.

FGF19 agonist

Fibroblast growth factor (FGF)19 is released after the activation of intestinal FXR, with similar downstream effects as those noted following FXR activation. NGM282 is a humanised analogue of FGF19, which acts on the same pathway as intestinal FXR agonists.[128] In a multicentre open label trial of NGM282, patients received subcutaneous NGM282 at 1 or 3 mg with paired biopsies at 12 weeks (n = 43).[129] NGM282 at 3 mg decreased fibrosis by ≥1 stage without NASH worsening in 42% of patients and improved NAS by ≥2 points without fibrosis worsening in 63% of patients. A 24-week phase IIb trial of NGM282 for the treatment of NASH is currently underway (NCT03912532).

Targeting apoptosis

Apoptosis has been shown to contribute to liver injury in NASH. The final reaction of cell death is mediated by caspases. In a recent study, a pan-caspase inhibitor emricasan was administered alongside regular chow or HFD in a murine model of NASH. Mice fed with HFD and treated with emricasan had a reduction of apoptosis, an improvement of serum transaminases, a reduction of liver inflammation and fibrosis (evaluated with the NAS score) and serum markers of inflammation such as IL-1β and TNF-α.[130] However, emricasan did not show the same positive effects in 2 studies in humans. In a double blind, placebo-controlled study, 318 patients with NASH and stage F1-F3 fibrosis were randomised to twice-daily treatment with emricasan (5 or 50 mg) or matching placebo for 72 weeks. In this negative trial, emricasan treatment did not improve liver histology and may have worsened fibrosis and ballooning.[131]

In the multicentre double-blinded study, 263 patients with NASH cirrhosis and baseline hepatic venous pressure gradient (HVPG ≥12 mmHg) were randomised to twice daily oral emricasan 5 mg, 25 mg, 50 mg or placebo for up to 48 weeks. There were no significant differences in HVPG improvement for any emricasan dose *vs.* placebo when adjusted for baseline HVPG, compensation status, and non-selective beta-blocker use. Decompensating rate (∼10% over median exposure of 337 days) and liver disease progression were similar between treatment groups.[132]

Another anti-apoptotic molecule is selonsertib – an inhibitor of apoptosis signal-regulating kinase 1 (ASK1). ASK1 is activated by intracellular TNFα and endoplasmic reticulum stress and it goes on to activate the P38/JNK pathway, resulting in cell death.[133] An open label phase II trial evaluated the efficacy of selonsertib alone or in combination with simtuzumab in patients with NASH without advanced fibrosis (F2/F3). Simtuzumab is a monoclonal antibody against the enzyme lysyl oxidase-like 2, which is responsible for the cross-linking of collagen and is overexpressed during fibrosis progression.[134] Given for 24 weeks, selonsertib improved liver fibrosis, liver inflammation and steatosis (evaluated by MRI). There was no difference between selosertib alone or selosertib with simtuzumab.[135]

However the phase III trial on selonsertib among patients with histologically proven NASH with stage 3 fibrosis (STELLAR 3) and cirrhosis (STELLAR 4) did not confirm these positive results. Patients were randomised to receive selonsertib 6 mg or 18 mg, or placebo once daily for 48 weeks. The primary endpoint was the proportion of patients with ≥1-stage improvement in fibrosis without worsening of NASH at the end of treatment. Unfortunately, 48 weeks of selonsertib had no antifibrotic effect in patients with stage F3 fibrosis or compensated cirrhosis due to NASH.[136]

Targeting fibrosis

As already mentioned, fibrosis is the most important determinant of mortality in patients with NASH. Thus, several antifibrotic agents have been evaluated for the treatment of NASH with moderate to extensive fibrosis. Cenicriviroc is an antagonist of CCR2/5 (C-C motif chemokine receptor-2/5) that has antifibrotic effects – through the inhibition of migration and activation of collagen-producing HSCs – and improves insulin resistance.[137] A phase IIb trial (CENTAUR study) reported an improvement of fibrosis without worsening of NASH after 1 year of treatment with cenicriviroc in 20% of patients compared to 10% for placebo. However, this was not supported in a phase III study in patients with grade 2/3 fibrosis, which has been terminated early (AURORA study; NCT03028740).

Another molecule involved in liver fibrosis is galectin-3; in mouse models, GR-MD-02 (belapectin), a galectin-3 inhibitor, improved liver fibrosis, with a reduction of collagen deposition and NASH inflammatory activity.[138] However, the recently published phase IIb, randomised trial of the safety and efficacy of GR-MD-02 in patients with NASH, cirrhosis, and portal hypertension did not report an improvement of portal hypertension (evaluated by hepatic venous pressure gradient) nor hepatic fibrosis (evaluated by liver biopsy) after 52 weeks of treatment.[139]

Conclusion

NAFLD is a complex disease, involving environmental and genetic factors, whose pathogenesis and progression are not completely understood. In this review we have discussed preclinical models and potential mechanisms of liver damage such as glucotoxicity and lipotoxicity. The complete comprehension of these mechanisms will help us to prevent the first hit and the progression of liver disease through effective treatment. Numerous molecules are currently being studied in phase II and III clinical trials and some agents have shown promise in terms of improving steatosis, inflammation and fibrosis.

Abbreviations

ACC, acetyl-CoA carboxylase; ASK1, apoptosis signal-regulating kinase 1; CAP, controlled attenuation parameter; ChREBP, carbohydrate responsive element–binding protein; FAS, fatty acid synthase; FFA, free fatty acid; FGF21, fibroblast growth factor-21; FXR, farnesoid X receptor; GGT, gamma glutamyltransferase; HCC, hepatocellular carcinoma; HFD, high-fat diet; HSC, hepatic stellate cells; HSL, hormone-sensitive lipase; HVPG, hepatic venous pressure gradient; IL-, interleukin-; JNK, c-Jun N-terminal kinase; LXR, liver X receptor; MCD, methionine- and choline-deficient; MUFA, monounsaturated fatty acids; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NEFA, non-esterified fatty acid; PPARα, peroxisome proliferator-activated receptor-α; Phf2, histone demethylase plant homeodomain finger 2; PY, persons/years; PUFAs, polyunsaturated fatty acids; RCT, randomised controlled trial; SCD1, stearoyl-CoA desaturase-1; SFA, saturated fatty acid; SREBP-1c, sterol regulatory element–binding protein-1c; TCA, tricarboxylic acid; TLR4, Toll-like receptor 4; TNF-α, tumour necrosis factor-α; VLDL, very low-density lipoprotein.

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Conflict of interest

LP, MR, HG and CP declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

The authors contributed equally for the draft of the article, for the critical revision and for the final approval of the article.

Supplementary data

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Author names in bold designate shared co-first authorship

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