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TOPIC HIGHLIGHT

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Translational approaches: From fatty liver to non-alcoholic steatohepatitis

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Abstract

Over the past few decades, non-alcoholic fatty liver disease (NAFLD) has become one, if not the most common, cause of chronic liver disease affecting both adults and children. The increasing number of cases at an early age is the most worrying aspect of this pathology, since it provides more time for its evolution. The spectrum of this disease ranges from liver steatosis to steatohepatitis, fibrosis and in some cases, hepatocellular carcinoma. NAFLD may not always be considered a benign disease and hepatologists must be cautious in the presence of fatty liver. This should prompt the use of the available experimental models to understand better the pathogenesis and to develop a rational treatment of a disease that is dangerously increasing. In spite of the growing efforts, the pathogenesis of NAFLD is still poorly understood. In the present article we review the most relevant hypotheses and evidence that account for the progression of NAFLD to non-alcoholic steatohepatitis (NASH) and fibrosis. The available *in vitro* and *in vivo* experimental models of NASH are discussed and revised in terms of their validity in translational studies. These studies must be aimed at the discovery of the still unknown triggers or mediators that induce the progression of hepatic inflammation, apoptosis and fibrosis.

Key words: Fatty Liver; Obesity; Metabolic syndrome; Inflammation; *In vitro*; Experimental model

Core tip: The molecular mechanism associated with the accumulation of fatty acids in the liver cells and the resulting molecular cascade leading to hepatic damage is far from being understood. Due to the development of reliable *in vitro* and *in vivo* models, we are starting to open the "black box". This will lead to a better understanding of the active clinical condition and hopefully to a more effective treatment. This article critically reviews what is known and what has still to be discovered about the link between the accumulation of fat within the liver and the resulting damage.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a complex spectrum of diseases ranging from benign steatosis (usually asymptomatic) to more severe alterations like non-alcoholic steatohepatitis (NASH), cirrhosis and, in some cases, hepatocellular carcinoma (HCC). The most serious aspect of the disease is the high incidence in pediatric and adolescent populations, providing longer time for evolution[1]. Day by day, social and medical operators witness the dramatic increase in the incidence of this phenomenon. The "global society" is driving us towards a global epidemic of obesity, type 2 diabetes mellitus (T2DM) and metabolic syndrome (MS). NAFLD and NASH are strictly linked to the presence of insulin resistance (IR) and are nowadays considered the hepatic manifestation of the MS[2]. Although most typical forms of NAFLD are overwhelmingly associated with IR and MS, it cannot be said that IR and MS are invariably associated with fatty liver[3]. Interestingly, NAFLD markers have also been associated with IR in type 1 diabetes[4], which is not closely related to the MS. Hepatology and Gastroenterology communities are facing a great challenge since within few years, NAFLD will be the most important chronic liver disease worldwide (Figure 1).

Lifestyle changes have occurred in the industrialized societies due to the introduction of modern technologies resulting in eating more and more importantly, moving less. According to the Food and Agriculture Organization of the United Nations (FAO http://www.fao.org/docrep/x0262e/x0262e23.htm), in the next 40 years the daily caloric requirements will decrease by 350 calories. Several epidemiological studies have linked NAFLD to unhealthy diet and sedentary behaviors[5-7], and the only effective treatment for NAFLD and NASH is to guide the patient to a healthier lifestyle[8] with lifestyle coaching including personalized diet, physical activity and cognitive-behaviour therapy[9]. However, the lack of patient compliance is the main limitation of this approach. Although to a lesser extent, NAFLD can also occur in non-obese populations[10], suggesting that dietary composition is not the only cause of fatty liver. Several sets of data reviewed by Caldwell *et al*[3] showed that both ethnicity and genetic polymorphisms play a major role in the development and progression of the disease, and different genetic profiles might be also responsible for the variations of steatosis in the MS.

It is therefore of pivotal importance to further develop a strong translational approach to understand the pathophysiology of this new disease and to translate it into clinical practice. In the present paper, we review the most recently published data on the pathophysiology of NAFLD in an attempt to amalgamate the available information in order to contribute to the understanding of the factors involved, including a critical analysis of the *in vitro* and *in vivo* models.

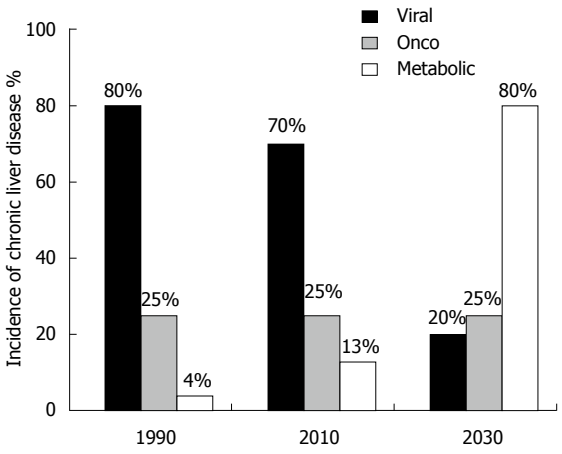

Figure 1 Estimation of the main etiological incidence of past, present and future chronic liver diseases according to the available data from the United States[130] and Europe[131,132].

PATHOGENESIS

The most accepted scheme to explain the development of NAFLD and the progression from simple steatosis to NASH is still based on theories. In 1998, Day[11] proposed the "two hits" theory. The "first hit" is characterized by the accumulation of lipids in hepatocytes due to an altered intrahepatic lipid metabolism, where insulin resistance seems to be the key pathogenic factor for the development of hepatic steatosis[12], while the "second hit" leads to hepatocyte injury, inflammation and fibrosis. Several factors were suggested to initiate the second hit such as: (1) proinflammatory cytokines and adipokines[13]; (2) mitochondrial dysfunction[13,14]; (3) oxidative stress; and (4) endoplasmic reticulum (ER) stress[15] with subsequent apoptosis. In 2010, a more complex, global and realistic model, the "multiparallel hits" hypothesis, was proposed to explain the pathogenesis of NAFLD[16]. In this model, the adipose tissue and gut-related factors play a key role in the initiation of hepatic inflammation, suggesting that simple steatosis and NASH might be two different disorders and pointing to new, non-hepatic players in the mechanisms of NAFLD and its progression.

Contributors to the development of insulin resistance

During the last few years, the interplay among gut microbiota, obesity and the metabolic consequences (liver sensitization) has become important. Some of the main factors involved in this process are summarized in Figure 2. Several clinical and experimental studies recently reviewed in detail[17] suggest that microbiotal factors may be the driving forces of IR[18], hepatic steatosis and subsequent inflammatory state. Changes in the composition of the gut microbiota might induce an increased permeability and translocation of bacterial endotoxins promoting a chronic inflammatory state. This condition can alter pathways such as insulin signaling, promoting the devel-

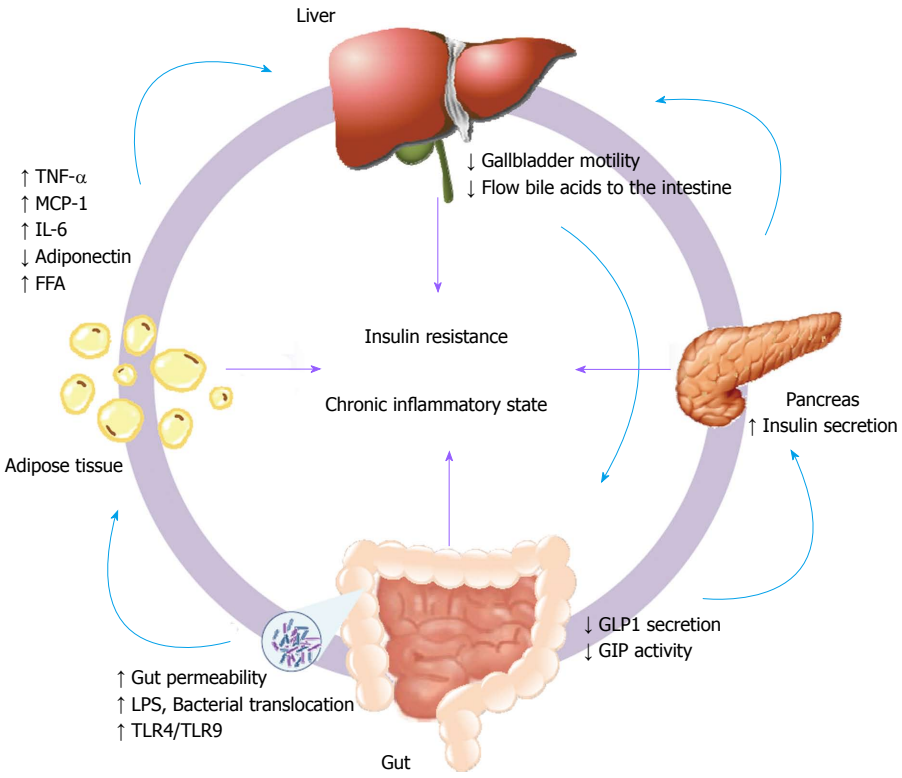

Figure 2 Extrahepatic factors involved in the pathogenesis of non-alcoholic fatty liver disease. The affected organs and their response are represented in a dynamic circle; in the center are indicated the main factors that contribute to the initiation/perpetuation of the hepatic injury (insulin resistance and chronic inflammatory state). The light blue arrows represent the organ-specific effects of each response. TNF-α: Tumor necrosis factor-α; MCP-1: Monocyte chemotactic protein 1; IL-6: Interleukin-6; FFA: Free fatty acids; LPS: Lipopolysaccharides; TLR: Toll-like receptor; GIP: Glucose-dependent insulinotropic peptide; GLP: Glucagon-like-peptide.

opment of IR. The molecular basis of IR is the result of multiple genetic[19] and non-genetic mechanisms. IR can initiate a dangerous vicious circle, involving inflammation and hypercoagulability, which increases atherogenesis[20]. Data from animal models indicate that IR develops in the vasculature well before these responses are detected in muscle, liver, or adipose tissue[21]. These findings could explain the high cardiovascular risk observed in subjects with MS. Moreover, disruption in the endothelial insulin signaling can promote the development of atherosclerosis in the absence of diabetes-related risk factors including hyperglycemia and hyperinsulinemia. The development of atherosclerosis is associated with a reduced bioavailability of nitric oxide and an excessive production of reactive oxygen species[22]. The endothelial dysfunction might be mediated by FoxOs transcription factors; FoxOs inhibition in endothelial cells has been shown to have promising atheroprotective effects[23]. Altogether these findings are in agreement with previous data[24], reinforcing the idea that hepatic IR and hepatic steatosis might precede the development of T2DM. Epidemiological evidence (reviewed in detail elsewhere[25]) also suggests an association between MS and the risk of developing chronic kidney disorders beyond the contribution of hyperglycemia and high blood pressure. The increased rates of chronic kidney disease (CKD) and cardiovascular disease are the most important clinical features associated with NAFLD. To date, there is a mounting body of evidence (reviewed extensively by Targher *et al*[26]) suggesting that patients with NAFLD have multiple risk factors of CKD and that NAFLD is associated with an increased prevalence and incidence of CKD both in patients with and without diabetes. Renal dysfunction may be promoted by a mosaic of effects such as: (1) inflammatory cytokines released by the adipose tissue (TNF-α (tumor necrosis factor-α), IL6, adiponectin[27], leptin[28]); (2) obesity-related mechanisms such as altered renal hemodynamics; (3) excess of renal sodium reabsorption; (4) activation of renin-angiotensin and sympathetic nervous systems; and (5) physical compression of kidneys by adipose tissue.

Over the last 14 years there has been a surge in the number of studies confirming that NAFLD is associated with IR (the "IR dogma"). Based on such studies, one could expect that, by correcting IR, NAFLD could be healed. Unfortunately, therapeutic studies[29] failed to confirm this expectation, suggesting a more complex interplay of factors involved in the pathogenic process.

Role of incretin hormones

Incretin hormones, such as glucose-dependent insulinotropic peptide (GIP) and glucagon-like-peptide 1 (GLP-1), are released by the gastrointestinal tract in response to nutrients that increase the glucose-mediated insulin secretion[31]. In patients with T2DM the incretin effect is severely reduced[32], due to an impaired secretion of GLP-1 and a decreased activity of GIP[33]. Recent *in*

vitro[34] and *in vivo*[35] data clearly show that hepatocytes express GLP-1 receptors, and the exposure to GLP-1 agonists leads to: (1) a reduction of intracellular fat load[36,37]; (2) enhanced fat oxidation[38]; and (3) an induction of macroautophagy[39], which is a critical process for the removal of toxic fatty acids from cells. Other important regulators of glucose homeostasis are the bile acids, which through various signaling pathways regulate cholesterol, fasting and mealtime glucose, and metabolism/energy homeostasis, as well as their own synthesis and blood levels in the enterohepatic circulation[40,41]. The composition of bile acids in T2DM has been shown to be altered[42] as a consequence of a reduced gallbladder motility resulting in a reduced secretion of bile acids to the intestine. A low bile acid concentration is associated with a reduction in the secretion of GLP-1 and consequently, an impaired glucose homeostasis with a decreased insulin secretion[43]. Paradoxically, patients with NAFLD can often present a hyperinsulinemic state. However, instead of a regulation of gluconeogenesis, insulin promotes *de novo* lipogenesis that exacerbates hepatic lipid deposition and accelerates the development of the disease. One possible mechanism to explain this situation could be the activation of sterol regulatory transcription factor element-binding protein-1c (SREBP1c), a master transcription factor regulator of lipid synthesis, through the stimulation of the target of rapamycin complex 1 (mTORC1)[44]. The regulation of incretin hormones represents a promising strategy for NAFLD. Therapy with GLP-1 agonists (like exenatide) in T2DM patients promotes a positive effect in the liver[45], since hepatocytes express GLP-1 receptor[34]. This compound might reduce or even reverse hepatic fat accumulation and reduce the triglyceride (TG) levels, most probably as a consequence of a reduced caloric intake, which is one of the main therapeutic contributions of this kind of drug[46]. Unfortunately, the success of bile acid interventions is limited in clinical practice and the results obtained are discordant from those observed in experimental models[47]. Hyperinsulinemia in NAFLD leads to upregulation of the production of insulin-like growth factor-1 (IGF-1) and activation of insulin receptor substrate (IRS)-1. This may activate several molecules and signaling pathways including p53, mitogen-activated protein kinases (MAPK), and phosphatidylinositol-3 kinase/Akt[48]. These pathways play a significant role in carcinogenesis by inducing cell proliferation and inhibition of cell apoptosis[49,50]. Thus, NAFLD and HCC appear to be regulated by similar signaling molecules and pathways related to inflammation. This evidence is particularly interesting to support the idea that NAFLD itself could promote HCC development in earlier stages, even in the absence of cirrhosis[51,52].

Alterations in hepatic lipid metabolism

TGs are the preferred nutritional storage to buffer fluctuations in energy demand and availability. TG physical properties allow their accumulation without adverse osmotic or colloidal effects. In higher organisms, TGs are stored mainly in adipocytes, and can be accumulated in other cell types only under particular circumstances. In this regard, an interesting example was presented by Cohen *et al*[53] in migratory birds that store large quantities of TGs in the liver as an energy source in preparation for prolonged seasonal flights. Like migratory birds, some humans who consume excess calories deposit fat in the liver, as a maladaptive process. The moiety of the intracellular fat has distinct toxic effects. As mentioned before, hepatic accumulation of neutral cholesterol esters and TG appears not to be a threat[54,55] (though this is still an open question[56]); however, the presence of the intermediate products seems to have a more deleterious effect on liver cells. An altered lipid metabolism leads to the accumulation of intermediate products such as diacylglycerol (DG) and phospholipids (sphingolipids and ceramides)[57-59], and these compounds account for the fatty acid-induced toxicity and for the hepatic IR (Figure 3). Moreover, these metabolites promote the activation of numerous kinases, including nPKC isoforms, MAPK, ERKs and c-Jun N-terminal kinase (JNK), S6K and inhibitor kappa beta kinase beta (IKKβ), that participate in the phosphorylation of the IRS inducing positive or negative effects on the insulin pathway[60]. Recent data suggest a connection between altered cholesterol homeostasis and hepatic free cholesterol (FC) accumulation as a trigger for the pathogenesis of NASH[61,62]. Most probably, FC accumulates within the ER membrane impairing its fluidity. The resulting stiffening of the ER membrane leads to an impaired activity triggering the ER stress and eventual unfolded protein response, cell apoptosis[63,64] *via* JNK signaling and to the release of RE Ca^{2+} stores. Adjacent mitochondria readily take up the released Ca^{2+}, and the acute Ca^{2+} overload results in changes in mitochondrial potential and opening of the permeability transition pores (PTPs)[65] ensuring a potent cellular cell signal[66]. Dysregulation in nuclear transcription factors SREBP-2[67], liver X-receptor (LXR)-α and farnesoid X receptor (FXR) might be the cause of cholesterol altered homoeostasis (extensively reviewed by Musso[68]). Interestingly, the incidence of NAFLD in the non-obese population has been associated with a high dietary cholesterol intake rather than intake of polyunsaturated fatty acids[69].

Chronic inflammatory state

Persistent IR associated with an excessive caloric diet and sedentary life style lead to obesity, now recognized as a chronic inflammatory disorder. Thus, inflammation is considered the major risk of obesity and is associated with white adipose tissue dysfunction. An altered adipokine profile has been suggested to play a pivotal role in the initiation and perpetuation of the pathological events[70,71]. In NAFLD, adipose tissue contributes to the systemic production of TNF-α[72], MCP1, IL6 and adiponectin; these mediators modify the hepatic inflammatory/immune system[56-59]. Furthermore, it has been reported that the adipose tissue of obese subjects presents an increased number of macrophages[73], and they

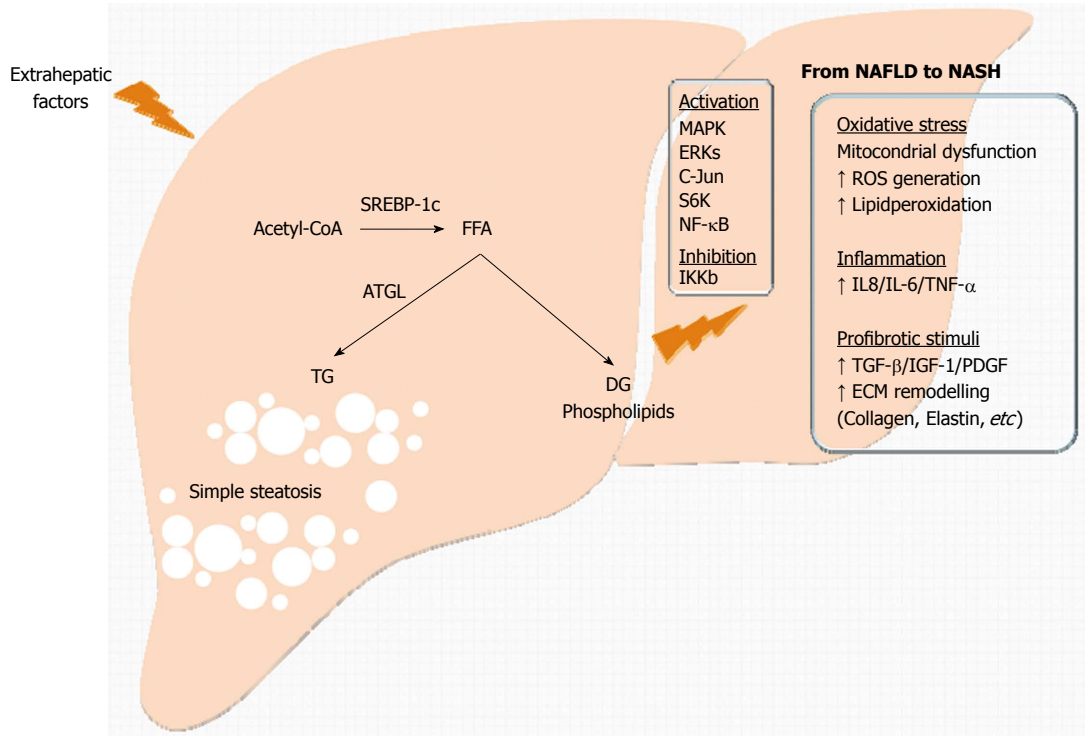

Figure 3 Effect of intracellular fat accumulation within the liver. Liver sensitization induces an alteration of the normal hepatic liver metabolism leading to simple steatosis with neutral triglyceride (TG) accumulation or in the more severe cases, to the production of intermediate products (DG and phospholipids) responsible for lipotoxicity. Alteration of several mediators of signaling pathways leads to the events observed during the progression from non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH) (hepatic insulin resistance, oxidative stress, inflammation, and fibrosis). IKKb: Protein Kinase-1-mediated IB Kinase; ROS: Reactive oxygen species; IL-6: Interleukin-6; TNFα: Tumor necrosis factor-α; IGF-1: Insulin-like growth factor-1; PDGF: Platelet-derivedgrowth factor; ECM: Extracellular matrix; MAPK: Mitogen-activated protein kinases; ERKs: Extracellular signal-regulated kinases; NF-κB: Nuclear factor κB.

might account for much of the adipose tissue inflammatory cytokine secretion. These cells presumably arise from peripheral blood monocytes that become activated by hyperinsulinemia and the abnormal levels of FFA encountered in individuals with IR. Monocytes have also been shown to be activated in poorly controlled type 1 diabetes, showing an increased ability to attach to the endothelial cells[74], one of the early stages in atherosclerosis. Moreover, it has been reported that monocytes are strongly correlated with glycated hemoglobin (HbA1c), explaining the association between monocytes and IR in type 1 diabetes[75]. Activation of these cells produces abundant quantities of cytokines such as TNF-α and IL6. Studies performed in human monocytes suggest that these cells might respond to the increased concentrations of saturated non-esterified fatty acids observed in IR conditions by producing high levels of IL6. This increased secretion of IL6 could prime these cells to generate a robust local or systemic inflammatory response contributing to the development of complications such as T2DM and atherosclerosis[76]. In the liver, fatty acid accumulation induces mainly the up-regulation of IL8, produced both by hepatocytes and non parenchymal cells[77-79]. It was reported that IL6 and TNF-α signaling *via* TNF-α receptor-1 are important in NASH-related development of HCC, and that hypoadiponectinemia accelerated hepatic tumor formation in the mouse model of NASH[80,81]. A detailed study of the role of the main cytokines in humans and animal models can be found in a recently published work from Braunersreuther *et al*[82]. Collectively, these data confirm the close relationship between lipid metabolism and liver cancer in animal experimental models, although there are still many doubts regarding human studies.

Contribution of oxidative stress

Several papers demonstrate that oxidative stress occurs during NAFLD, especially due to mitochondrial dysfunction[14,83-86]. It has been reported that activated hepatic mitochondrial metabolism[87] is a common characteristic of NAFLD in both human subjects[88] and animal models[89]. However, the regulatory connection linking FFA to altered mitochondrial function is still undefined. Currently, there are two competing views on the role of lipid beta-oxidation in the development of NAFLD[88,89]. One view holds that impaired or incomplete beta-oxidation leads to hepatic steatosis and accumulation of lipid intermediates that inhibit insulin signaling. The other view holds that increased supply of FFA to the liver results in excessive beta-oxidation that fuels reactive oxygen species (ROS) accumulation and inflammation. The loss of electrons from complexes I and Ⅲ in the mitochondrial electron transport chain can combine with oxygen to generate ROS, powerful oxidizing agents that indiscriminately damage many important components of the cell including DNA, lipid membranes and proteins. ROS are

known to activate pro-apoptotic pathways and initiate programmed cell death. However, it has also been reported that ROS-related lipoapoptosis appears to be cell-type dependent[90,91]. Altogether, the role of specific FFA metabolic pathways in promoting ROS accumulation and damage remain largely unclear. The oxidative stress observed in NAFLD subjects might probably be a bystander consequence of a sensitized liver, rather than the main cause of the disease.

Progression of NAFLD to NASH

The progression from simple steatosis to NASH is determined by the initiation of the fibrotic response. Understanding the regulation of the initiation, progression and perpetuation of fibrosis will be very important, particularly from a therapeutic viewpoint. Hepatic stellate cells (HSC) are the main regulators of extracellular matrix (ECM) production and play an essential role in the development of fibrosis (extensively reviewed elsewhere[92,93]). Under normal conditions, HSC have a quiescent phenotype and constitute a third of the non-parenchymal cell population; 85% of hepatic vitamin A is dissolved and stored within quiescent HSC[94]. However, these cells can be activated by noxious stimuli triggered by damaged hepatocytes. When activated, HSC undergo several phenotypic and functional changes. A decrease in the retinoid content is accompanied by a strong increase in the production of extracellular components and cell proliferation. During the initial fibrogenic process, there is a cross-talk between injured hepatocytes and HSC, which is further stimulated in a paracrine mode by the infiltrated leukocytes and activated Kupffer cells (KC). The initial process is followed by the perpetuation of the fibrogenic response. The master regulator of this process is TGF-β[95], a pro-fibrotic cytokine released by almost all the involved cells, whose effect is cell-type dependent. For instance, in mature hepatocytes TGF-β is responsible for inhibition of cell proliferation and participates in the induction of apoptosis[96], while in HSC it promotes cell activation[97] and enhanced production of ECM (collagen, elastin, proteogycans, among others) associated with a decreased degradation by inhibition of the activity of matrix metalloproteinases.

From 1980 when Ludwig *et al*[98] first defined the condition, great efforts have been dedicated to elucidate the underlying mechanisms involved in this multifactorial and frequent disorder. In spite of data obtained in clinical settings, animal models and *in vitro* systems, the molecular causes of NASH remains mostly speculative, and further investigations are needed.

In vivo and in vitro experimental models

Due to ethical considerations, mechanistic studies are difficult (or impossible) to be conducted in humans. Consequently, the development of experimental models able to mimic the human condition becomes a necessary tool in the study of the pathophysiology and progression from NAFLD to NASH. Over the last two decades, several animal models have been established and proposed as preclinical platforms for the study of NASH development and the definition of therapeutic options. Table 1 summarizes the most used animal models and their characteristics. The main advantage of this approach is the possibility to define pathogenic pathways in a cause-effect response. The goals these models need to fulfill are: (1) that the pathological pattern of liver injury reflects human steatohepatitis; and (2) that the model should reproduce the context in which human NASH develops. The most used models are genetically modified animals (see for detailed reviews[99-102]) such as the *ob/ob* mouse with a mutation in the leptin gene[73] or the *db/db* mouse which lacks the leptin receptor. However, to develop fibrosis and consequent NASH, both models require a methionine and choline deficient (MCD) diet[54]. A controversy still exists about the validity of this diet, since MCD feeding in normal animals induces weight loss and insulin sensitivity[103] despite the impairment of hepatic receptor signaling[104]. Moreover, few human diets are deficient in methionine and choline. Another genetic model consists of animals with deletions in acyl-CoA oxidase (ACOX). Although at an initial stage these animals present severe steatosis and liver inflammatory infiltration with hepatocyte apoptosis, after 6-8 mo they become resistant to steatosis with (PPAR)-α dependent liver regeneration, limiting the utility of this model for the study of steatohepatitis. Deletions in methionine adenosyltransferase (MAT)-1A (MATO mice)[105] and liver-specific *pten* lead to the development of steatohepatitis but without MS. Sterol regulatory element binding protein (SREBP)-1c transgenic mice, which present an overexpression of this protein in adipose tissue, show IR secondary to impaired adipose differentiation leading to severe hepatic steatosis with the histological features of steatohepatitis[106]. Conversely, these transgenic mice exhibit decreased adipose mass limiting its application to NAFLD/NASH, where adipose tissue is the storage compartment that contributes to perturbations of whole-body lipid homeostasis. An alternative genetically modified animal model is the KK-A^y mouse in which there is a heterozygous mutation of the agouti gene (*KK-Ay/a*). Interestingly, these animals present impaired hypothalamic appetite suppression[107] and consequently, they are hyperphagic and develop an obese phenotype. They also present hepatic steatosis in conjunction with IR. However, the main limitation of this model is that NASH does not occur spontaneously, and a MCD diet is required for the induction.

The use of diet-induced models, extensively reviewed elsewhere[102], is another strategy in the study of NASH development. Different diets for small animals have been characterized[108-110] with good results in the development of steatosis and inflammation, but marginal results in generating fibrosis. Different effects depending on the composition of the diet have been reported. High-carbohydrate diets stimulate moderate hepatic lipogenesis in rats, whereas animals fed with high-fat diets present a strong inhibition of this anabolic pathway. The plasma TG levels

Table 1 Summary of the major findings obtained among the most widespread *in vivo* models

Model	Genetic manipulation	Diet modifications	Obesity	Metabolic syndrome (IR)	Hepatosteatosis	Steatohepatitis	Fibrosis
ob/*ob* mice	Leptin Deficient	No Yes MCD	Yes Variable (loss weight in some)	Yes Yes	Yes Yes	Yes (in males) Yes	No (protected) Yes
db/*db* mice	Mutation on leptin receptor	No Yes MCD	Yes Variable (age-related weight gain)	Yes No	Yes Yes	No Yes	No Yes
AOX null mice	Nullizygous for acycil - CoA oxidase	No	No	No	Yes [before 6-8 mo Resistant (after 8 mo)]	Yes (before 6-8 mo) Resistant (after 8 mo)	No
MATO null mice	Nullizygous for (MAT)-1A	No	No	No	Yes	Yes	Yes
pten null mice	Liver specific pten deletion	No	No	No	Yes	Yes	Yes
(SREBP)-1c transgenic mice	SREBP-1c overexpressed in adipose tissue	No	No	Yes	Yes	Yes	Yes
KK-Ay mice	Heterozygous mutation on agouti gene (KK-Ay/a)	No Yes MCD	Yes Yes	Yes Yes	Yes Yes	No Yes	No No
LIRKO mice	Liver-specific Leptin receptor KO	No	No	Hepatic IR	No	-	-
C57Bl/6J	No	Yes HFHC HF	Yes	Yes	Yes	Yes	Yes (mild)
Cholesterol-Cholate (Atherogenic diet)	No	Yes Cholesterol Cholate	No	No (only hepatic IR)	Yes (over 1-6 mo)	Yes (over 1-6 mo)	Yes (over 1-6 mo)

MCD: Methionine choline deficient diet; HFHC: High fat-high carbohydrate diet; HF: High fructose diet; KO: Knock-out; IR: Insulin resistance.

are higher in the high-carbohydrates diets, whereas the high-fat diet determines an accumulation of TG in the liver. However, both diets induce an increase of plasmatic levels of glucose and insulin[111]. Regarding the generation of fibrosis, promising evidence has emerged from mice fed with an atherogenic diet containing 1.25% cholesterol and 0.5% cholate[112]. Under these dietary conditions, a progressive formation of steatosis is observed associated with an evident inflammatory response, induction of oxidative stress and development of fibrosis in 6-24 wk. However, these animals are systematically insulin-sensitive, albeit they develop hepatic IR and surprisingly, they show a weight loss. This makes the cholesterol-cholate model substantially different from human NASH, severely limiting its application. A valid tool for the study of hepatic IR and the effect of insulin on leptin homeostasis is represented by LIRKO mice, a liver-specific insulin receptor knock-out[113]. These animals present abnormal glucose metabolism and progressive liver dysfunction, and display focal dysplasia and hyperplastic nodules. However, serum TG levels are decreased, most probably by the inability of insulin to promote TG synthesis in the liver and by reduced lipolysis in adipose tissue. In spite of the hyperinsulinemia and IR, these animals are not obese[114]. A promising approach is the administration of a high-fat diet associated with high fructose to male C57Bl/6J mice, which induces results similar to those observed in human NASH[115]. In spite of the promising results, substantial objections remain: (1) the long term exposure required for observing the pathological phenotype[116]; (2) the inclusion of only male animals excluding the application of this approach to the female population; and (3) rodents might adapt to high-fat feeding and become resistant to the development of obesity[117].

Worthy of attention is the fact that under specific experimental settings, animals can develop NASH from simple steatosis. However, the data fail to explain why in humans only some individuals develop NASH while others can live with NAFLD with no complications[118]. This crucial issue is still an open question, and most probably may be related to a different response of the cell to fat storage[119,120].

Contrary to other liver diseases in which *in vitro* models are important tools in research, convincing data are still missing in NAFLD and NASH. One of the reasons may be related to the use of a rather simplistic set-up to tackle the multistep process of the development of NASH. The use of an *in vitro* approach presents several advantages and disadvantages, as recently reviewed in detail[121]. A broad spectrum of *in vitro* validated possibilities is available, such as the use of primary cell culture, immortalized cell lines, or an even more sophisticated

system such as precision-cut slices of perfused liver. The main obstacle of the *in vitro* system is the extrapolation of the results to the much more complex human environment. A good example of this limitation is the choice of free fatty acids (FFA), since it has been reported that individual FFA have distinct inherent steatotic and toxic activities, the saturated FFA presenting the highest toxicity[122]. In normal and in NAFLD subjects, the most abundant FFAs in liver triglycerides are oleic acid (18:1) and palmitoleic acid (16:1) for unsaturated, and palmitic acid (16:0) and stearic acid (18:0) for saturated FFAs[123]. The relative concentration has been demonstrated to be a determinant in their hepatic accumulation and toxicity[124]. For instance, different effects of oleic and palmitic acid were reported on lipid accumulation and on the induction of apoptosis. Oleic acid was shown in several hepatic cell lines to be more steatogenic than palmitic acid[125] but less toxic that the latter. Long chain FFAs are highly insoluble in the aqueous phase, and for this reason are carried in blood associated with serum albumin. Whereas under physiological conditions the FFA: albumin ratio is around 2:1[78] under pathological states, the ratio can be as high as 7.5:1[126]. This simple, but fundamental, detail is often disregarded in several studies. In addition, since the development of NAFLD and the progression to NASH involve several cell types, another crucial point is the cell type used in the experimental system. The vast majority of the published data has been obtained in hepatocyte cultures, but for the study of the progression to fibrosis, other cell types such as HSC and KC must be considered. The crucial role played by the interaction among the different cell types points to the need of much more controlled experimental setups to provide a comprehensive approach to the molecular mechanisms involved. For this reason, the establishment of co-culture systems has been acknowledged to be promising in the last few years[77,127-129] with regard to the study of the different intracellular mechanisms.

In any case, in spite of the progress in the molecular biology of NAFLD/NASH, the main limitation of these *in vitro* approaches remains the different models and experimental variables used in the different laboratories. This makes each study somewhat unique and independent from the others. A better definition of the experimental conditions and standardized models would greatly contribute to improving the possibility of achieving solid results.

CONCLUSION

A translational approach to NAFLD and NASH is just at the beginning. The disease is rather new and is still based on a negative definition, but we now know that it is linked to a metabolic dysfunction of the glucose and/or lipid hepatic pathways. The number of patients affected by this disorder is exponentially growing worldwide, and NAFLD diagnosis must be performed early to prevent the progression to NASH, cirrhosis and HCC or to cardiovascular diseases, and to adopt effective preventive strategies. We now have the experimental models to investigate the still unknown reasons why only some types of sugars and lipids induce progressive hepatic inflammation, apoptosis and fibrosis. We hope they will help us to understand the inner mechanism of the damage and design better drugs that will combine with a much healthier lifestyle to fight this plague.

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