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Recent Insights into the Pathogenesis of Nonalcoholic Fatty Liver Disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is a burgeoning health problem worldwide and an important risk factor for both hepatic and cardiometabolic mortality. The rapidly increasing prevalence of this disease and of its aggressive form nonalcoholic steatohepatitis (NASH) will require novel therapeutic approaches based on a profound understanding of its pathogenesis to halt disease progression to advanced fibrosis or cirrhosis and cancer. The pathogenesis of NAFLD involves a complex interaction among environmental factors (i.e., Western diet), obesity, changes in microbiota, and predisposing genetic variants resulting in a disturbed lipid homeostasis and an excessive accumulation of triglycerides and other lipid species in hepatocytes. Insulin resistance is a central mechanism that leads to lipotoxicity, endoplasmic reticulum stress, disturbed autophagy, and, ultimately, hepatocyte injury and death that triggers hepatic inflammation, hepatic stellate cell activation, and progressive fibrogenesis, thus driving disease progression. In the present review, we summarize the currently available data on the pathogenesis of NAFLD, emphasizing the most recent advances. A better understanding of NAFLD/NASH pathogenesis is crucial for the design of new and efficient therapeutic interventions.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a clinicopathological entity that comprehends a liver disease spectrum that spans from noninflammatory isolated steatosis, defined by the presence of triglyceride (TG) accumulation in hepatocytes (conventionally defined as the presence of lipid droplets within the cytoplasm in more than 5% of hepatocytes), to nonalcoholic steatohepatitis (NASH), a more aggressive form of the disease, which is characterized by steatosis, inflammatory changes, and hepatocyte cell ballooning associated with varying degrees of liver fibrosis (1, 2) (**Figure 1**). By definition, the abnormalities develop despite the absence of excessive alcohol consumption (typically defined as <20 g per day in women and <30 g per day in men) (2). NAFLD usually develops in the context of the metabolic syndrome (MetS) and is strongly associated with obesity, insulin resistance (IR), type 2 diabetes mellitus (T2DM), and dyslipidemia (3). Patients with NAFLD exhibit an increased overall mortality compared to the general population, mainly linked to T2DM and cardiovascular risk factors (4), and those with NASH also have an

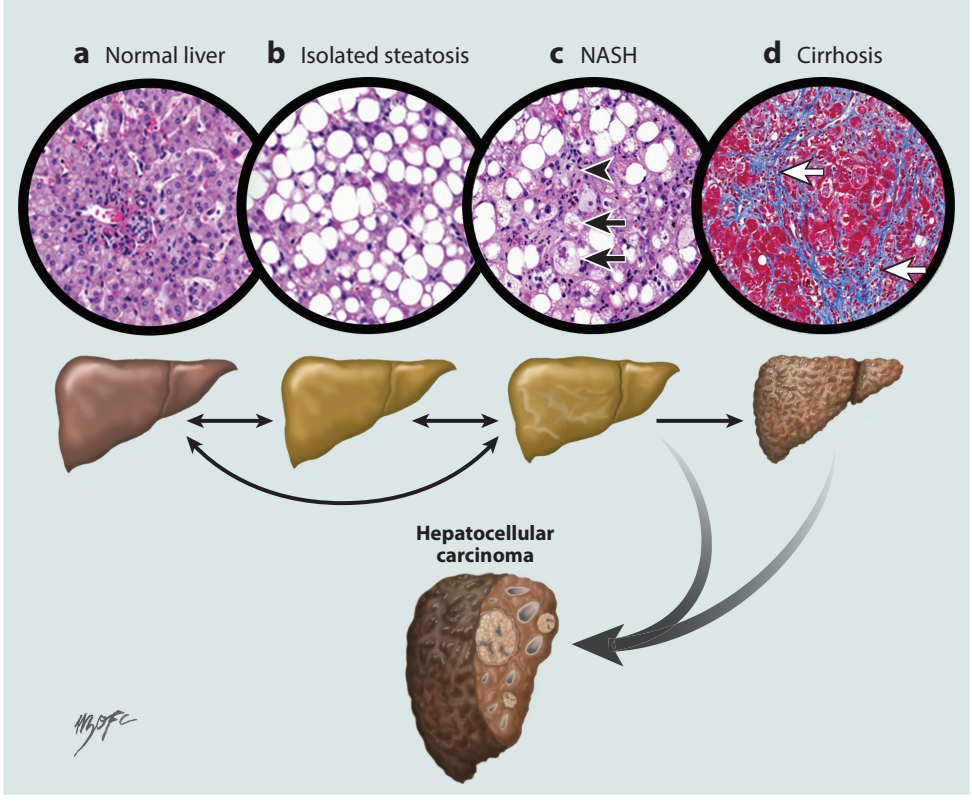

Figure 1

Spectrum of nonalcoholic fatty liver disease (NAFLD). NAFLD encompasses a spectrum of disease, including steatosis in which there is noninflammatory isolated fat accretion in hepatocytes; nonalcoholic steatohepatitis (NASH), a more aggressive form of the disease, which is characterized by steatosis, inflammatory changes (*arrowhead*), and hepatocyte cell ballooning (*black arrows*) associated with varying degrees of liver fibrosis; and cirrhosis with its characteristic collagen bands surrounding liver nodules (*white arrows*). Hepatocellular carcinoma can arise from both precirrhotic NASH and cirrhosis. The different stages of disease can regress or progress as illustrated by the arrows in the middle panel. Also, a fast track pathway may determine that a full-blown NASH develops from the beginning of the disease.

increased liver-related mortality due to the progression to fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (5, 6). In line with the obesity epidemic and sedentary lifestyles, NAFLD has become increasingly common worldwide over the last decades. In a recent meta-analysis, the global prevalence of NAFLD was found to be 25.24% (similar to the prevalence found in the United States, 24.13%), with the highest prevalence rates found in the Middle East (32%) and South America (31%) and the lowest prevalence in Africa (14%) (7). NAFLD can be detected by ultrasound in approximately 90% of obese patients [body mass index (BMI) $\geq$ 30 kg/m^2], 65% of overweight patients (BMI 25.0–29.9 kg/m^2), and 25% of normal-weight patients (BMI 20.0–24.9 kg/m^2) (8, 9). Similarly, the overall prevalence of NAFLD in patients with T2DM is up to 70% (10). Thus, NAFLD is currently considered the most common cause of chronic liver disease, and NASH is the second leading etiology of cirrhosis among adults awaiting liver transplantation in the United States (11) and is expected to be the first leading etiology within a decade (12). Moreover, emerging data suggest that the recent increase in the incidence of HCC is driven by NAFLD, particularly in Western countries (13). A major concern is the increasing incidence of HCC in precirrhotic NASH, which accounts for as much as 30–40% in some series (13–15).

The pathogenesis of NAFLD is multifactorial and its understanding is still incomplete. Although knowledge of the cellular and molecular mechanisms underlying disease development and progression has grown significantly in recent years, the exact contribution of environmental and genetic factors as well as that of extrahepatic and intrahepatic events in determining the disease phenotype remains ill defined (16). This review summarizes and integrates the current knowledge about the pathogenesis of NAFLD and its progression and presents new insights that open the possibility of targeting specific pathophysiological pathways in the treatment of this serious global health problem.

PATHOGENETIC MECHANISMS IN NONALCOHOLIC FATTY LIVER DISEASE

NAFLD is a complex disease, and factors that promote steatosis development and those that trigger hepatic inflammatory responses and fibrogenesis may be at play in a parallel or sequential fashion and with different hierarchies along the whole spectrum of the disease. In 1998, a two-hit hypothesis of the disease was proposed (17). This hypothesis considered steatosis as a first hit to the liver, which then would require a second hit to progress to NASH/fibrosis. This concept turned out to be too simplistic as the different factors influencing disease development and progression were unveiled. Nowadays, a multiple-hit hypothesis that implicates a myriad of factors acting in a parallel and synergistic manner in individuals with genetic predisposition is the more accepted view to explain the different phenotypes observed clinically. Although progression from isolated steatosis (also called NAFL) to NASH is generally conceived as a sequential process, the marked heterogeneity that exists in terms of clinical outcomes of these two subgroups has led to the concept that NAFL and NASH may correspond to two different entities. Thus, hepatocyte injury occurs in NASH, leading to cell death, local inflammation, and fibrogenesis, which would occur very early in the disease triggered and sustained by many different factors, such as IR, adipokines, nutritional factors, and gut microbiota (GM), as well as genetic and epigenetic factors (**Figure 2**). Alternately, significant hepatocyte injury and death would not occur in NAFL, perhaps due to a higher capacity of liver cells to deal with fatty acid (FA) overload. In this case, the histological picture would remain as bland steatosis due to the absence of lipotoxicity (17). Subsequently, NAFL and NASH may also be seen as entirely separated entities. Although plausible, this view is not generally accepted because the progression from NAFL to NASH has been documented in

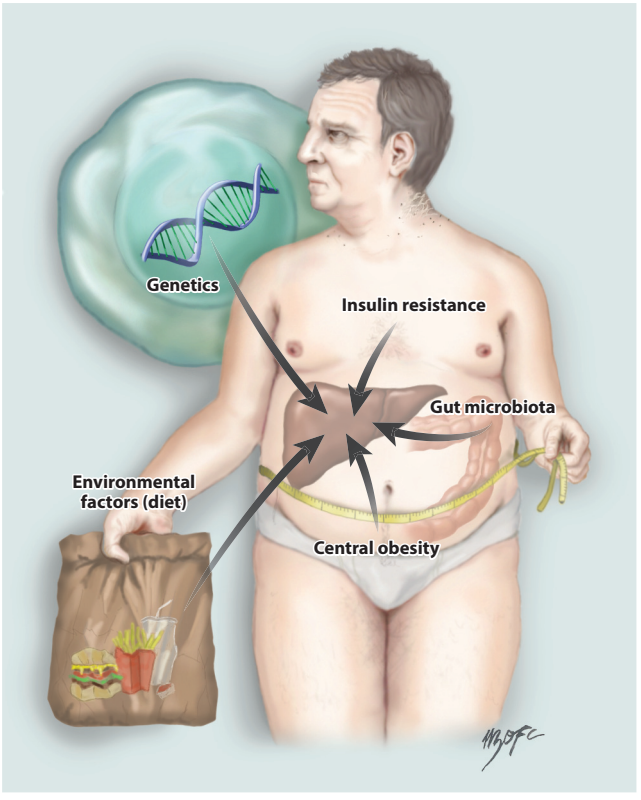

Figure 2

Schematic representing the factors contributing to the pathogenesis of nonalcoholic fatty liver disease (NAFLD). NAFLD is a multifactorial disease; several factors such as insulin resistance, central obesity, environmental or nutritional factors, and gut microbiota, as well as genetic and epigenetic factors, are linked to its pathogenesis.

a large proportion of patients (18). In the following paragraphs, factors contributing to steatosis development and the occurrence of liver cell injury, inflammation, and fibrosis are summarized.

Mechanisms of Steatosis Development

Hepatic lipid homeostasis is tightly regulated by a complex system of signaling/transcriptional pathways governed by hormones, transcription factors, and nuclear receptors with insulin signaling playing a pivotal role (19). TG accumulation is likely the first step in the pathophysiology of NAFLD and results from an imbalance between TG synthesis and utilization. Impairment in insulin signaling at the level of the adipose tissue (AT) and the liver seems to be a very early event (20). In patients with obesity or T2DM, the development of IR leads to an uninhibited lipolysis in the AT, resulting in an excessive influx of nonesterified fatty acids (NEFAs) to the liver where they are taken up by hepatocytes in a facilitated manner by fatty acid transport protein 2 (FATP2), FATP5 (21), and other transport proteins like FA binding protein and caveolin-1 (22, 23). CD36 (also called FA translocase) also facilitates NEFA uptake and intracellular trafficking in several cell types (macrophages, hepatocytes, adipocytes, enterocytes, and myocytes). CD36 has been shown to increase in murine models of hepatic steatosis and is a common target of liver

X receptor (LXR), pregnane X receptor (PXR), and peroxisome proliferator-activated receptor gamma (PPARγ) (24). The role of CD36 in human disease is not well defined, but in morbidly obese patients with NAFLD, messenger RNA levels of CD36 have been correlated with liver fat content and apoptosis (25, 26).

Dietary fat and de novo lipogenesis (DNL), which is enhanced in NAFLD (27), are the other two contributing sources for hepatic fat accumulation. Hepatic FA synthesis is a process catalyzed mainly by two enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), and controlled at the transcriptional level via the activation of sterol regulatory element-binding protein 1c (SREBP-1c) by insulin and via the activation of carbohydrate-responsive element-binding protein (ChREBP) by glucose. In turn, the activity of both transcription factors is controlled by LXR (28). LXR regulates both response elements and directly induces ACC and FAS (29). DNL is markedly increased in NAFLD mainly due to the coexistent hyperinsulinemia and excessive intake of simple sugars, such as fructose (see below), that activate SREBP-1c and ChREBP, respectively (27, 30). Interestingly, in NAFLD patients DNL is not suppressed in the fasting state, underscoring the important role for this pathway in hepatic steatosis.

Attempts to dissect the origin of hepatic TG in NAFLD have been made using labeled stable isotopes and indicate that serum FA (derived from AT lipolysis) can account for up to 60% of hepatic TGs in NAFLD patients, while the contribution of DNL, which has been shown to be up to threefold higher in NAFLD patients compared to controls, has been estimated in 25% in NAFLD patients, with a dietary contribution of approximately 15% (27, 31).

Impaired TG utilization related to disturbed hepatic FA oxidation or impaired synthesis or altered secretion of very low-density lipoprotein (VLDL) can also contribute to TG accumulation. Hepatic FA oxidation occurs in the mitochondria, peroxisomes, or microsomes, leading to a high-yield energy production. During fasting, the main source of energy is the β-oxidation of FAs, where FAs enter into the citric cycle after being broken down into acetyl-CoA. In order to be used for β-oxidation, long-chain fatty acids (LCFAs) need to be transported to the mitochondria by activation of acyl-CoA-synthetase to acyl-CoA in the cytosol. LCFAs are transported and catalyzed in the outer mitochondrial membrane by carnitine palmitoyl transferase 1 (CPT1). CPT1 is inhibited by malonyl-CoA (a key intermediate in DNL) and insulin (32) and activated by PPARα (33). During states of FA excess, acetyl-CoA can undergo ketone body conversion instead of entering the citric acid cycle (34) and has a CYP4A-dependent ω-oxidation in the endoplasmic reticulum (ER) (35). The role of mitochondrial beta oxidation in NAFLD is not clear because contradictory reports have been published with studies showing both reduced and increased rates of FA oxidation in NAFLD, with some studies demonstrating an impairment with progression to NASH (see below) (36). As for VLDL export, it has been shown that the rate of TG secretion as VLDL in NAFLD is increased, although this cannot compensate the increased rate of TG synthesis.

Dietary caloric and specific nutrient intake is pivotal for the development of hepatic steatosis. Among dietary factors, fructose is of central importance since it is both a substrate and a powerful inducer of hepatic DNL through the activation of key transcription factors such as SREBP-1c and ChREBP. Of note, hepatocytes are exposed to higher concentrations of fructose than cells of other tissues since, after absorption, fructose is directly delivered to the liver via the portal vein (30). In addition, carbohydrates can also regulate hepatic FA metabolism contributing to lipogenesis by entering the Krebs cycle to produce acetyl-CoA for DNL or by providing the glycerol backbone for TG synthesis via triose-phosphate. Finally, glucose also induces the transcription of pyruvate kinase. Pyruvate can be processed in the metabolic citric acid cycle or be used for DNL in an anabolic pathway (19).

Insulin signaling plays a central role in hepatic steatosis. Obesity and the impairment of insulin action in adipocytes lead to a failure in lipolysis suppression, adipocyte stress, and recruitment and

infiltration by macrophages in the AT, with a consequent release of proinflammatory adipocytokines, mainly tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), resistin, and plasminogen activator inhibitor-1 (PAI-1). These adipocytokines contribute to the disruption of insulin signaling by nuclear factor κβ (NF-κβ) and c-Jun N-terminal kinase (JNK) pathways (37) creating a vicious cycle in the AT and promoting IR in other insulin-sensitive tissues. Alternately, protective adipokines (those that reduce IR) such as adiponectin are reduced in NAFLD patients, and the administration of recombinant adiponectin has shown improvement in insulin sensitivity and steatosis in murine models of NAFLD, probably through AMP-activated protein kinase (AMPK) and PPARα pathways (38, 39). Additionally, the accumulation of lipids in myocytes leads to IR and impairs glucose uptake. Thus, the development of peripheral IR and hyperinsulinemia contributes to the increased FA influx to the liver. In the liver, activation of the insulin signaling pathway increases DNL without suppression of gluconeogenesis. Furthermore, diacylglycerides contribute to increasing hepatic IR by increasing translocation of the protein kinase C (PKC) isoform ε, which can inhibit the intracellular domain of the insulin receptor, providing more mechanisms to TG accumulation in the liver. Diacylglyceride content and PKCε have been associated with hepatic IR in a study of obese patients (40).

Factors Involved in Disease Progression Toward Nonalcoholic Steatohepatitis

Although most patients have noninflammatory isolated steatosis, approximately one-third will develop NASH, which is the necroinflammatory form of the disease that confers further risk of progression toward more advanced liver fibrosis, cirrhosis, and cancer. Inflammation develops when the influx of FAs toward the liver overwhelms physiologically adaptive mechanisms, leading to reactive oxygen species (ROS) formation, ER stress, and hepatocellular dysfunction and injury in a process named lipotoxicity (41, 42). Cellular injury leads to immune and apoptotic pathway activation, resulting in cell death, which is one of the major and most important pathogenic events that, over time, drives fibrosis and cirrhosis development (43, 44).

TG synthesis from FAs is considered a key mechanism to avoid lipotoxicity. The key enzymes involved in this protective pathway are stearoyl-CoA desaturase 1 (SCD1), diglyceride acyltransferase 1 (DGAT1) and DGAT2, ACC, and FAS. Of note, disturbed activity of these enzymes may result in increased lobular inflammation and fibrosis. This has been shown experimentally in mice undergoing DGAT2 knockdown and in SCD1 knockout mice fed a methionine-choline deficient diet that accumulate less TG but exhibit more hepatocyte apoptosis and liver injury (45–47). Thus, TG synthesis serves as an adaptive response to the intracellular accumulation of lipotoxic FAs, which are partitioned into inert lipids.

The excessive cellular load of FAs also leads to mitochondrial adaptations that in turn lead to enhanced FA oxidation and upregulation of the mitochondrial respiratory chain. However, this results in ROS overproduction that exceeds normal antioxidative mechanisms, leading to oxidative stress. This event likely plays a significant role in the initiation of NASH. Notably, mitochondrial function seems to decrease with advanced stages of NASH (which correlates with ultrastructural mitochondrial defects), impairing energy output and aggravating ROS overproduction (48). More recently, the role of increased mitochondrial cholesterol content as a relevant factor in determining mitochondrial dysfunction has gained attention (49). In addition, β-oxidation also occurs in peroxisomes while ω-oxidation takes place in the ER. These pathways can participate in the adaptive response to lipid accumulation, contributing to the production of ROS and generation of oxidative stress (50). Of note, CYP2E1 and CYP4A have been suggested as major sources of oxidative stress in NAFLD (51). In turn, oxidative stress results in DNA damage, phospholipid membrane disruption by lipid peroxidation, and secretion of proinflammatory cytokines (52). Lipid peroxidation

of polyunsaturated fatty acids (PUFAs) generates toxic products, including malondialdehyde and hydroxynonenal, which are highly toxic and proapoptotic (53).

The role of iron in the progression of NASH remains controversial, but there are data suggesting that iron may have a role in the pathogenesis of NASH. In a murine model of db/db genetically obese mice, iron overload results in hepatic oxidative stress, immune cell activation, and hepatocellular ballooning injury, leading to NASH (54). In humans, it has been reported that subjects with NASH have increased duodenal iron absorption through upregulation of divalent metal transporter 1 (55) and that hepatocellular iron deposition in NASH contributes to disease progression through increased ROS generation and higher rates of hepatocyte apoptosis (56). Finally, hepatocellular iron deposition has also been associated with an increased risk of hepatic fibrosis (57).

The ER, which is an intracellular organelle where most of the secreted and membrane proteins are folded, is also sensitive to free fatty acid (FFA) injury, leading to the accumulation of unfolded or misfolded proteins. This accumulation activates the unfolded protein response, which is an adaptive response to reestablish homeostasis; if this response fails, other stress sensor proteins, such as inositol-requiring enzyme 1 (IRE1), activating transcription factor 6, and protein kinase R–like ER kinase (PERK), trigger autophagy pathways (58, 59). IRE1 splices and activates the transcription factor XBP1 (60). XBP1 interacts with various inflammatory cascades by the activation of JNK and inhibitor of κB (IκB) kinase (IKK)-NFκB signaling and the production of ROS (61).

The innate immune system also plays a role in disease pathogenesis and progression. Bacterial products are identified by the immune system through recognition of pathogen-associated molecular patterns (PAMPs), which are a limited and defined set of conserved molecular patterns carried by all microorganisms of a given class (62). The most studied gut-derived PAMP is the bacterial endotoxin known as lipopolysaccharide (LPS), which is found in the outer membrane of gram-negative bacteria. Once bacterial products reach the liver, they can activate immune cells through pattern recognition receptors such as the membrane-bound Toll-like receptors (TLRs) and the cytoplasmic nucleotide-binding oligomerization domain-like receptors (NLRs). TLRs are transmembrane proteins that play a key role in the innate immune system, usually expressed in sentinel cells such as macrophages and dendritic cells; however, TLRs have been identified in the response to LPS of hepatic nonimmune cells, including hepatic stellate cells (HSCs) and endothelial cells (63, 64). TLRs recognize structurally conserved PAMPs and activate an inflammatory response (65, 66). There are 10 subtypes of TLRs (67). One subtype is TLR4, which activates the innate immune response to LPS through the coreceptors CD14 or MD-2 (65, 68). Signaling downstream of TLR4 may be either MyD88-dependent or -independent (69). The MyD88-dependent pathway involves nuclear translocation of NF-κB (70). NF-κB activation induces the release of proinflammatory cytokines such as TNFα, IL-6, and IL-1β (71). The MyD88-independent pathway implies the phosphorylation of interleukin regulatory factor 3, which leads to the induction of type I interferon (72, 73). Other TLRs of relevance are TLR2 and TLR9, which are activated in response to peptidoglycan and unmethylated CpG motifs, respectively (74, 75). TLRs can also be activated by damage-associated molecular patterns released from injured cells or tissues (76). This fact may lead to sterile inflammation and plays a role in the pathogenesis of NAFLD (65, 77). TLR4 serves as a receptor for both endotoxin and FAs and has been linked to ER stress. X-box-binding protein 1 (XBP1) is a key transcription factor that mediates the unfolded protein responses in ER stress. TLR4 mediates the transition of benign steatosis to steatohepatitis by the reactive oxygen-species-dependent activation of XBP-1, thereby leading to NF-κB activation and proinflammatory cytokine production. The inactivation of either TLR4 or XBP-1 has been shown to protect against diet-induced steatohepatitis and liver injury in ApoE$^{-/-}$ mice (78). In

addition to the activation of innate immunity, changes in adaptive immunity, such as the activation of Th17, are increasingly recognized. Differentiation of a naïve T cell into a Th17 cell leads to proinflammatory cytokine and chemokine production with subsequent myeloid cell recruitment leading to an inflamed tissue (79).

Recently, several research groups have shown that lipotoxicity in hepatocytes is linked to an increased inflammatory response through the release of cell-derived extracellular vesicles (EVs). These EVs contain several bioactive molecules (including proteins, lipids, and nucleic acids) that serve as cell-to-cell messengers and may modulate the progression of NASH-activating resident mononuclear cells and increase the liver homing of immature myeloid cells (for a recent in-depth review on this topic, see 80). EVs may also signal to other cells, such as endothelial cells activating angiogenesis and HSCs inducing a profibrogenic phenotype (81). The release of EVs from hepatocytes is a complex process induced by toxic lipid overload and mediated by the TNF-like apoptosis-inducing ligand (TRAIL) receptor 2 (TRAIL-R2) signaling cascade and Rho-associated protein kinase 1 (80). Interestingly, it has been proposed that EVs may serve as biomarkers of NASH, but further research is needed to determine if EVs in serum may discriminate patients with NASH from those with simple steatosis.

In addition to inflammation, apoptosis and the activation of cell death pathways are distinctive in the pathophysiological features of NASH. Cell death can occur as a result of a programmed process with intact plasma membrane (apoptosis) or an accidental process with lysis of the cellular membrane (necrosis). In the context of lipotoxicity, this process has also specifically been referred to as lipoapoptosis. Apoptosis is tightly regulated and follows the extrinsic or intrinsic pathways. The extrinsic pathway is mediated by death receptors on the cell surface, such as FAS, TNF receptor 1 (TNF-R1), and TRAIL receptors. The intrinsic pathway is mediated by the activation of ER and mitochondria stress mechanisms (82). FAs can upregulate cell death receptors, and those receptors are more expressed in NASH patients compared to patients with steatosis (83, 84). Furthermore, FFAs can induce lysosomal permeabilization, mitochondrial dysfunction, and caspases activation, leading to apoptosis (85). Since apoptosis is a key feature in NASH, markers of apoptotic activity, such as cleaved cytokeratin-18 fragments, have been correlated with disease activity and severity and could be used as noninvasive markers for NASH (86, 87).

In addition to apoptosis and necrosis, there are other forms of cell death that have a role in NASH. Necroptosis is the term used to refer to cell death induced by receptor-associated signaling but is caspase independent. Necroptotic signaling is transduced by the necrosome, a complex of proteins [mainly receptor-interacting protein 1 (RIP1) and RIP3], which has been seen to be upregulated in NASH (88). Pyroptosis is a caspase-1-dependent form of programmed cell death, mediated by NLR pyrin domain-containing 3 (NLRP3) inflammasomes. Animal models of NASH have demonstrated the importance of NLRP3 inflammasome in hepatocyte pyroptosis (89).

Finally, autophagy, which is an important degradative cellular pathway of the autodigestion of cellular proteins and organelles to obtain energy, has been suggested to play a role in NAFLD/NASH, although information is still fragmentary. Autophagy seems to play critical functions in both hepatocytes and nonparenchymal cells (i.e., macrophages and HSCs) influencing insulin sensitivity, lipid accumulation, hepatocellular injury, and the innate immune response (90). Toxic FAs, such as palmitic acid, suppress autophagy, while oleic acid has been shown to promote it (91). Mice with genetic ablation of *Atg7*, a critical autophagy mediator, have shown increased hepatic fat content accumulation, related mainly to a reduction in the secretion of TGs that were packaged in VLDL particles (92). Also, the autophagic degradation of intracellular lipid droplets (termed lipophagy) may play a role in buffering FA toxicity and maintaining hepatic lipid homeostasis in the background of FA overload (93, 94). Thus, published data suggest that the

pharmacological activation of lipophagy could be an attractive strategy to export hepatic lipids and prevent human NAFLD, but more data are needed. Of note, Kim et al. (95) showed that mouse models with skeletal muscle–specific deletion of *Atg7* were protected from obesity and IR by inducing Fgf21, warranting more studies in this regard.

Mechanisms of Fibrosis in Nonalcoholic Steatohepatitis

As in most chronic liver diseases, ongoing liver injury in NASH is associated with the occurrence of hepatic fibrogenesis, ultimately leading to liver cirrhosis. Liver scarring is the consequence of a wound-healing response of the liver to injury and consists of the deposition of high-density extracellular matrix (ECM) proteins that distort the liver architecture and form regenerative nodules. Basic studies had unveiled the major role of HSCs as crucial mediators of the fibrogenic response (96). HSCs are vitamin A–storing resident mesenchymal cells that lie in the space of Disse and undergo phenotypic and genotypic changes upon the occurrence of hepatocyte damage (97). The activation of HSCs engages a complex cascade of events that involves paracrine stimulation by neighboring cells [Kupffer cells (KCs), hepatocytes, bile duct epithelial cells, platelets, and sinusoidal endothelium], which are mediated by several cytokines [particularly transforming growth factor beta 1 (TGF-β1)], which are then perpetuated by autocrine stimuli. After activation, HSCs lose their retinoid stores, acquire contractile properties, produce tissue inhibitors of metalloproteinases (TIMPs), and proliferate and produce ECM components. Thus, an enlarged hepatic population of HSCs then leads to the progressive accumulation of scarring proteins. Excellent reviews of the general mechanisms of hepatic fibrosis summarizing these advances can be found elsewhere (98–100). Here we summarize recent information regarding the potential disease-specific mechanisms of fibrosis at play in NAFLD/NASH (101, 102). Indeed, the profoundly disturbed metabolic milieu of NAFLD may critically influence fibrogenesis. For instance, hyperinsulinemia is universally present in NAFLD, and insulin promotes profibrogenic signals in the HSCs of connective tissue growth factor, which mediates profibrotic activity either directly or as a cofactor of TGF-β, a key cytokine mediating the induction and promotion of fibrogenesis (103, 104). Also, dysglicemia and T2DM, which are commonly present in NAFLD, determine the occurrence of nonenzymatic glycation and oxidation of proteins and lipids, resulting in advanced glycation end product (AGE) formation. HSCs express a receptor for AGEs and undergo activation when exposed in vitro to glyceraldehyde-derived AGEs (101). AGEs are also able to promote fibrogenesis through the modulation of TNFα, converting enzyme activity (105). These observations likely explain why the degree of IR is associated with disease severity and the fact that patients with T2DM affected by NASH may have rapidly progressive disease and a poorer prognosis (106).

The role of adipokines in hepatic fibrosis development in NAFLD/NASH is also relevant (see the section titled Role of Adipokines and Hepatokines) (107). These AT-derived hormones exert a myriad of regulatory functions that influence insulin sensitivity, inflammation, and lipogenesis. With regard specifically to hepatic fibrogenesis in NAFLD, the two key adipokines are leptin and adiponectin, which appear to act as opposite substances, with leptin being a profibrogenic molecule and adiponectin having strong antifibrotic properties (108, 109). The leptin receptor is expressed in both KCs and HSCs. In KCs, it upregulates the expression of TGF-β, which likely contributes to HSC activation in a paracrine manner. In HSCs, leptin stimulates the production of TIMP-1 and the expression of collagen 1 and represses the expression of matrix metalloproteinase 1 (MMP1). In addition, leptin upregulates microRNA 21, which is closely linked to the profibrogenic TGF-β/smad pathway. MicroRNA 21 targets the inhibitory smad protein, smad-7, and promotes SMAD2/3-SMAD4 colocalizations in the nucleus. Finally, leptin also maintains the

activated phenotype of HSCs via the upregulation of the hedgehog (Hh) pathway, which has been extensively studied in experimental models of NAFLD/NASH and seems to be critically involved in fibrogenesis in this setting. Of note, Hh ligands and Hh-responsive cells are increased in NASH, and overactivation of the Hh pathway is profibrogenic in animal models. Regarding adiponectin, it has been shown that upon binding its cognate receptors, adiponectin receptors 1 and 2, this adipokine can critically influence hepatic fibrosis development through multiple mechanisms, including direct antifibrotic effects on HSCs and indirect antifibrotic roles related to its potent anti-inflammatory activity. Serum levels of adiponectin are usually reduced in NAFLD, whereas leptin is increased, contributing to the profibrotic milieu of fibrosis. Consistently, a correlation between hypoadiponectinemia and the presence of liver fibrosis has been reported (110).

Other factors that may act as drivers of fibrosis in NAFLD/NASH are the accumulation of free cholesterol (FC) as a result of increased dietary intake and absorption and a disturbed cellular cholesterol homeostasis in fat-laden hepatocytes (49). While promoting apoptosis and necrosis in hepatocytes (111), FC accumulation in KCs and HSCs contributes to both inflammation and fibrogenesis. It has been shown that mice fed a high-fat high-cholesterol diet develop NASH and accumulate intracellular cholesterol crystals in KCs, which is associated with increased inflammasome activation (112) and secretion of proinflammatory mediators (e.g., IL-6 and -8 and TNFα) and profibrotic factors, particularly TGF-β (49). Cholesterol crystals have been implicated in the activation of the inflammasome and macrophages in atherosclerotic lesions; thus, these findings suggest that a similar process may be at play in NASH (113, 114). In HSCs, Tomita et al. (115) showed that high FC levels upregulate TLR4 by suppressing the endosomal-lysosomal degradation pathway of this receptor and sensitizing HSCs to TGF-β, resulting in increased liver fibrogenesis. The same group also reported that the enzyme acyl-CoA:cholesterol acyltransferase 1 (ACAT1), which catalyzes the conversion of FC to cholesterol ester, markedly influences hepatic fibrogenesis as ACAT1 deficiency aggravates liver fibrosis in mice (116).

Finally, GM can influence hepatic fibrogenesis in NASH (see below). It has been shown that NAFLD/NASH is associated with a disruption of the bacterial gut community (also termed dysbiosis), which has been clinically linked with disease development and progression (117–119). Work from De Minicis et al. (120) elegantly demonstrated that dysbiosis contributes to fibrogenesis in experimental NAFLD, documenting changes in the microbiota composition (i.e., an increase in the percentage of gram-negative Proteobacteria and a reduced ratio between Bacteroidetes and Firmicutes) in high-fat diet–fed mice and a profibrogenic activity of this microbiota upon transplantation into mice fed a control diet and subjected to a fibrogenic challenge. The mechanisms at play are still ill-defined but likely involve the occurrence of a more robust inflammation and activation of HSCs. Since HSCs express TLRs, gut-derived bacterial products can indeed contribute to their activation in NASH, as shown recently by Bigorgne et al. (121). Also, the role of inflammasome activation in both the liver and intestine is linked to liver injury and fibrosis in NASH. Henao-Mejia et al. (122) showed that mice genetically deficient in inflammasome components exhibit higher susceptibility to developing dysbiosis and more liver damage. Of note, a high-fat diet seems to downregulate inflammasomes in the gut and upregulate this pathway in the liver. Along the same line, mice lacking *Tlr2* had reduced inflammasome activation with less inflammation and fibrosis upon dietary induction of NASH, and pharmacological blockade of inflammasomes reduces fibrosis in experimental NASH (123).

Role of Adipokines and Hepatokines

Adipokines are cytokines secreted by AT. The most relevant are leptin, adiponectin, adipsin, resistin, visfatin, TNFα, and PAI-1. Some of these adipokines, such as leptin and adiponectin, have

been shown to have a role in NAFLD. Animal models of leptin knockout mice (*ob/ob* mice) have shown reduced fibrogenesis, which increases with leptin administration (124). Notably HSCs, KCs, and sinusoidal endothelial cells express leptin receptors (125, 126). Leptin can activate quiescent stellate cells (stimulating proliferation, collagen deposit, and ROS generation) and stimulate TGF-β production by KCs (127). In humans, leptin levels showed an inverse relationship with the degree of steatosis in a small study of patients undergoing bariatric surgery (128).

Adiponectin (also called apM1, ACRP30, AdipoQ, and GBP28) is a 274-amino-acid-long polypeptide that modulates a number of metabolic processes, including glucose regulation and FA oxidation (129). Adiponectin is induced by PPARγ and has been shown to have antisteatotic, anti-inflammatory, and antifibrotic effects. Adiponectin transcription is tightly regulated by PPARγ via direct binding to conserved *cis*-acting regulatory DNA elements. Adiponectin inversely correlates with hepatic steatosis, necroinflammatory activity, and MetS in humans, and levels are increased in mice and in humans after treatment with PPARγ agonist agents (130). The effects of adiponectin are mediated by membrane receptors called AdipoR1, which is abundantly expressed in skeletal muscle, and AdipoR2, which is predominantly expressed in the liver (131). Adiponectin activates AMPK and PPARα; this leads to lowered IR as a result of decreased TGs in muscles and liver (132). Furthermore, adiponectin inhibits endothelial NF-κβ signaling and proinflammatory cytokines such as IL-6 and TNFα, while stimulating anti-inflammatory cytokines such as IL-10 (133, 134). The antisteatotic effect of adiponectin is in part mediated by its ability to increase CPT1 activity, thereby enhancing hepatic FA oxidation, while reducing the activities of ACC and FAS, and expression of SREBP-1c (39, 135). Adiponectin-deficient mice are protected against chronic carbon tetrachloride (CCl4) injury. This modulation of HSCs is produced by inhibition of proinflammatory pathways (NF-κB), reduced TGF-β-induced profibrogenic gene expression, and increased caspase-mediated apoptosis. HSCs express adiponectin receptors, and HSC antifibrotic action is mediated through AMPK-mediated pathways (136, 137). Through these combined immunometabolic effects, adiponectin is a central player in the pathogenesis and progression of NASH.

The term hepatokines is used to define proteins exclusively or predominantly produced by the liver with both paracrine and endocrine activities. It has been shown that the liver may affect the lipids and glucose metabolism by hepatokines released into the blood, and NAFLD seems to be associated with altered hepatokine production. Fetuin-A (also known as α2-HS-glycoprotein), fibroblast growth factor-21 (FGF-21), selenoprotein P, sex-hormone-binding globulin (SHBG), angiopoietin-related growth factor (also known as angiopoietin-related protein 6), and leukocyte derived chemotaxin-2 (LECT2) are considered to be the most important hepatokines. Fetuin-A might have a major role in regulating insulin sensitivity in the pathophysiology of T2DM and cardiovascular disease in humans. The production of fetuin-A is increased in steatotic and inflamed liver, but not in expanded and dysregulated AT. FGF-21 has been shown to increase energy expenditure, decrease IR, and increase β-cell survival (138).

Gut-Liver Axis and Microbiota

The intestine and the liver are in a close anatomical and functional relationship. The GM comprise a wide spectrum of microorganisms (mainly bacteria) that contribute to digestion, the synthesis of vitamins, energy extraction, and resistance to colonization. The microbiota may also stimulate the immune system in the gastrointestinal tract and decrease pathogens by competing for nutrients and space (139). GM and bacterial translocation play an important role in the pathogenesis and progression of chronic liver diseases, including NAFLD, which has been associated with qualitative and quantitative (overgrowth) changes in GM (140). Due to dysbiosis (defined as

the imbalance between protective and harmful bacteria), impairment of the intestinal barrier, and altered immunity status, bacterial products can reach the liver through the portal vein, where they are recognized by specific receptors, activate the immune system, and induce pathways such as stress-activated protein kinases, JNK, p38, interferon regulatory factor 3, and NF-κβ, leading to a proinflammatory response, IR, obesity, hepatic steatosis, fibrosis, and NASH development and progression, through multiple interactions with the host's immune system and other cell types (122).

In animal models, a pro-obesity microbiota class has been described that has the ability to extract more nutrients from the diet. Furthermore, this pro-obesity phenotype is transmissible to lean germ-free mice, which become obese (141). Microbiota can influence energy absorption by fermenting resistant starch and nonstarch polysaccharides to short-chain fatty acids, mainly acetate, propionate, and butyrate, and by making them absorbable by the intestinal epithelium (142). Moreover, microbiota dysbiosis can increase lipoprotein lipase activity, increase TG accumulation, and promote NASH by either reducing choline levels or increasing methylamine levels (143). Microbiota can also affect bile acids (BAs), regulate farnesoid X receptor (FXR) signaling (144), and alter endogenous alcohol production. NASH may be associated with specific changes in microbiota composition that are different from simple steatosis and may serve as a biomarker. Some of the bacteria associated with the progression to NASH or fibrosis are associated with the production of endogenous ethanol or changes in BA composition (145).

The intestine has a highly specialized epithelial membrane that regulates transport across the mucosa (146). Several studies have shown an altered intestinal permeability in patients with NAFLD and its potential role in NASH development (147). Tight junction proteins, such as zonula occludens and claudins, normally seal the junction between intestinal endothelial cells at their apical aspect and thus have a vital role in preventing translocation of harmful substances from the gut into the portal system. Murine models of high-fat diets have shown dysbiosis and increased intestinal permeability, with translocation of bacterial LPS from gram-negative bacilli, and an association with NASH severity (148). A recent study by Rahman et al. (149) showed that loss of junctional adhesion molecule A (JAM-A) promotes severe steatohepatitis in mice on a diet high in saturated fat, fructose, and cholesterol. These authors also showed that colon tissues from patients with NAFLD have lower levels of JAM-A and higher levels of inflammation than those from subjects without NAFLD. Other authors have shown increased gut permeability in NAFLD patients, which is associated with the disruption of intercellular tight junctions in the intestine, evaluated by immunohistochemical analysis of zona occludens-1 protein (150). Finally, some dietary compounds (e.g., fructose) and certain specific receptors [e.g., G protein–coupled chemokine receptor (CX3CR1)] may regulate intestinal permeability in NAFLD, contributing to disease progression (151, 152). Thus, altered intestinal permeability seems to play a role in NAFLD and may also be a target of future therapies.

Lastly, it has been shown that the NLRP3 inflammasome pathway is important in modulation of microbiota in the intestine. Defective NLRP3 inflammasome pathways result in dysbiosis with an increased translocation of endotoxins and thus more inflammation in the liver (122). Thus, restoration of intestinal barrier integrity and manipulation of GM might be developed as therapeutic strategies for patients with NASH.

Role of Bile Acids

BAs are no longer considered to be only detergents that stimulate hepatic bile flow and biliary excretion and aid the digestion and absorption of fats from the intestinal lumen. In fact, BAs

have emerged as relevant signaling molecules that act on both hepatic and extrahepatic tissues to regulate lipid and carbohydrate metabolic pathways as well as energy homeostasis. Activation or modulation of specific BA-activated receptors, including members of the nuclear receptor superfamily (FXR, NR1H4), a vitamin D receptor (NR1I1), PXR (NR1I2), members of the G protein–coupled receptor superfamily (TGR5 and sphingosine 1 receptor 2), and transporters such as ileal apical sodium-dependent bile acid transporter (ASBT), appears to affect both insulin sensitivity and NAFLD/NASH pathogenesis at multiple levels, and these approaches hold promise for exploitation for novel therapies (144).

FXR is a key BA nuclear receptor highly expressed in the liver, small intestinal mucosa, and kidneys with effects on glucose and lipid metabolism. Mice with FXR deletion exhibit decreased insulin sensitivity. Conversely, treatment with the selective, nonsteroidal FXR agonist GW4064 improved IR and glucose homeostasis in obese *ob/ob* and diabetic *db/db* mice (153) and also increased hepatic glycogen synthesis, probably mediated by FGF-15 (human ortholog FGF19) (154). Thus, BA-mediated regulation of hepatic glucose metabolism is complex although most studies support the concept of restitution of insulin sensitivity when FXR is activated. The effect of FXR over lipid metabolism has received increasing attention. Mice deficient in FXR have a marked elevation of serum, hepatic cholesterol, and TGs (155). Also, the administration of an FXR agonist decreased plasma cholesterol, TG, and FFA levels in *db/db* and wild-type mice but not FXR knockout mice (153). The main mechanisms of action of FXR on steatosis are inhibition of SREBP-1c by small heterodimer partner (SHP) (156) and FXR-dependent induction of PPARα (and its target carnitine palmitoyl transferase 1) (157, 158) and several other genes related to VLDL and TG metabolism (158, 159). FGF15/19 also decreases hepatic lipogenesis and indirectly stimulates mitochondrial FA oxidation (154, 158).

Another BA receptor of relevance is G protein–coupled bile acid receptor 1 [GPBAR1, also known as transmembrane G protein–coupled receptor 5 (TGR5)], which has a role in regulating energy expenditure, glucose metabolism, and immunity. TGR5 activation has been shown to activate thermogenesis in brown AT and with that induces energy expenditure. TGR5 also has an effect on glucose metabolism over its action on enteroendocrine L cells in the intestine (160), which release glucagon-like peptide-1 (GLP-1), and on inflammation through the inhibition of NF-κβ and the suppression of cytokine release. Activation of FXR in the ileal enterocytes after active intestinal BA uptake also has important metabolic implications via FXR-stimulated local production of FGF15 (FGF19 in humans) (161). In hepatocytes, FGF15/19 binds FGF receptor 4 (FGFR4) and functions as a major regulator of BA synthesis by directly inhibiting cholesterol 7α-hydroxylase (CYP7A1) expression (154). In addition, FGF15/19 also decreases hepatic lipogenesis and indirectly stimulates mitochondrial FA oxidation (154, 158). Finally, BA transporters such as ASBT (SLC10A2) could play metabolic roles by controlling BA flux and influencing intracellular BA levels and BA signaling in the enterocyte, having an important role in FXR activation in the intestine (162).

Current Understanding of the Role of Genetics

It is currently known that genetic polymorphisms can influence NAFLD development and progression, mainly by alterations in hepatic FFAs, oxidative stress, and inflammation. In 2008, Romeo et al. (163) reported results of the first genome-wide association study (GWAS) in patients with NAFLD and controls, finding a frequent nonsynonymous variant of patatin-like phospholipase 3 (*PNPLA3*) gene (rs738409 C/G, I148M) strongly associated with an increase in hepatic fat content and elevated serum levels of alanine aminotransferase and aspartate aminotransferase. PNPLA3, alternatively known as adiponutrin-ADPN and calcium-independent phospholipase A2 epsilon

(iPLA2-ε), belongs to a novel group of lipid metabolizing enzymes known as the patatin-like phospholipase domain-containing family (PNPLA). The expression of PNPLA3 is up to tenfold in the human liver compared to AT and shares its greatest homology with ATGL (PNPLA2). Shortly afterwards, Sookoian et al. (164) established that Argentinian patients with a *PNPLA3* risk allele (G) showed higher inflammation and fibrosis degree in the liver histopathology, independent of age, gender, BMI, and insulin sensitivity. These findings have been further supported by several cross-sectional studies (165, 166). The *PNPLA3* gene encodes for a protein called adiponutrin, which mediates triacylglycerol hydrolysis in adipocytes. *PNPLA3* risk allele polymorphism has been shown to be associated with lower DNL and SREBP-1c expression but with significantly higher hepatic TG content, less VLDL secretion, and without a change in hepatic β-oxidation (167, 168). *PNPLA3* polymorphism confers not only a risk of NAFLD [odds ratio (OR) 3.41] and NASH (OR 4.44) but also an additive risk of HCC (adjusted OR 2.26) (169).

Multiple studies have shown differences in the prevalence of NAFLD among ethnic populations. Latino ancestry confers the highest predisposition to NAFLD, whereas African Americans are the least predisposed. This *PNPLA3* variant is also known to be more common in Latinos compared with other ethnicities (170). The study by Romeo et al. (163) reported an allelic frequency of the I148M variant at 0.49, 0.23, and 0.17 in Hispanics, European Americans, and African Americans, respectively. The severity of NAFLD also may be greater in Hispanic subjects. The increased prevalence and severity of NAFLD in patients of Latino origin are likely related to the interplay of several factors such as genetic ancestry, access to health care, or the prevalence of other chronic diseases (170, 171). Interestingly, knockout mouse models for *PNPLA3* showed no phenotype, whereas I148M overexpression in hepatocytes or I148M knockin resulted in hepatic steatosis development but did not explain the acceleration of liver inflammation and fibrosis observed in human clinical studies (172–174). Recent data in human HSC cell lines indicate that *PNPLA3* is highly expressed in HSCs and is directly required for HSC activation (175). Its genetic variant I148M potentiates the proinflammatory and profibrogenic features of HSCs, now providing a molecular mechanism for the higher risk of progression and severity of liver diseases conferred to patients carrying the I148M variant (175).

Another relevant genetic variant is the transmembrane 6 superfamily member 2 (*TM6SF2*) rs58542926 variant of the gene. The *TM6SF2* gene encodes a protein that promotes VLDL secretion, and this polymorphism is associated with loss of function, hepatic steatosis, elevated aminotransferases, and advanced hepatic fibrosis (176, 177). Interestingly, carriers of this variant are protected against cardiovascular disease (178).

Epigenetics is defined as an inheritable but reversible phenomenon that affects gene expression without changing the DNA sequence, which includes DNA methylation, histone modifications, and microRNAs, and also can play a role in NAFLD pathogenesis. A GWAS study by Xu et al. (179) showed that during NAFLD progression, genes related to lipid biosynthesis, cellular apoptosis, and inflammation were increased, while those related to DNA damage response, cholesterol biosynthesis, and carbohydrate metabolism were decreased. Other studies have shown correlations between the methylation or acetylation status of different histones and mitochondrial proteins and inflammatory activity in NASH (180). Additionally, hepatic expression of different microRNAs, such as miR-122, miR-335, miR-29c, miR-34a, miR-155, and miR-200b, has been implicated in the pathogenesis of NAFLD and could be potentially used as a biomarker (181).

PROGRESSION TO LIVER CANCER

Obesity and T2DM are known precarcinogenic conditions; however, they usually coexist with NAFLD, so the magnitude of the neoplastic potential of this disease may be underestimated.

HCC (in men) is considered to be the highest cancer risk among all obesity-driven cancers (four-fold). Patients with NAFLD can present with HCC, which is most often associated with advanced fibrosis or cirrhosis but also is a complication in earlier precirrhotic stages of the disease. In these cases, the specific mechanism is not completely known and is probably related to the pathogenesis of the underlying disease rather than to the fibrotic process alone. Such pathogenetic aspects could include direct signaling effects of lipids or lipid intermediates (in particular FAs), resulting in activation of proinflammatory pathways NF-κB, activator protein 1 (AP-1), JNK, and STAT3 and downregulation of *PTEN*. Overall, the relative HCC risk and mortality rate in NASH-related cirrhosis seem to be lower than in viral or alcohol-related cirrhosis (182). However, HCC in patients with NASH is the most common indication for HCC-related liver transplantation (183).

The mechanisms of HCC development in a cirrhotic liver have been extensively described. These include chronic injury with hepatocellular destruction and the cyclic compensatory regeneration and proliferation, which mainly favor tumor development (184). Patients with NAFLD generally also have IR, which, together with hepatic steatosis and low-grade chronic inflammation, promotes an auspicious environment for tumor growth. IR and hyperinsulinemia promote a hormonal imbalance, which leads to AT-derived inflammation, oxidative stress, lipotoxicity, and overstimulation of the IGF-1 axis. Proinflammatory cytokines such as TNFα and IL-6 enhance JNK/NF-κB and JAK/STAT3 pathways while leptin activates PI-3K/Akt signaling, leading to the expression of genes involved in cell proliferation, migration, and survival. Alternately, some tumor suppressor factors, such as PTEN and SOCS3, are downregulated, failing in the control of protumorigenic signaling (182). Lipid metabolites of SCD activity are associated with aberrant palmitate signaling in aggressive HCC samples (185). Also, it has been shown that cholesterol-associated mitochondrial aberrations contribute to chemotherapy resistance by increasing membrane order (186).

Additionally, dietary factors, GM, and genetic factors, such as the *PNPLA3* rs738409 variant, contribute to an increased risk of HCC. High-fat and high-fructose diets can increase DNL and lipoperoxidation in the liver and increase the release of proinflammatory cytokines. Changes in GM associated with obesity promote the translocation of bacterial products (such as endotoxins, LPS, and deoxycholic acid), which reach the liver and favor the senescence-associated secretory phenotype in HSCs, which in turn secrete various inflammatory and tumor-promoting factors in the liver (187).

PATHOGENESIS-BASED THERAPEUTIC PERSPECTIVES

The basis of all therapeutic interventions is lifestyle changes with diet and exercise, which have been shown to reverse steatosis, inflammation/NASH, and even fibrosis. The target weight loss must be approximately 5–10% of the original body weight to achieve clinical and histopathological benefits. The AGA, AASLD, and ACG guideline (188) advises a weight loss of at least 3–5% to recover from steatosis, but a weight loss greater than 10% is recommended to observe changes in necrosis and inflammation. Exercise (aerobic or anaerobic) should be recommended to all NAFLD patients too, since it has been shown to have independent beneficial effects (189).

Inhibition/Prevention of Steatosis

IR has a pivotal role in the pathogenesis of NAFLD and is responsible for lipolysis and FFA overload, so targeting it has been tested in multiple trials. One logical approach is the use of metformin; however, no trials have shown benefits on steatosis beyond weight loss (190). Some

antidiabetic drugs have been tested in NAFLD. The two most important groups of these drugs are the PPAR agonists and the glucagon-like peptide-1 (GLP-1) agonists. PPARs are a nuclear receptor superfamily with multiple roles in the regulation of lipid, glucose, and energy metabolism and also have a role in the modulation of inflammatory pathways (particularly inhibiting NF-κB pathways). There are three main isotypes of PPARs, α, δ, and γ, and different combinations of ligands are being tested in NAFLD. The first trials with fibrates (a PPARα agonist) were not able to show improvement in NAFLD. Currently, dual PPARα/δ agonists such as elafibranor are being tested in phase 3 trials (ClinicalTrials.gov, NCT02704403, RESOLVE-IT), with promising preliminary phase 2 results in metabolic, anti-inflammatory, and antifibrotic effects (191). The phase 2b GOLDEN-505 trial (192) showed histological improvement at 52 weeks of treatment with the highest dose (120 mg) without any relevant side effect, and phase 3 studies are currently ongoing (ClinicalTrials.gov, NCT02704403). Another dual agonist, the PPARα/γ agonist Saroglitazar, have been shown to reduce steatosis and necroinflammatory activity in murine models of NASH, and it is currently under phase 3 trial evaluation (ClinicalTrials.gov, NCT02265276). PPARγ agonists, thiazolidinediones, have beneficial, even antifibrotic effects, if tested in the right populations (i.e., patients with prediabetes or T2DM) (193). One of the most emblematic studies in NASH is the PIVENS trial, which showed the beneficial effect of vitamin E. It was not able to show statistically significant benefits of pioglitazone over placebo for improvement in the histologic features of NASH, as assessed by the use of a composite of standardized scores for steatosis, lobular inflammation, hepatocellular ballooning, and fibrosis; however, both pioglitazone and vitamin E significantly improved steatosis, lobular inflammation, and NAFLD activity score (194).

GLP-1 is an incretin secreted by L cells in the intestine after eating and regulates insulin secretion in the pancreas and insulin sensitivity. In general, GLP-1 also has central effects reducing appetite and delays gastric emptying. In animal models, GLP-1 agonists (such as exenatide and liraglutide) have been shown to reduce steatosis and necroinflammation. Interestingly, direct effects on HSCs have been shown, improving HSC phenotype (195). The phase 2 LEAN trial (196) showed resolution of NASH at 48 weeks in 39% (9 out of 23) of patients in the liraglutide group compared with 9% (2 of 22) in the placebo group and 26% (6 of 23) of improvement in fibrosis compared with 14% (3 of 22) in the placebo group. Liraglutide also decreased body weight by 5.5% but with significant gastrointestinal side effects (such as diarrhea, constipation, and bloating) and the need for subcutaneous administration. Currently, a trial is underway to compare liraglutide to bariatric surgery in NASH in obese Asian adults (phase 3 CGH-LiNASH, ClinicalTrials.gov, NCT02654665). Dipeptidyl peptidase 4 (DPP-4) degrades incretins; thus DPP-4 inhibitors have also been tested in NASH. The main advantage is that these drugs are orally administrated. Some preliminary data have shown improvement in liver steatosis, but further trials are urgently needed. In a recent randomized controlled trial (RCT), sitagliptin was shown to not be more effective than placebo in improving liver fat and fibrosis in patients with NASH (197).

Another approach to treat steatosis has been the direct targeting of the key enzymes involved in lipogenesis. ACC inhibition appears to be beneficial via the inhibition of DNL and the promotion of mitochondrial β-FA oxidation (indirectly by reducing levels of malonyl-CoA, which inhibits the CPT1 mitochondrial FA transfer protein) (198, 199). Targeting of other key enzymes such as SCD1 or DGAT is currently under evaluation.

Drugs aimed at lowering serum lipids have been the focus of extended research because they could reduce hepatic steatosis, inflammation, and cardiovascular risk at the same time. This is of paramount importance since cardiovascular disease is the main cause of death in patients with

NAFLD. Currently available lipid-lowering options are statins, omega-3 PUFAs, and ezetimibe. Additionally, the GLP-1 agonist Liraglutide also has shown beneficial effects on LDL cholesterol.

Statins are broadly used medications. In the United States, more than one-quarter of adults aged 40 and over use cholesterol-lowering medications, as do almost half of those over age 75 (200). However, statins are underprescribed in NAFLD patients, mainly because of the fear of hepatotoxicity. Statins can increase serum aminotransferases levels in about 1–3% of patients, but this increase rarely persists or leads to more severe injury. (Drug-induced liver injury by statins is seen in less than 1 in 100,000 cases.) Post-hoc analysis from large trials (GREACE, IDEAL, and ATTEMPT) and high-quality RCTs has shown that statins are safe even in patients with compensated cirrhosis; moreover, withholding statins from patients with increased serum aminotransferases is associated with worsening cardiovascular outcomes (201, 202). Although statins are an interesting potential therapy for NAFLD, to date no studies have been able to show improvement in steatosis or any other liver outcome. Only case series with small numbers of patients have shown improvement in aminotransferases and liver steatosis, but there are no data from larger RCTs with proper follow-up and biopsies to evaluate outcomes. Of note, a new role for statins in NAFLD has emerged in the prevention of HCC. Retrospective studies have shown a 26% reduction in HCC in statin users compared with nonusers. Further data are urgently needed in this regard (201).

Omega-3 PUFAs are important for normal metabolism. The three types of omega-3 PUFAs involved in human physiology are eicosapentaenoic acid and docosahexaenoic acid (both commonly found in marine oils) and α-linolenic acid (ALA) (found in vegetable oils). Omega-3 PUFAs have been shown to reduce inflammation (probably mediated by arachidonic acid metabolism) and lower serum TGs. Observational studies have shown that subjects with NAFLD consume less omega-3 and more omega-6 PUFAs compared to healthy controls. For this reason, studies attempting to supplement NASH patients with omega-3 PUFAs have shown some effect on steatosis but have not shown any effects on steatohepatitis and apoptosis (203, 204). A similar disappointing effect has been shown with ezetimibe on NAFLD (205).

Another approach aiming to regulate steatosis has been with the modulation of BA receptors. As previously described in this review, FXR function is pivotal for glucose and lipid metabolism. Obeticholic acid (OCA; 6-ethoxy-chenodeoxycholic acid) is a potent FXR agonist with an activity 100 times higher than that of chenodeoxycholic acid, the natural BA with the highest affinity to FXR. OCA has been shown to improve insulin sensitivity and inflammatory markers. In 2015, the FLINT study, which randomized patients with NASH to receive OCA or placebo, showed that 45% of patients receiving OCA attained the primary histological response (a reduction of the NAFLD activity score by ≥2 points without worsening of fibrosis) at week 72, compared with 21% in the placebo group. Additionally, 35% of patients in the OCA group had improvement in fibrosis, compared to 19% in the placebo group. A note of concern arises from the observation that there was an increase in total cholesterol, LDL, and a reduction in HDL in the OCA group compared to the placebo group, along with a deterioration of IR (HOMA index). The long-term implications of these changes, in terms of cardiovascular risk, need to be assessed. Additionally, OCA was associated with pruritus in some patients (206). Currently, OCA is being studied in a phase 3 trial (REGENERATE; **https://clinicaltrials.gov/ct2/show/NCT02548351**). There are also ongoing studies with selective, nonsteroidal FXR ligands, GS-9674 (**https://clinicaltrials.gov/ct2/show/NCT02854605**) and LJN-452 (**https://clinicaltrials.gov/ct2/show/NCT02855164**). Other drugs targeting BAs receptors are INT-767, a dual FXR and TGR5 agonist, which has been shown to improve NASH in mouse models, and Aramchol, a synthetic conjugate of cholic acid and arachidic acid (a saturated FA),

which has been shown to reduce steatosis in humans and is currently in clinical trials (phase 2 Aramchol003; ClinicalTrials.gov, NCT01094158) (201).

FGF-19 agonists, such as NGM-282 (NGM Biopharmaceuticals), are being tested (phase 2, NCT02443116) since FGF19 activation reduces endogenous BA synthesis, protects hepatocytes from BA toxicity, and promotes secretion of BAs. One note of concern is that FGF19 has been associated with the development of HCC in animal models and, potentially, in humans. FGF-21 has also been proposed as an inhibitor of HSCs, thus reducing hepatic fibrogenesis. FGF-21 is a secreted protein that belongs to the same family of FGFs as FGF-23 and FGF-15/19. FGF family members possess broad mitogenic and cell survival activities, including effects on glucose and lipid metabolism. BMS-986036, an FGF-21 agonist, is being assessed for hepatic steatosis in a phase 2 study (NCT02413372) in addition to various pegylated forms of FGF-21.

Finally, another therapeutic approach is called mitochondrial medicine. It has been shown that a controlled-release mitochondrial protonophore produces mild hepatic mitochondrial uncoupling, improving diabetes and NASH in a rat model (207).

Inhibition of Inflammation

NASH, like alcoholic steatohepatitis, is an inflammatory process mainly driven by sterile inflammation and aseptic necrosis. It involves the recruitment of immune cells and activation of proinflammatory signaling pathways such as TNFα, NF-κβ, AP-1, TLR4, and NLRP3. Therapies aimed at inhibiting TNF production such as pentoxifylline (a phosphodiesterase inhibitor with antioxidant properties) had been tested in NASH, with encouraging preliminary and animal data, but without evidence of improvement in subsequent RCTs (208, 209). Therefore, currently pentoxifylline is not a recommended option for treatment of NASH, and further studies are needed.

The release of proinflammatory cytokines and chemokines leads to the recruitment of immune cells and perpetuation of the inflammatory process within the liver. Chemokines (CC, CXC, CX3C, and C) activate leukocytes through chemokine receptors, which have been described to be upregulated in NASH (CCR2 and CCR5) (210). Animal data have suggested that inhibition of these pathways could lead to improvement in inflammation and fibrosis (211, 212). Currently, trials with Cenicriviroc, a dual CCR2/CCR5 antagonist, are underway (phase 2, CENTAUR, NCT02217475, and NCT03059446; phase 3, AURORA, NCT03028740) and have shown promising results during interim analysis. Interestingly, this analysis revealed improvement of fibrosis without beneficial effects on steatosis/NASH, pointing toward a potential dissociation of these two phenomena (213).

Modulation of GM has also been assessed to inhibit inflammation in NASH. The use of prebiotics (products that promote the growth of beneficial intestinal microbiota), probiotics (specific quantities of beneficial microbial strains), or antibiotics such as rifaximin have been shown to be of benefit in preclinical studies, but high-quality RCTs in humans with histological end points are needed (214). Currently, there are trials to evaluate this topic (NCT00808990 and NCT02764047).

Other pathologically oriented treatments that could modulate inflammation in NASH include vitamin D, which has been shown to affect serum high-sensitive C-reactive protein and malondialdehyde in NASH (NCT01571063 and NCT01623024); resveratrol, an antioxidant polyphenol with protectant properties through inhibition of AKT and NF-κB pathways (NCT01464801 and NCT02030977); Viusid, another antioxidant containing vitamins, trace minerals, and glycyrrhizinic acid (extracted from licorice root) (phase 3, NCT00509418); and Protandim, a botanical extract with antioxidant properties from *Bacopa monnieri*, milk thistle, Ashwagandha powder, green tea, and turmeric (phase 2, NCT00977730).

Inhibition of Fibrosis

Since fibrosis is the stereotypical response that leads to the advanced stages of all liver disease and appears to be the key prognostic factor of NAFLD (6), it is of maximum interest to develop therapies targeting the pathogenetic mechanisms of fibrosis in NAFLD. Lysyl-oxidase-like 2 (LOXL2) protein is a key enzyme in the biogenesis of connective tissue, encoding an extracellular copper-dependent amine oxidase that catalyzes the formation of crosslinks between collagens and elastin, being essential in hepatic fibrogenesis. Phase 2 RCTs have evaluated simtuzumab (GS-6624), a humanized monoclonal anti-LOXL2 antibody, in patients with NASH, significant fibrosis and cirrhosis, but it showed no superiority to placebo (NCT01672866 and NCT01672879). Simtuzumab was also studied in combination with Selonsertib (GS-4997) [an oral molecule that inhibits apoptosis signal-regulating kinase 1 (ASK1)] in patients with NASH and advanced fibrosis (phase 2, NCT02466516). Preliminary data obtained with elonsertib are promising (215), and this approach is currently being explored in patients with NASH and bridging fibrosis (F3) (STELLAR3, phase 3, NCT03053050) or cirrhosis (STELLAR4, phase 3, NCT03053063).

GR-MD-02 and GM-CT-01 are two galectin inhibitors that have been shown to reduce fibrosis in animal models of NAFLD. Galectins are glycoproteins on the cell surface that can mediate cell migration, matrix interaction, and inflammatory signaling. GR-MD-02 is currently being tested in patients with NASH and advanced fibrosis (NCT01899859) or cirrhosis (NASH-CX study, NCT02462967).

Inhibition of Apoptosis

Hepatocyte apoptosis is a hallmark of NASH. Preclinical and preliminary studies in humans with pan-caspase inhibitors [VX-166, Vertex Pharmaceuticals; Emricasan (IDN-6556), Conatus Pharmaceuticals] have shown improvement in aminotransferases, apoptosis, inflammation, and fibrosis (216, 217). Phase 2 studies with GS-9450 (Gilead), a selective caspase 1, 8, and 9 inhibitor, and emricasan have shown reduction in serum aminotransferases and CK-18 levels (218, 219). Currently, three phase 2 studies with emricasan are being conducted in patients with NAFLD and raised aminotransferases (NCT02077374), severe portal hypertension (ENCORE-PH, NCT02960204), and fibrosis (ENCORE-NF, NCT02686762).

SUMMARY AND OUTLOOK

NAFLD is a rising health problem worldwide, affecting between 25 and 30% of the general population (5, 7). Overall estimates of NAFLD prevalence vary according to the selected population (ethnicity and comorbidities) and diagnostic method used. In high-risk populations such as those with T2DM and obesity, the prevalence of NAFLD may be as high as 70–90% (220). The prevalence of NAFLD is increasing in parallel with the prevalence of obesity; both processes are closely linked to IR. Although most patients have noninflammatory isolated steatosis, around one-third will develop NASH, which, in one-third of cases, can progress to advanced fibrosis or cirrhosis.

Key pathophysiological mechanisms involve IR; lipotoxicity; environmental factors such as diet, obesity, and microbiota; genetics, ER stress; autophagy; apoptosis; and, ultimately, HSC activation and fibrogenesis (**Figure 3**). Currently, several trials targeting one or more of these pathomechanisms are underway or awaiting results, and hopefully, we will have new therapeutic options in the future. Since NAFLD/NASH is a complex disorder, it may be necessary to combine several therapeutic targets.

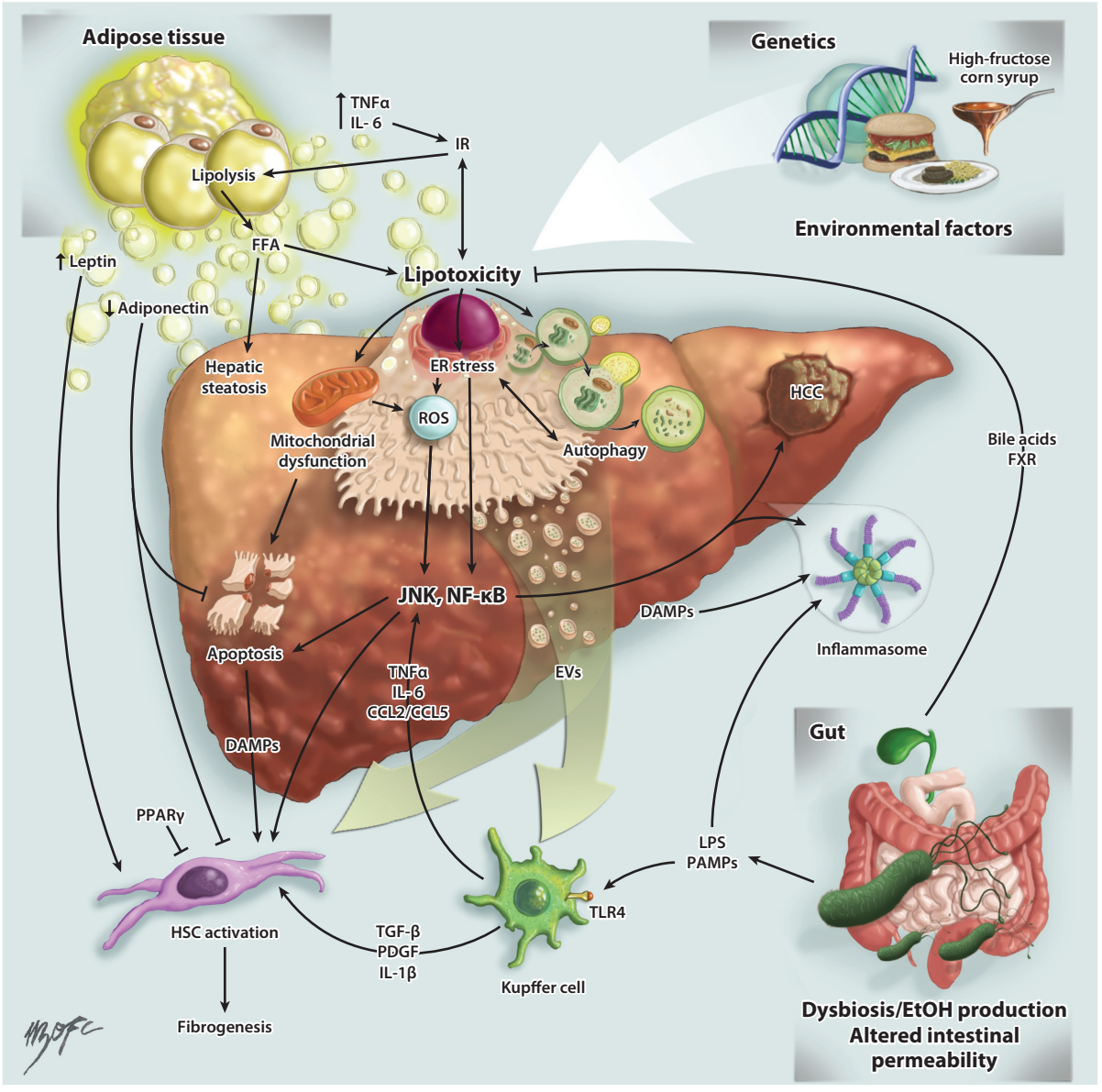

Figure 3

Schematic summary of NAFLD pathogenesis. The main components for the development and progression of the disease are diet, genetics, obesity with adipose tissue expansion and enhanced lipolysis due to IR, resulting in lipotoxicity, mitochondrial dysfunction, production of ROS, ER stress, autophagy, apoptosis, inflammasome activation, release of EVs, and ultimately HSC activation and fibrogenesis. Abbreviations: CCL2/CCL5, C-C motif chemokine ligand 2/5; DAMPs, damage-associated molecular patterns; ER, endoplasmic reticulum; EtOH, ethanol; EVs, extracellular vesicles; FFAs, free fatty acids; FXR, farnesoid X receptor; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; IL-6, interleukin 6; IR, insulin resistance; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; NAFLD, nonalcoholic fatty liver disease; NF-κB, nuclear factor kappa B; PAMPs, pathogen-associated molecular patterns; PDGF, platelet-derived growth factor; PPAR, peroxisome proliferator-activated receptors; ROS, reactive oxygen species; TGF-β, transforming growth factor beta; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor α.

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Annual Review of Pathology: Mechanisms of Disease

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Contents

Errata

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