Nonalcoholic Fatty Liver Disease: Pathogenesis and Disease Spectrum

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver dysfunction in the Western world and is increasing owing to its close association with obesity and insulin resistance. NAFLD represents a spectrum of liver disease that, in a minority of patients, can lead to progressive nonalcoholic steatohepatitis (NASH), fibrosis, and ultimately hepatocellular carcinoma and liver failure. NAFLD is a complex trait resulting from the interaction between environmental exposure and a susceptible polygenic background and comprising multiple independent modifiers of risk, such as the microbiome. The molecular mechanisms that combine to define the transition to NASH and progressive disease are complex, and consequently, no pharmacological therapy currently exists to treat NASH. A better understanding of the pathogenesis of NAFLD is critical if new treatments are to be discovered.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of liver disease that includes simple steatosis [triacylglycerol (TAG) infiltration in >5% of hepatocytes], fatty infiltration plus inflammation, and hepatocellular ballooning degeneration [nonalcoholic steatohepatitis (NASH)], progressing to fibrosis and ultimately cirrhosis, in the absence of excessive alcohol consumption (a typical threshold being <30 g per day for men and <20 g per day for women) (1). NAFLD is strongly associated with features of metabolic syndrome, including obesity, insulin resistance (IR) or type 2 diabetes mellitus (T2DM), and dyslipidemia; it is thus considered the hepatic manifestation of this syndrome (2, 3). NAFLD is associated with an increased overall mortality compared with the general population (4), and NASH is associated with a greater than tenfold increased risk (2.8% versus 0.2%) of liver-related death and a doubling of cardiovascular risk (5). NAFLD has rapidly become the most frequent cause of liver dysfunction in many developed and developing countries, in line with increasing prevalence of obesity and IR, as lifestyles have become increasingly sedentary and dietary patterns have changed (6, 7). In addition, NAFLD is projected to be the primary indication for liver transplantation in many countries within a decade (8, 9), as the incidence of cirrhosis from chronic viral hepatitis decreases whereas that of NAFLD increases (10).

The pathogenesis of NAFLD remains incompletely understood, despite substantial clinical and basic research; the events determining progression of disease are a focus of intense research. This review aims to present the current understanding of these processes.

PREVALENCE OF NAFLD

Estimates of NAFLD prevalence vary by both the population studied (e.g., patients with different ethnicities, sex, and comorbidities) and the sensitivity of the diagnostic technique used (1, 4, 11). To reliably distinguish simple steatosis from NASH, a liver biopsy remains the diagnostic gold standard, although there are drawbacks to this approach; studies based on histology are subject to sampling error, variation in pathological interpretation, and selection bias as biopsy is predominantly used in tertiary care settings (12, 13). The more sensitive noninvasive modalities, such as computerized tomography, proton magnetic resonance spectroscopy (^{1}H-MRS), and magnetic resonance imaging, give quantitative assessment of hepatic triglyceride (TG) content (14, 15). Unfortunately, they cannot distinguish simple steatosis from NASH or detect fibrosis that falls short of advanced cirrhosis. These modalities are also resource-intensive and expensive (14). Thus, the first-line imaging modality currently recommended in routine practice (1, 6, 16) is ultrasonography, which provides a qualitative and subjective assessment of hepatic fat content, although it is only sensitive when >33% of hepatocytes are steatotic (14, 17, 18).

Liver biochemistry correlates weakly with the presence of NAFLD and with disease severity (19, 20); studies using biochemical screening consistently report a lower prevalence of NAFLD (3–12%) than imaging- or biopsy-based studies (4). A community study based in the United Kingdom determined that NAFLD detected by ultrasonography was the most common etiology for asymptomatic abnormal liver biochemistry, accounting for 26.4% of cases (21).

In unselected populations using ^{1}H-MRS, 31% of 2,349 US adults in the Dallas Heart Study were found to have hepatic steatosis (22, 23); this was not distributed evenly through ethnic groups, with 45% of Hispanics, 33% of Caucasians, and 24% of African Americans being affected (22, 23). Further evidence for ethnic variation was provided by a study that revealed a significantly higher prevalence of NAFLD in Hispanics than in non-Hispanic whites even after controlling for obesity and body fat distribution (24). Histology-based studies in apparently healthy candidates for living liver donation found the prevalence of NAFLD was 12–18% in Europe (25, 26) and 27–38% in the United States (25, 27, 28).

The prevalence of NAFLD rises substantially when groups with known metabolic risk factors are studied. The close association with obesity was demonstrated in the European DIONYSOS study cohort in which NAFLD detected by ultrasound was present in 94% of obese patients [body mass index (BMI) ~30 kg/m^2], 67% 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) (13, 29). Similarly, the overall prevalence of NAFLD in people with T2DM is believed to be much greater than in the general population, on the order of 40–70% (13, 30–33).

The prevalence of NASH is difficult to determine owing to the fact that diagnosis still requires invasive liver biopsy. The results of a systematic review in 2011 placed the prevalence of NASH at 3–5% (20); an earlier autopsy study in Canada estimated the prevalence of NASH at 6% (34). In at-risk populations, NASH is thought to be present in 25–30% of patients with obesity or T2DM and in 35% of severely obese patients with T2DM (4, 35, 36). Data from healthy living liver donors estimate the prevalence of NASH at 6–15% (25, 28). Taken together, the available evidence suggests that NAFLD is present in 12–38% and NASH in 3–15% of the general population (20, 25, 28, 34).

HISTOPATHOLOGY

Although the diagnosis of NAFLD can be made following demonstration of hepatic steatosis on ultrasound, examination of liver biopsy tissue remains the gold standard to confirm a diagnosis of NAFLD, as well as for documenting disease activity, fibrosis stage, and architectural veracity (37). Currently, no single noninvasive test is ready for widespread use, or to replace histological examination; recent developments in the field of magnetic resonance imaging for hepatic fat content and fibrosis are encouraging, but the published literature falls short and must be considered experimental (38, 39). The current research into noninvasive testing in NAFLD/NASH, its role in risk stratification, and indications for liver biopsy is beyond the scope of this review and has recently been summarized elsewhere (40). The focus of this review is the histological characterization of NAFLD/NASH.

Steatosis

The histological hallmark of NAFLD is steatosis, the accumulation of hepatic TGs. Usually, mixed large (macrovesicular) and small (microvesicular) droplets are contained within hepatocytes; true microvesicular steatosis is rare (41). The most reproducible semiquantitative method for histological assessment of steatosis relies on the acinar architecture and refers to the percentage of liver parenchyma containing steatotic hepatocytes: 0–33%, 33–66%, or >66% (42). In adults, steatosis is initially localized to zone 3, or present in a diffuse, panacinar distribution (41).

Steatohepatitis

The histological diagnostic criteria for NASH include steatosis, hepatocellular injury, and lobular inflammation, usually occurring in zone 3, with or without fibrosis. However, once fibrosis progresses and architectural remodeling occurs, the lesions may lose acinar localization (41). Hepatocellular injury occurs in the form of ballooning, but apoptotic bodies and lytic necrosis may also be present. Hepatocyte ballooning describes the presence of enlarged, swollen hepatocytes, with rarefied cytoplasm that may have a reticulated appearance or contain Mallory-Denk bodies. Loss of the normal distribution of keratins 8 and 18 within affected hepatocytes, demonstrated by immunohistochemistry, has been proposed for identification of ballooned hepatocytes

(43). Lobular inflammatory infiltrates are usually composed of lymphocytes, macrophages, eosinophils, and occasional neutrophils. Portal inflammation occurs in varying degrees in NASH, with increasing disease severity correlating with increasing severity of activity and serological features of IR in adult and pediatric biopsies (44). Fibrosis in adults with noncirrhotic NASH is perisinusoidal, usually seen initially in acinar zone 3 (45). Progressive scarring can develop in zone 3 perisinusoidal fibrosis followed by bridging fibrosis and cirrhosis (45). The term cryptogenic cirrhosis has historically been applied to cases in which steatosis and other lesions have not persisted; indeed, NASH is probably underdiagnosed in the setting of advanced liver disease, where it is thought to be the underlying cause of liver disease in 30–75% of cases of cryptogenic cirrhosis (6). Recent interest has been stimulated by other histological lesions associated with NASH. The ductular reaction (i.e., the presence of hyperplastic ductular structures accompanied by varying amounts of inflammation and connective tissue at the portal tract interface) is thought to arise from hepatic progenitor cells and to be related to portal and advanced fibrosis (46).

Grading and Staging NAFLD

A semiquantitative evaluation of the lesions associated with NASH, proposed in 1999 by Brunt et al. (45), introduced grading activity and staging fibrosis, as was done for other chronic liver diseases. A central feature of the original scoring was that no single characteristic could be relied on to assess disease activity; rather, the combinations of steatosis, inflammation, and ballooning determined the grade. The NASH Clinical Research Network (CRN), sponsored by the National Institute of Diabetes and Digestive and Kidney Diseases, proposed and validated an updated scoring system to aid clinical trials of the CRN and encompass the whole spectrum of NAFLD (42). The NAFLD Activity Score (NAS) is therefore derived from single scores for steatosis, lobular inflammation, and hepatocyte ballooning and quantifies disease activity (42). Staging of fibrosis follows a five-tier (0–4) method indicating progression of fibrosis from zone 3 perisinusoidal to portal, bridging, and cirrhosis (45).

A NAS >5 has been subsequently used to diagnose NASH in both clinical trials and medical practice. However, after a review of 976 liver biopsies, a NAS >5 was present in only 75% of biopsies with definite NASH and 28% of biopsies with borderline NASH; even 7% of biopsies without NASH had a NAS >5 (47). Indeed, it has been repeatedly cautioned that NAS cannot be used for diagnostic purposes; however, patients with NAS >5 have been selected for inclusion in clinical trials.

Additionally, although the basic features of NASH are agreed upon, there exists interobserver variability among pathologists (48), mainly due to the different histopathological criteria used by various groups (49).

With this in mind, the Fatty Liver Inhibition of Progression (FLIP) consortium devised a simplified SAF (steatosis-activity-fibrosis) score and FLIP algorithm with the intention of improving interobserver variability (50). Indeed, further simplification of the NASH CRN score has been endorsed by liver pathologists and hepatologists (51). Key differences from the NASH CRN score include a dissociation of steatosis from activity and a clear description of normal hepatocytes adjacent to ballooned hepatocytes. Thus, an SAF score of steatosis (S = 0–3), activity (A = 0–4) combining lobular inflammation and ballooning, and fibrosis (F = 0–4) is derived; an A > 2 correctly distinguished all patients with NASH in a study of 679 obese patients, whereas no patients with A < 2 had NASH (50). In a separate study, in two groups of patients with metabolic syndrome, concordance (κ) was substantial in both groups for steatosis, 0.61; activity, 0.75; and fibrosis, 0.83 (52); thus, the SAF score and FLIP algorithm are true diagnostic scores (**Figure 1**).

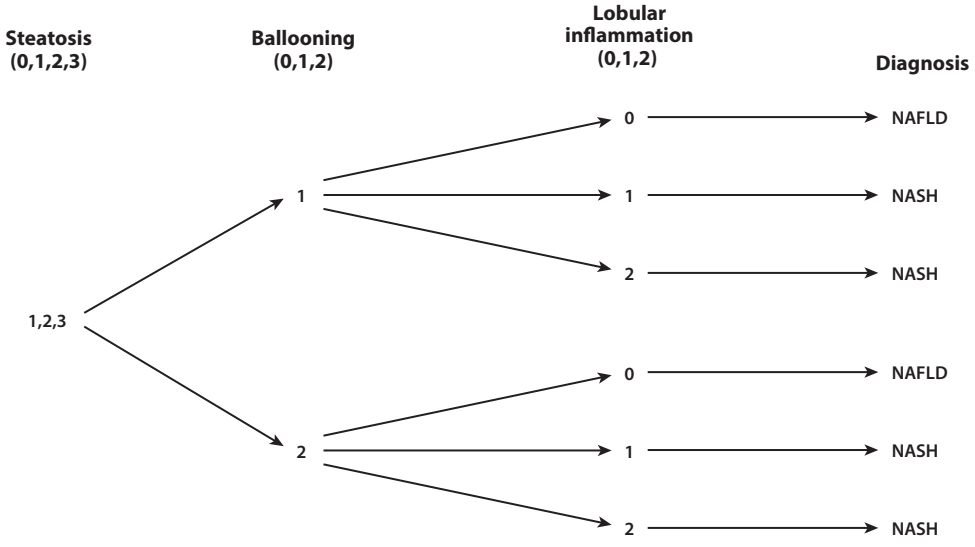

Figure 1

A modified diagnostic Fatty Liver Inhibition of Progression (FLIP) consortium algorithm using the SAF (steatosis-activity-fibrosis) score. An A < 2 excludes nonalcoholic steatohepatitis (NASH). Abbreviation: NAFLD, nonalcoholic fatty liver disease. Figure adapted with permission from Reference 52.

NATURAL HISTORY

Long-term mortality studies in NAFLD patients during 15 years of follow-up show a 26% risk of death in these patients, 34–69% higher than in the general population of the same age and sex (53). Overall, NAFLD is associated with an increased risk of both cardiovascular disease (CVD) and liver-related mortality (4). Whereas most patients with NAFLD exhibit only steatosis, a minority of patients progress to NASH, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Histological subtype analysis suggests that patients with simple steatosis are at exceptionally low risk for the development of progressive disease. NASH is usually associated with excess liver-related mortality; however, data are emerging to suggest that some patients with steatosis can graduate to NASH and a more severe outcome, especially if they develop intercurrent T2DM (54, 55). As liver fibrosis progression takes many years to develop, high-quality prospective data on the progression of NAFLD are limited, especially in primary care, where disease activity or progression cannot be readily and accurately monitored by liver biochemistry alone (19).

A Swedish cohort study demonstrated that although simple steatosis was not associated with increased risk of all-cause death and death related to CVD or liver disease, NASH was associated with greater than tenfold increased risk of liver-related death (2.8% versus 0.2%) and death from CVD (15.5% versus 7.5%) over a mean follow-up period of 13.7 years (5). Other cohort studies and a meta-analysis of five community-based studies support these data; causes of death from NASH in order of decreasing frequency include CVD, malignancy, and liver-related disease (4, 56–58). This may be explained by the greater propensity of patients with NASH to have or develop cirrhosis compared with patients with simple steatosis. Indeed, several recent studies indicate that the presence and severity of fibrosis are the key histological determinants of long-term prognosis, and that the reported distinction between NASH and simple steatosis is largely due to the greater likelihood of fibrosis being present in patients with NASH. In a Swedish study of 118 NAFLD patients followed for a median of 21 years (56), there was no difference in overall or liver-related

mortality between those with and without definitive NASH (as classified according to NASH CRN scores). In contrast, patients who died were more likely to exhibit some degree of fibrosis (89%) compared with survivors (70%, $p < 0.02$) and had a greater incidence of advanced fibrosis ≥F2 stage (68%, versus 28%, $p < 0.001$). Another study following 209 patients over a median of 12 years found that the presence of NASH did not correlate with liver mortality unless fibrosis was included in the definition. When the individual histological features of NASH were analyzed, only stage F3 portal fibrosis (i.e., seen in patients with bridging fibrosis/cirrhosis) was independently associated with liver-related mortality (hazard ratio 5.6, 95% CI 1.5–21.5) (48). That noninvasive scoring systems to assess degree of hepatic fibrosis predict liver-related events, transplantation, and death in patients with NAFLD further supports the view that fibrosis is a key determinant of outcome (59).

Despite the potential for selection bias, studies using paired-biopsy in tertiary care centers offer the best available data on natural history (5, 60–67). A recent meta-analysis of 11 studies totaling 411 patients with histologically characterized NAFLD (150 steatosis, 261 NASH) and 2,145.5 person-years of follow-up overall found that 33.6% exhibited progression of fibrosis, 43.1% had stable disease, and 22.3% had some regression of fibrosis (68). This study estimated the rate of fibrosis progression to be 0.07 stages (95% CI 0.02–0.11) per annum in those with steatosis but no fibrosis at baseline, approximately doubling for patients with NASH to 0.14 stages per annum (95% CI 0.07–0.21). Put another way, patients with lone steatosis progressed on average one stage of fibrosis in 14.3 years (95% CI 9.1–50.0) and patients with NASH progressed one stage in 7.1 years (95% CI 4.8–14.3). The proportion of fibrosis progressors who progressed from stage 0 fibrosis to stage 3 or 4 (rapid progressors) was identical in the two histological subgroups (17% of steatosis and 18% of NASH patients), and in the four studies that examined it, there was no association between the severity of necroinflammation and risk of progressive fibrosis. Importantly, patients with steatosis tended to have lower levels of fibrosis than patients with NASH (steatosis 90% F0–1, 10% F2 versus NASH 61% F1, 21% F2, 18% F3–4), a finding consistent with the view that the greater liver-related mortality in patients with NASH compared with those with steatosis that has been frequently demonstrated may be attributed to the generally higher degree of fibrosis found in patients with NASH.

A single-center study in the United Kingdom further supports this assertion (55). Describing 108 NAFLD patients undergoing repeat liver biopsy at a median interval of 6.6 years (range 1.3–22.6), we found that 45 (42%) patients had fibrosis progression, 43 (40%) had no change in fibrosis, and 20 (18%) had fibrosis regression. The mean annual rate of fibrosis progression was 0.08 ± 0.25 stages. Importantly, no significant difference in the proportion exhibiting fibrosis progression was observed (37% versus 43%) when patients with steatosis at index biopsy were compared with those with NASH. It is noteworthy that all those patients with steatosis that exhibited progressive fibrosis had also developed NASH and 80% had developed T2DM by the time of follow-up liver biopsy (55). As in the studies included in the meta-analysis, those patients with NASH had more fibrosis at baseline than the steatosis patients. Consistent with another similar study (54), steatosis patients with higher steatosis scores [2(2–3) versus 1(1–2), $p = 0.01$] and/or mild inflammation versus bland steatosis (60% versus 24%, $p = 0.07$) were more likely to have fibrosis progression.

Overall, based on the currently available evidence, fibrosis progression is generally slow, taking approximately 8 years to progress from stage F0 to F1. However, a subgroup of patients exists that may rapidly progress three to four stages within 2–6 years. An important finding, that the presence or absence of NASH on baseline histology has little impact on risk of progression, suggests that there may be a more dynamic bidirectional flux between steatosis and NASH than was previously appreciated. That being the case, higher stages of fibrosis seen in patients with NASH reflect

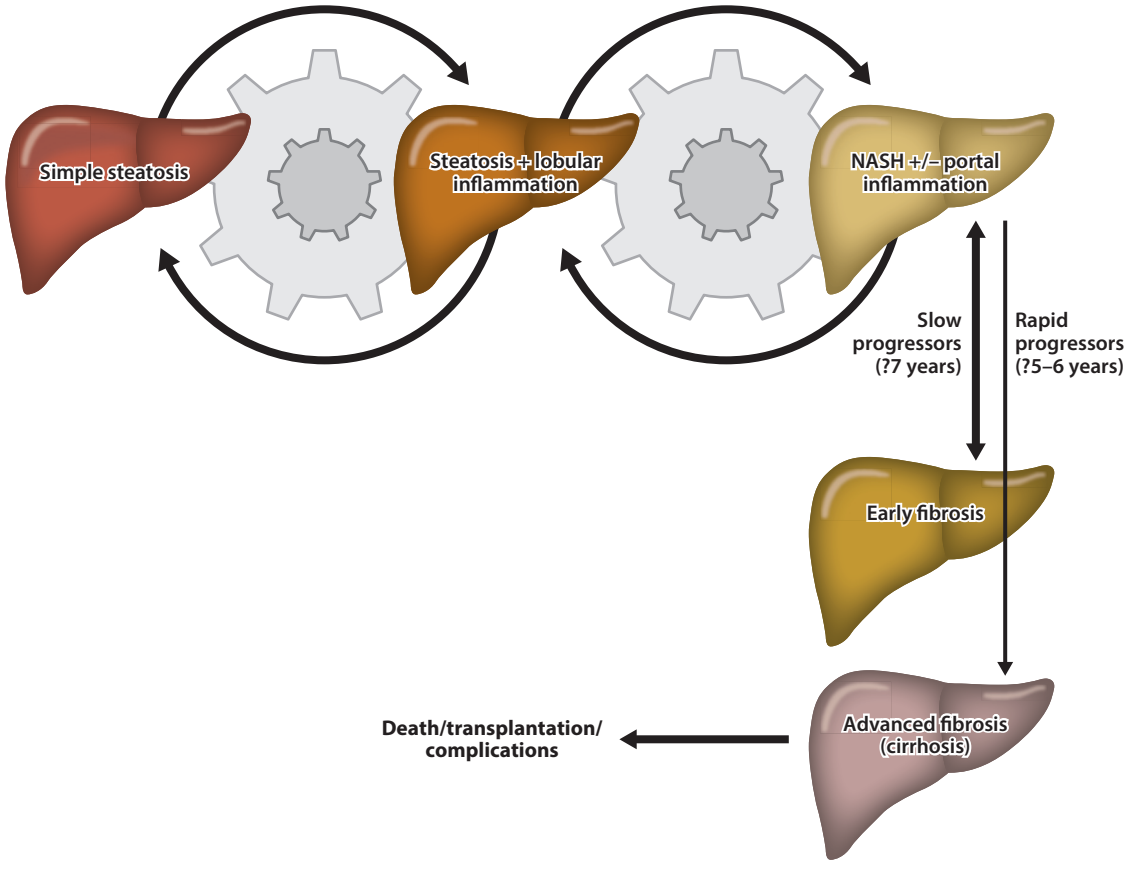

Figure 2

Natural history of nonalcoholic fatty liver disease. Susceptible patients cycle through steatosis, mild lobular inflammation (LI), and full-blown nonalcoholic steatohepatitis (NASH), with the duration of LI/NASH increasing with age as anti-inflammatory mechanisms fail. Inflammation/NASH triggers fibrosis in those who are susceptible, eventually leading to cirrhosis, hepatic carcinoma, or death.

longer overall disease duration and thus a greater length of time accrued in a steatohepatitic state in which hepatocyte damage and stellate cell activation occur (**Figure 2**). Supporting this assertion, patients with NASH were 9 years older than those with steatosis in the most recent study (55) and 44% of the steatosis patients had developed NASH after a median 8-year follow-up period, suggesting that NASH usually develops after steatosis. Patients with mild/moderate steatosis in the absence of all inflammation do, however, seem to be at the lowest risk of progression.

The incidence of HCC worldwide has risen over the last decade; although most cases are related to chronic viral hepatitis infection, over half of cases in developed countries occur in patients not infected with viral hepatitis, reflecting both advances in therapy for viral hepatitis and increased prevalence of NAFLD (69–71). Cirrhosis is present in 80% of patients with HCC regardless of etiology (72). In NAFLD the prevalence of HCC is estimated to be 0.5% but increases to 2.8% in patients with NASH (71). A study based in the United States involving 4,406 patients reported that NAFLD was the most common etiological factor in patients with HCC (58.5%), with T2DM being the second most common (35.8%) (69). The association was not simply through synergy

with other liver diseases but was the most common etiological factor in patients with a single risk factor for HCC (69). In a prospective study in patients with NASH cirrhosis, the annual incidence of HCC in NAFLD was 2.6% (versus 4% in chronic hepatitis C) (73). Several recent studies assessing the outcome of patients with NASH cirrhosis demonstrated that during 6.5 years of follow-up, approximately 7% will progress to HCC (74–77).

A small but growing number of case reports and patient series have described HCC in NAFLD patients who do not have cirrhosis. This is a particularly interesting finding because obesity and metabolic syndrome, although known risk factors for NAFLD, have been associated with HCC and cancers outside the liver (78, 79). The American Cancer Society conducted a large, prospective study that showed obese men and women (BMI >35 kg/m^2) had a 4.52-fold and 1.68-fold greater relative risk of mortality from HCC than sex-matched people with a normal BMI (80). A meta-analysis comprising 5,037 overweight and 6,042 obese patients and normal-weight controls demonstrated an increased odds ratio (OR) for developing HCC of 1.17 and 1.89, respectively (81). Obesity has been shown to increase the risk of developing HCC even in patients already considered high-risk, such as those with cirrhosis; obesity increased the odds of developing HCC by 11-fold in patients with alcoholic cirrhosis and threefold in those with cryptogenic cirrhosis (82). Studies suggest that diabetes alone can increase the risk of developing HCC two- to threefold (79, 83); IR correlated with increased risk of HCC in patients with chronic hepatitis C (84). Several studies have introduced data suggesting that HCC can develop in noncirrhotic NASH; in particular, a recent cross-sectional study of 87 biopsy-proven Japanese NASH patients showed that nearly half (49%) of HCCs developed in noncirrhotic NASH (28% F2; 21% F3) (85). Another single-center study with 10 years of follow-up reported that cases of HCC due to NAFLD rose the most dramatically (41/118) and 22.8% of HCCs in NAFLD were in noncirrhotic patients (86).

ANIMAL MODELS OF NAFLD

Vital insights into the pathogenesis and underlying mechanisms of NAFLD have been gained from the study of existing animal models; detailed discussion is outside the scope of this article and is reviewed elsewhere (87) and summarized in **Table 1**. The majority of mechanistic work in NASH has been developed in animal models that rely on genetic [leptin-deficient mouse (*ob/ob*)] or dietary manipulation [methionine-choline–deficient (MCD) diet]. However, these models differ significantly from the human NAFLD phenotype and have pathogenically distinct mechanisms, leading researchers to question how reliably results from these models reflect human disease.

ob/ob Mouse

The *ob/ob* mouse carries a spontaneous mutation in the leptin gene, rendering them leptin-deficient; this autosomal recessive trait renders the animals hyperphagic, indolent, and severely diabetic with marked hyperglycemia, giving them a propensity to overeat, become obese and develop hepatic steatosis (88). Unlike humans with NAFLD, *ob/ob* mice do not spontaneously progress from steatosis to steatohepatitis; they require a "second hit" to trigger progression, usually in the form of low-dose lipopolysaccharide (LPS), ethanol exposure, or hepatic ischemia-reperfusion challenge. Although the model is useful for studying the transition of steatosis to NASH, progression to fibrogenesis is poorly modeled; *ob/ob* mice are protected from hepatic fibrosis (89). Two unmitigated limiting factors of this model are that *ob* gene mutations are not prevalent in humans and leptin levels do not correlate with human NAFLD (90).

Table 1 Commonly used animal models in NAFLD research

Model	Description of model	T2DM/IR	Obese	Hepatic inflammation	Fibrosis
Genetic					
ob/ob	Leptin-deficient, naturally occurring mutation; possible progression from steatosis to NASH with addition of LPS	Yes	Yes	No	No
db/db	Leptin-resistant, inherited mutation in leptin receptor gene; possible progression from steatosis to NASH with addition of "second hit"	Yes	Yes	No	No
Pten knockout	Liver-specific *Pten* knockout mice and *AlbCrePten flox/flox* mice; progression through steatosis, NASH, and hepatocellular adenomas	Yes	Yes	Yes	Yes
SREBP-1c	Overexpression of *SREBP-1c* in adipose tissue in transgenic mice	Yes	No	Yes	Yes
PPARα knockout	Impaired mitochondrial β-oxidation; development of steatosis	No	No	Yes	No
Dietary					
MCD	Impaired mitochondrial β-oxidation; development of steatosis, NASH, and fibrosis	No	No	Yes	Yes
CDAA	Lowered methionine levels and altered amino acid composition, causing a steady reduction in choline, impaired mitochondrial β-oxidation, and hepatic lipid accumulation; development of steatosis, NASH, fibrosis, and IR	Yes	Yes	Yes	Yes
ALIOS	High-fat chow (30% trans fats), fructose and glucose in drinking water, activity restriction	Yes	Yes	Yes	No
HFHC	Medium-chain trans fats; fructose in drinking water	Yes	Yes	Yes	Yes
Lieber-DeCarli HF liquid	Sprague-Dawley rats fed a high-fat, liquid diet (71% of energy from fat, 11% from carbohydrates, 18% from protein)	Yes	Yes	Yes	No
Combined					
db/db + MCD	Leptin-resistant plus second hit	Yes	Yes	Yes	Yes

Abbreviations: ALIOS, American lifestyle–induced obesity syndrome; CDAA, choline-deficient L-amino acid–defined; HF, high-fat; HFHC, high-fat, high-cholesterol; IR, insulin-resistance; LPS, lipopolysaccharide; MCD, methionine-choline–deficient; NASH, nonalcoholic steatohepatitis; PPARα, peroxisome proliferator–activated receptor α; PTEN, phosphatase and tensin homolog on chromosome 10q; SREBP-1c, sterol regulatory element–binding protein 1c; T2DM, diabetes mellitus.

db/db Mouse

Mutations in the diabetes gene (*db*) result in a rodent phenotype similar to that of the *ob/ob* mouse, yet *db/db* mice have normal or elevated levels of leptin but are resistant to its effect; the *db* gene encodes the leptin receptor Ob-R, of which there are two isoforms. The Ob-Rb isoform has a long intracytoplasmic region that contains signal transduction motifs that activate the JAK-STAT (Janus kinase–signal transducer and activator of transcription) protein kinase signal transduction cascade; C57BL/6K-*db/db* mice contain an insertion mutation that prematurely terminates the intracytoplasmic signaling domain rendering it inactive with the resultant leptin-resistant

phenotype (91). These animals develop steatohepatitis and liver fibrosis, although a second hit (MCD diet) is needed for both.

PTEN-Null Mouse

PTEN (phosphatase and tensin homolog on chromosome 10q) is a tumor-suppressor gene, whose major substrate is phosphatidylinositol-3,4,5-trisphosphate (PIP3), a lipid second messenger molecule generated by the action of phosphoinositide 3-kinases (PI3Ks). PIP3 activates the serine-threonine kinase protein kinase B (PKB/Akt) (92); these pathways regulate apoptosis, proliferation, and oncogenesis. Liver-specific *Pten* knockout mice (*AlbCrePten flox/flox* mice) develop hepatomegaly and steatohepatitis similar to human NASH at 40 weeks, and by 78 weeks HCC develops (93). A disadvantage is that the mice are insulin-sensitive.

American Lifestyle–Induced Obesity Syndrome Model

Recent efforts have, therefore, led to the development of the American lifestyle–induced obesity syndrome (ALIOS) model that more closely reflects the nutritional composition of the American fast-food diet and sedentary lifestyle that are risk factors for the human NASH phenotype. Nongenetically modified mice kept in cages without exercise racks and given unlimited access to a high-fat (30% trans fats) diet and high-fructose corn syrup for 16 weeks develop obesity, impaired glucose tolerance, hyperinsulinemia, and steatosis with necroinflammatory liver injury but only an early profibrogenic response at the molecular level (94); however, if fed longer, they will develop liver fibrosis and HCC after approximately 1 year of feeding.

A second group showed that significant fibrosing steatohepatitis can be produced in nongenetically modified mice fed medium-chain trans fats (as opposed to long-chain trans fats) and high-fructose, high-sucrose drinking water. The phenotype may develop from increased hepatic reactive oxygen species (ROS) and proinflammatory macrophages driving collagen deposition (95).

High-Cholesterol Diet

The role of dietary cholesterol has been investigated in animal models; a cholate- and cholesterol-enriched, atherogenic diet induced the progressive formation of steatosis, inflammation, ballooning, and fibrosis over 6–24 weeks. Pathological lesions developed at 12 weeks when 60% cocoa butter was added; furthermore, oxidative stress was induced (96). Accumulation of hepatic free cholesterol (FC) was observed in obese, hyperinsulinemic mice, mediating the transition from NAFLD to NASH (97).

Methionine-Choline–Deficient Diet

The MCD model is still considered the most reliable model for studying the inflammatory and fibrotic aspects of the NAFLD spectrum. The MCD diet induces much greater ROS production, mitochondrial DNA damage, and apoptotic cell death compared with other models of NAFLD (98); mice fed an MCD diet develop inflammation and hepatic fibrosis, in addition to hepatocellular TAG accumulation. The intensity of this effect varies with species, strain, and sex of the animals studied (99). Studies suggest that MCD impairs mitochondrial β-oxidation leading to induction of CYP2E1 (100). MCD also impairs hepatic very low density lipoprotein (VLDL) secretion. The principal disadvantage of this model is its dissimilarity to the pathogenic mechanisms operating in metabolic syndrome–related NAFLD in humans. In contrast, animals fed an MCD diet are

cachectic (losing 50% of body weight compared with control mice after 10 weeks), have low plasma TG levels, have low liver weight/bodyweight ratios, and are not overtly insulin resistant.

Choline-Deficient L-Amino Acid–Defined Diet

The choline-deficient L-amino acid–defined (CDAA) diet causes histopathological features similar to those seen with the MCD diet (steatosis, fibrosis, cirrhosis) but without the severe cachexia associated with the MCD diet. The features of fibrosing steatohepatitis develop after 24 weeks, with neoplastic foci developing at approximately 60 weeks and adenoma and HCC by approximately 84 weeks (101). Also, mice fed a CDAA diet develop peripheral IR after 1 month of treatment, and a progressive increase in body weight (102). Overall, this more closely resembles the human NAFLD condition and progression.

PATHOGENESIS

The current understanding of NAFLD pathogenesis has been advanced through both clinical research and the translational study of specific animal models (103). It is generally accepted that the initiating events in NAFLD are dependent on development of obesity and IR at the level of the adipose tissue and liver (104). Together, these conditions produce an increased free fatty acid (FFA) flux within the liver, derived from the nonesterified fatty acid pool (NEFA) via dysregulation of peripheral lipolysis, de novo lipogenesis (DNL), and dietary fats. Accumulation of lipotoxic intermediates such as diacylglycerol (DAG) causes hepatic IR (105). Increased FFA flux to the liver in turn places hepatocytes under considerable metabolic load and promotes hepatocyte lipotoxicity and endoplasmic reticulum (ER) stress (106, 107). The accumulation of TAGs within the cytoplasm of hepatocytes (steatosis) is an histologically apparent epiphenomenon reflecting these metabolic changes and is best considered an early adaptive response through which potentially lipotoxic FFAs are partitioned into inert intracellular TAG for storage (108). Ultimately, these insults combine with the additive effects of endotoxin–Toll-like receptor 4 (TLR4)-induced cytokine release by Kupffer cells and immune-mediated hepatocellular injury to induce cellular damage and activate cell death pathways, marking the transition to steatohepatitis (NASH) (109–111). When these processes persist, stellate cell activation, collagen deposition, and hepatic fibrosis occur (112).

Hepatic Lipid Homeostasis

Lipid handling in the liver is maintained by a delicate balance among delivery of fatty acids (FAs) to the liver, its usage by either esterification or oxidation, and turnover; this is orchestrated by a complex interplay of hormones, nuclear receptors, intracellular signaling pathways, and transcription factors and is summarized in **Figure 3**. Insulin signaling is crucial for the integration of lipid and glucose metabolism; it controls DNL via activation of sterol regulatory element–binding protein 1c (SREBP-1c) and Akt-regulated production of VLDL. Peroxisome proliferator–activated receptors (PPARs) promote lipid oxidation and expression of fatty acid transport proteins (FATPs).

Dietary fats are first absorbed in the intestinal lumen. The liver is essential for enterocyte hydrolyzed lipid absorption via bile acids (BAs); once absorbed, lipids are esterified and packaged into nascent chylomicrons, and released into the circulation via the lymphatic system (113). Once in the circulation, nascent chylomicrons mature by gaining apolipoprotein E (apoE) and apolipoprotein C2 (apoC2); gain of apoC2 activates lipoprotein lipase, hydrolyzing TAGs into glycerol and

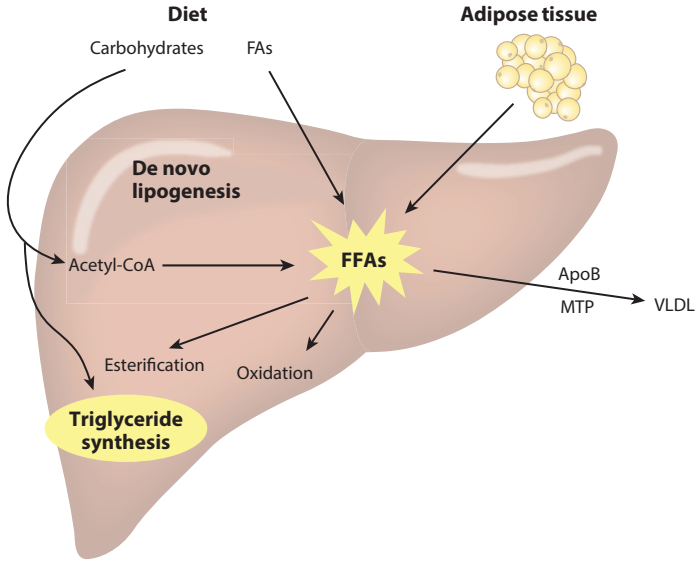

Figure 3

Lipid handling by the liver. Free fatty acids (FFAs) are delivered to the liver by diet, adipose tissue, and de novo lipogenesis (DNL). Dietary carbohydrates contribute to lipogenesis both by entering the Krebs cycle to produce acetyl-CoA for DNL and by providing the glycerol backbone via triose-phosphate.
Abbreviations: acetyl-CoA, acetyl coenzyme A; ApoB, apolipoprotein B; FAs, fatty acids; MTP, microsomal transfer protein; VLDL, very low density lipoprotein.

FAs (114); and FAs are partially taken up by adipose tissue with the remainder transported in chylomicron remnants and taken up by the liver after binding with the apoE receptor (115).

Hepatic lipogenesis. Liver FAs are sourced from DNL, the NEFA pool bound to albumin via uptake into the hepatocyte, and dietary fat. Once in the hepatocyte, FAs are further processed to form TAGs for storage, oxidized by mitochondria to create energy and ketones, added to lipoproteins for secretion as VLDL, or used to synthesize phospholipids, depending on ongoing metabolic requirements.

Acetyl-coenzyme A (acetyl-CoA) and malonyl-coenzyme A (malonyl-CoA) are important metabolic intermediates of DNL within the liver; acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) are two predominant enzymes that catalyze hepatic FA synthesis. DNL is tightly controlled on a transcriptional level by insulin and glucose, via SREBP-1c and carbohydrate-responsive element–binding protein (ChREBP), respectively. The activity of both SREBP-1c and ChREBP is controlled by another nuclear receptor, liver X receptor (LXR) (116). LXR binds to response elements in the promoters of gene targets such as CYP7A1; LXRα-null mice show decreased SREBP-1c and less lipogenesis (117). LXR directly induces DNL enzymes ACC and FAS and can be activated indirectly through insulin and glucose, further linking glucose and lipid metabolism (118, 119).

FAs are stored as inert TAGs that consist of a glycerol backbone with three esterified FAs; TAGs are then stored within the hepatocyte as a lipid droplet or packaged into VLDLs (120). SREBP-1c inhibits VLDL secretion by decreasing expression of microsomal transfer protein (121).

Hepatic FAs are also sourced via the NEFA pool; in an insulin-resistant state the NEFA pool is greater due to increased adipocyte lipolysis. FAs are taken up, not only passively but in a

facilitated manner by FATP2 and FATP5 (122); FATP5 knockout mice have decreased hepatic FFA uptake and TAG storage (123, 124). Other transport proteins include fatty acid binding protein and caveolin-1. Fatty acid translocase (CD36/FAT) is expressed on macrophages, adipocytes, myocytes, enterocytes, and hepatocytes, facilitating the uptake and intracellular trafficking of FFA and TG esterification in myocytes. In rodents with hepatic steatosis, CD36/FAT expression is increased and is a common target of LXR, pregnane X receptor, and PPARγ (125). Its role in human liver disease is not well clarified; however, in morbidly obese NAFLD patients, hepatic CD36/FAT mRNA levels were positively correlated with liver fat content and apoptosis in NASH (125–127).

Hepatic fatty acid oxidation. FAs may be oxidized in the mitochondria, peroxisomes, or microsomes and represent the most efficient energy yield for homeostasis, compared with other macronutrient subtypes. β-oxidation of FAs in the mitochondria is the predominant energy source in the fasted state and is the process by which FAs are broken down into acetyl-CoA, which then enters the citric cycle. However, for FAs to be used in β-oxidation pathways, they need to be transported from the cytoplasm into the mitochondria; although short- and medium-chain fatty acids simply diffuse across the mitochondrial membrane, long-chain fatty acids (LCFAs) are activated by acyl-CoA-synthetase to acyl-CoA in the cytosol. LCFAs are shuttled across and catalyzed by carnitine palmitoyl transferase 1 (CPT-1) on the outer mitochondrial membrane. Malonyl-CoA, a key intermediary of DNL, is an inhibitor of CPT-1, as is insulin (128). This step is promoted by PPARα, which also upregulates FA transport proteins and enzymes related to apolipoprotein B (apoB) metabolism (129). Under normal circumstances, short-, medium-, and long-chain fatty acids undergo β-oxidation in the mitochondria, whereas very long chain fatty acids undergo oxidation in the peroxisomes. When FAs are in abundance, CYP4A-dependent ω-oxidation occurs in the ER (130). Similarly, in FA excess, acetyl-CoA can be converted into ketone bodies rather than enter the citric acid cycle (131).

Hepatic Glucose Metabolism

Carbohydrate intake can also influence FA metabolism in the liver; excess glucose is normally stored as glycogen, under the influence of insulin. Excess glucose can provide the glycerol backbone via triose phosphate or acetyl-CoA (via the Krebs cycle), which can be further esterified into TAGs or VLDL via DNL.

After feeding, glucose is delivered to the hepatocyte by the portal vein and is taken up by glucose transporter type 2, independent of insulin signaling. Once in the hepatocyte, glucose is phosphorylated to glucose-6-phosphate by liver glucokinase (L-GCK); mutations in the GCK gene have been implicated in NAFLD pathogenesis and are discussed below.

Glycolysis and glycogen synthesis. Insulin regulates glycolysis, a 10-step process that metabolizes glucose to pyruvate with a net gain of two ATP and two NADH molecules per glucose molecule; enzymes involved in regulation are L-GCK, phosphofructokinase, AMP, and pyruvate kinase. Pyruvate kinase is activated by phosphoenolpyruvate and limited by ATP abundance. ChREBP induces transcription of pyruvate kinase with glucose; insulin, adrenaline, and glucagon regulate pyruvate kinase via the phosphoinositide 3-kinase (PI3K) pathway (132). Pyruvate undergoes decarboxylation to acetyl-CoA, which is then processed in the metabolic citric acid cycle or utilized in anabolic pathways such as DNL, as mentioned above. Insulin also activates glycogen synthesis via repression of protein kinase A (PKA), an inactivator of glycogen synthase, the key enzyme catalyzing uracil-diphosphate glucose to glycogen.

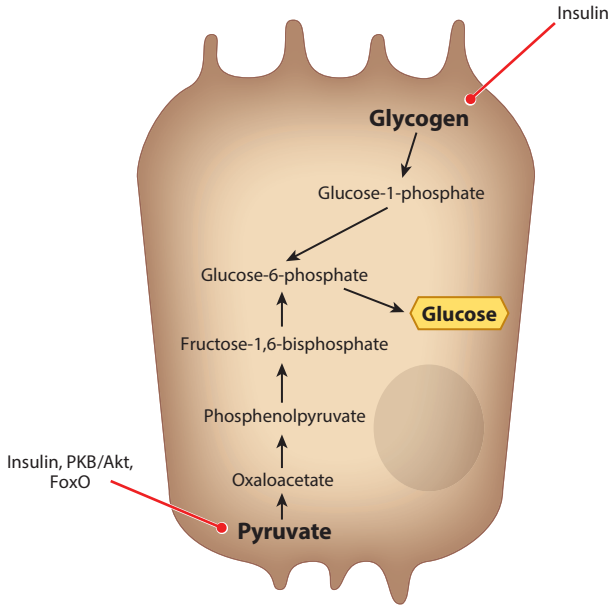

Figure 4

Hepatic glucose metabolism, when fasted. Glycogenolysis (*top*) and gluconeogenesis (*bottom*) provide glucose-6-phosphate as a substrate for the synthesis of glucose in the fasted state. Both pathways are normally repressed by insulin.

Glycogenolysis and gluconeogenesis. When glucose levels start to wane, the liver provides the body with energy by breaking down and converting glycogen into glucose; during prolonged fasting, hepatic gluconeogenesis occurs, a process beginning in the mitochondria and summarized in **Figure 4**.

Insulin Resistance and Hepatic Steatosis

Hepatic steatosis occurs when insulin signaling is impaired, with the development of IR at the level of adipose tissue and liver and a sustained excess delivery of FAs to the liver. In patients with NAFLD, the majority of hepatic fat comes from the NEFA pool (59%); the remainder comes from DNL (26%), which is increased in NAFLD, and from the diet (15%), as shown by isotope studies (133) and summarized in **Figure 5**. **Figure 6** summarizes the main processes contributing to IR and hepatic steatosis. Specifically, impairment in insulin-mediated suppression of lipolysis in adipocytes due to obesity, recruitment of macrophages to adipocytes, and increased secretion of proinflammatory adipocytokines (TNFα) leads to the increased NEFA pool in NAFLD and thus, the accumulation of intramyocellular lipids in skeletal muscle, which interferes with insulin signaling and impairs glucose uptake. This scenario leads to the development of peripheral IR and a compensatory hyperinsulinemia, resulting in increased delivery of FAs to the liver. Hyperinsulinemia leads to overstimulation of the transcription factors SREBP-1c and ChREBP leading to increased DNL. Gluconeogenesis is not suppressed despite hyperinsulinemia, providing yet more substrate for DNL. Both β-oxidation and VLDL assembly are inhibited in patients with NAFLD (131), leading to further buildup of TAGs in the liver. FA metabolites such as DAG further amplify hepatic IR.

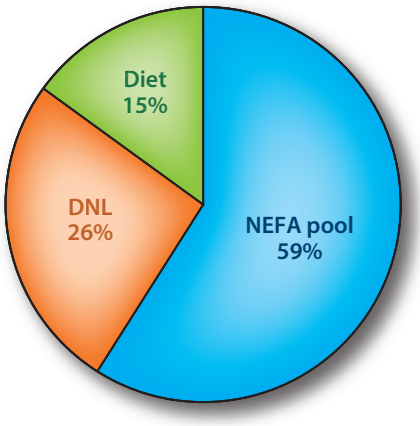

Figure 5

Contribution of free fatty acids (FFAs) to hepatic steatosis. In patients with nonalcoholic fatty liver disease, the majority of FFAs come from the nonesterified fatty acid pool (NEFA). Data from Donnelly et al. (133). Other abbreviation: DNL, de novo lipogenesis.

Hepatic insulin resistance. Hepatic IR has been described in NAFLD, with studies in animal models implicating cytokine-mediated (TNFα/Il-6) and ER stress-activated, c-Jun N-terminal kinase (JNK)-mediated IR. However, recent data suggest that lipid metabolites themselves can cause IR. DAG is an intermediate in the conversion of FA oleate into TAG. Accumulation of hepatic DAG within cytosolic lipid droplets is associated with increased translocation of the primary novel protein kinase C (PKC) isoform in the liver, PKCε, to the plasma membrane where it binds and inhibits the intracellular kinase domain of the insulin receptor (134, 135). In a recent translational study in a group of obese, nondiabetic patients undergoing bariatric surgery, although all patients were obese, there was significant variation in IR; DAG content and PKCε activation were the strongest predictors of hepatic IR and accounted for 60% of the variability in hepatic insulin sensitivity (136).

Adipose tissue insulin resistance. Adipose tissue is a complex, metabolically active organ that secretes adipocytokines that play a crucial role in regulating insulin sensitivity. As obesity increases, so does expansion of ectopic fat, that is, expansion of adipocytes at sites such as the omentum (visceral fat). Ectopic fat is considered dysfunctional; it is more likely to be infiltrated with macrophages, undergo inflammation, and secrete inflammatory cytokines such as tumor necrosis factor α (TNFα), Il-6, monocyte chemoattractant protein 1 (MCP-1), resistin, and plasminogen activator inhibitor 1, all of which blunt adipocyte insulin sensitivity. TNFα is expressed more in ectopic fat than subcutaneous fat, and activates 2 proinflammatory pathways: the nuclear factor κB (NF-κB) pathway and JNK pathway (137). TNFα-induced disruption of insulin signaling occurs via phosphorylation of insulin receptor substrate 1 mediated by JNK (138). MCP-1 is an important chemokine for macrophage migration into adipose tissue, which is increased in ectopic fat (139).

Conversely, adipokines that repress IR and steatosis show decreased secretion in ectopic fat. Adiponectin acts to modulate lipid metabolism and decrease inflammation via AMP-activated protein kinase (AMPK) and PPARα pathways (140); it is also insulin-sensitizing as a result of mitogen-activated protein kinase (MAPK)-mediated improvement in FA oxidation and decreased liver gluconeogenesis (141). Patients with NAFLD have been reported to have low adiponectin

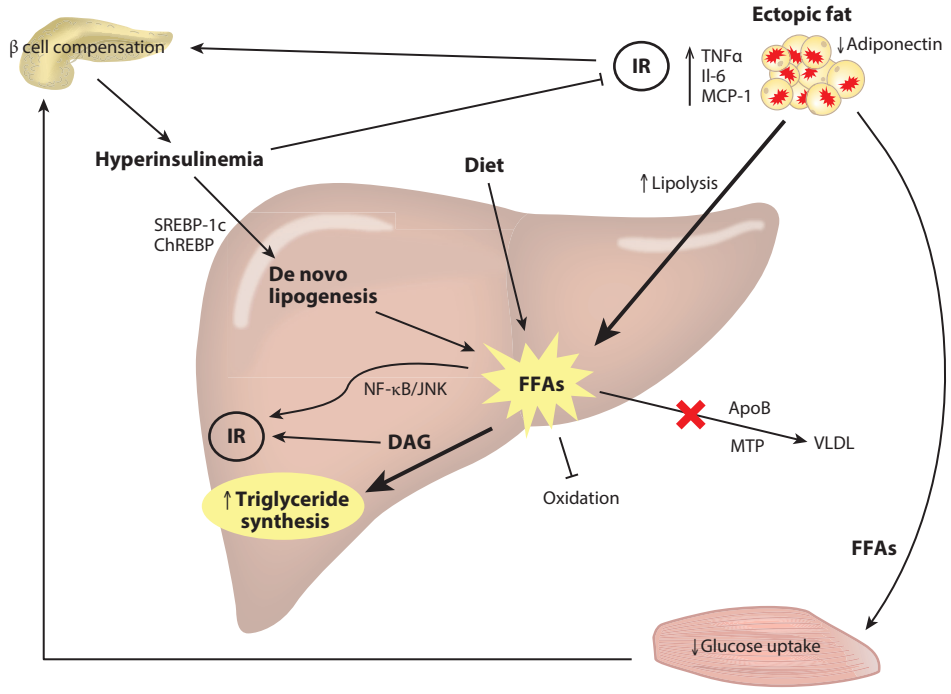

Figure 6

Interorgan links among insulin resistance (IR), dysregulation of hepatic free fatty acid (FFA) flux, and the development of hepatic steatosis. Obesity and the deposition of ectopic fat lead to the recruitment of macrophages; dysregulated adipokines abrogate insulin signaling. Thus arises an impairment of insulin-mediated suppression of lipolysis, leading to increased flux of FFAs from adipocytes to other tissues (60%). Also, impaired glucose tolerance develops secondary to increased FFA flux to muscles and suppression of glucose uptake. Pancreatic β cells compensate by increasing insulin secretion, leading to hyperinsulinemia. De novo lipogenesis is stimulated in the liver, contributing 25% of the altered FFA pool in hepatic steatosis; dietary FFAs contribute the remainder. Diacylglycerol (DAG), a triacylglycerol intermediate, also contributes to hepatic IR. Abbreviations: ApoB, apolipoprotein B; ChREBP, carbohydrate-responsive element–binding protein; Il-6, interleukin 6; MCP-1, monocyte chemoattractant protein 1; MTP, microsomal transfer protein; NF-κB/JNK, nuclear factor κB/c-Jun N-terminal kinase; SREBP-1c, sterol regulatory element–binding protein 1c; TNFα, tumor necrosis factor α; VLDL, very low density lipoprotein.

levels, and mice with steatotic livers have improved insulin sensitivity, decreased steatosis, and lower levels of TNFα when treated with recombinant adiponectin (142, 143).

Leptin is another adipocytokine that is purported to have a role in hepatic steatosis, as leptin-deficient mice (*ob/ob*) become obese, hyperphagic, and diabetic and develop marked steatosis, indicating a role for leptin in prevention of fatty liver either indirectly through central neural pathways or directly via hepatic activation of AMPK (144, 145). In contrast to animal models, obese patients have a fatty liver despite high levels of leptin, suggesting resistance to leptin mediated by overexpression of suppressor of cytokine signaling-3, a molecule that inhibits leptin signaling leading to reduced AMPK activation (146).

Bile acids in steatosis. Important regulators of both glucose and lipid homeostasis, BAs absorbed from the ileum act as ligands for a number of nuclear hormone receptors, with farnesoid X receptor

(FXR) being the first identified and most dedicated to BA signaling (147, 148). In the liver, FXR is a key negative regulator of BA synthesis, interfering with CYP7A1-initiated conversion of cholesterol to primary BAs by upregulating a protein called small heterodimer partner (SHP); it also facilitates the absorption of BAs in the distal ileum. FXR also negatively regulates glycolysis through direct inhibition of ChREBP (149) and lipogenesis through the SHP–SREBP-1c axis (150). Activation of hepatic FXR leads to reduced FA and TAG synthesis, increased β-oxidation of FFAs via the induction of PPARα (151), and decreased expression of apolipoprotein C3, involved in VLDL assembly (152). As such, FXR-deficient mice develop moderate hepatic steatosis (153).

Steatohepatitis

Until recently, steatosis was considered the "first hit" in the pathogenesis of NAFLD/NASH but is now believed by many to be an epiphenomenon reflecting changes in hepatocyte FFA flux and cellular stress responses (154); thus, steatosis can be considered an early adaptive response to hepatocyte stress through which lipotoxic FFAs are partitioned into relatively stable, inert intracellular TAG stores. An elegant study showed that silencing expression of diacylglycerol acyltransferase 2, a key enzyme mediating the conversion of FFA to TAG, increased hepatic inflammatory activity, fibrosis, oxidative stress, and hepatocellular apoptosis in leptin receptor-deficient (*db/db*) mice fed an MCD diet, rather than ameliorating steatohepatitis by reduction in TAG synthesis (108).

Thus, discussion of pathogenesis and progression of NAFLD/NASH should consider the combined effects of several fundamental biochemical and immunological processes rather than abiding by the sequential two hit hypothesis (154). Lipotoxicity develops when adaptive mechanisms that mitigate the deleterious effects of FA in the liver are overwhelmed, leading to the generation of ROS, ER stress, and cellular dysfunction. Ultimately, cellular damage triggers a mixture of immune-mediated hepatocellular injury and both necrotic and apoptotic cell death pathways; once these persist, stellate cell activation and fibrogenesis ensue (112) (**Figure 7**).

Direct hepatocyte lipotoxicity. A surplus of lipid metabolites may enter harmful pathways leading to cell dysfunction (lipotoxicity); recent data link the overloading of hepatocytes with FFAs to hepatocyte apoptosis and liver injury (155). The key role of stearoyl-CoA desaturase 1 (SCD1), the enzyme that converts saturated fatty acid to monounsaturated fatty acid, was demonstrated in vivo and in vitro; SCD-1 knockout mice fed an MCD diet accumulated less TAG compared with wild-type mice but had increased hepatocyte apoptosis and liver injury (155).

Another lipid metabolite, FC has been shown to sensitize hepatocytes to TNF and Fas-induced apoptosis (156). Mari et al. (156) showed that in TAG- or FC-loaded hepatocytes, TNF treatment caused apoptosis and ROS in livers with increased FC content, due in part to glutathione reduction in mitochondria. It has been reported that FC in the liver increases with disease progression from steatosis to NASH (157).

Oxidative stress. Oxidation of FAs in the hepatocyte is a primary source of ROS; under normal physiological conditions, short-, medium-, and long-chain fatty acids undergo β-oxidation, a "safe" processing mechanism. In the event of hepatocyte FFA overloading, physiologically minor pathways such as β-oxidation in the peroxisome, and cytochrome P450-4A- and P450-2A1-mediated ω-oxidation in the ER further increase hepatocyte ROS production. Indeed, β-oxidation of LCFAs within peroxisomes and ω-oxidation in ER are upregulated in NASH, contributing to ROS formation (158). This could be secondary to inhibition of mitochondrial β-oxidation due to inhibition of CPT-1 by an accumulation of malonyl-CoA (133, 159). Oxidative stress is defined

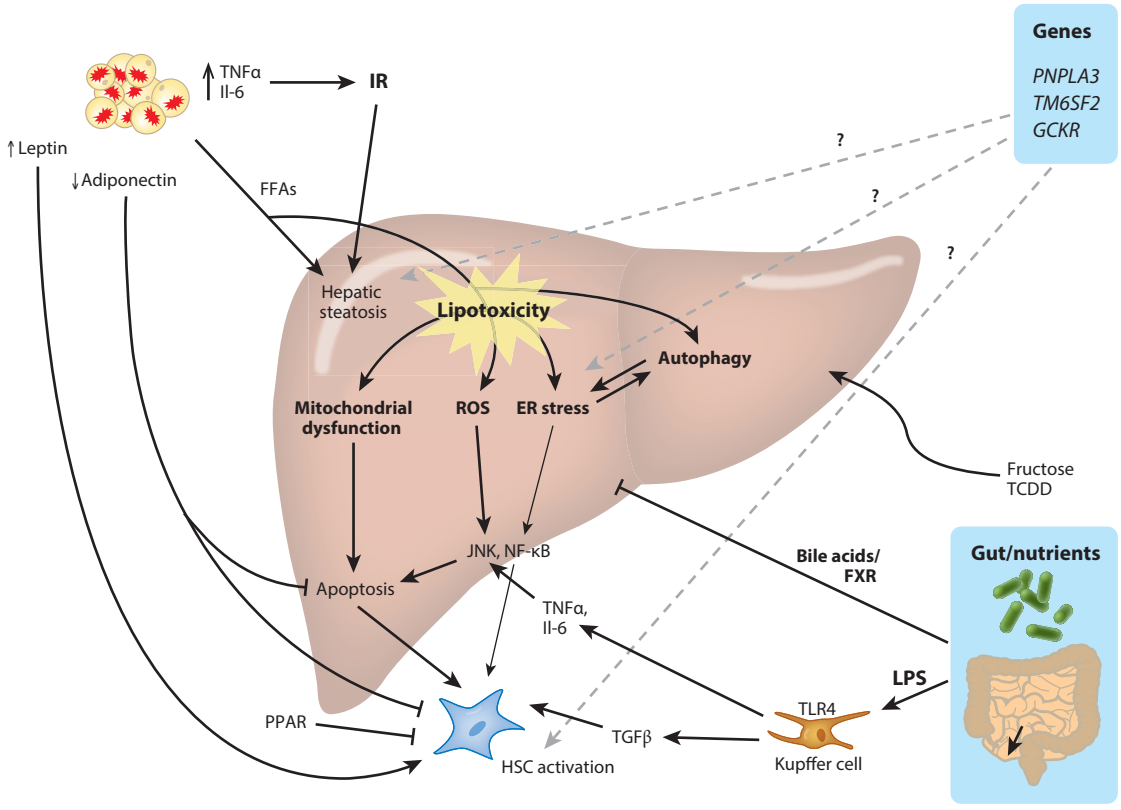

Figure 7

A schematic synthesizing nonalcoholic steatohepatitis pathogenesis and integrating lipotoxicity, diet, obesity, insulin resistance (IR), endoplasmic reticulum (ER) stress, autophagy, apoptosis, and ultimately hepatic stellate cell (HSC) activation and fibrogenesis. Microbiota and genetics (*light blue areas*) are modifiers of these processes. Abbreviations: FFAs, free fatty acids; FXR, farnesoid X receptor; *GCKR*, glucokinase regulator; Il-6, interleukin 6; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; *PNPLA3*, patatin-like phospholipase domain containing 3; PPAR, peroxisome proliferator–activated receptors; ROS, reactive oxygen species; TCDD; 2,3,7,8-tetrachlorodibenzodioxin; TGFβ, transforming growth factor beta; TLR4, Toll-like receptor 4; *TM6SF2*, transmembrane 6 superfamily 2; TNFα, tumor necrosis factor α.

as an imbalance between ROS production and that of protective antioxidants and is a common final pathway in FFA lipotoxicity (160). Oxidative stress results in nuclear and mitochondrial DNA damage, phospholipid membrane disruption by lipid peroxidation, and the release of proinflammatory cytokines. Sanyal et al. (160) demonstrated that a marker for oxidative stress, 3-nitrotyrosine, was elevated in liver biopsies with NASH. Lipid peroxidation of polyunsaturated fatty acids (PUFAs) generates toxic aldehyde by-products, including malondialdehyde and hydroxynonenal; malondialdehyde and hydroxynonenal are more pervasive than ROS and so can damage more distant intracellular organelles, causing cell death (161).

Endoplasmic reticulum stress. ER stress responses in NAFLD have created significant interest. The ER is an intracellular membranous network where the majority of secreted and membrane proteins are folded. Unfolded proteins can accumulate due to a variety of cellular responses. Indeed, the ER is highly sensitive to lipids, and when FFAs are in excess, the development of misfolded or unfolded proteins may ensue. These proteins form aggregates and an adaptive mechanism called

the unfolded protein response (UPR) is triggered (162); this response can lead to the induction of autophagy (107). The UPR is an orchestrated response to reestablish normal homeostasis by cell cycle arrest, transient attenuation of global protein synthesis, folding catalysts, induction of ER-localized chaperone proteins, and ER-associated protein degradation. If this response fails, stress-sensor proteins, including activating transcription factor 6, inositol-requiring enzyme-1 (IRE-1), and Protein Kinase R–like ER kinase (PERK), can trigger apoptosis (163, 164). IRE-1 possesses endoribonuclease activity and thus can excise a 26-nucleotide fragment from XBP1 mRNA and translate an active splice of transcription factor XBP1s (164). This interacts with various inflammatory cascades by activation of JNK and inhibitor of κB (IκB) kinase (IKK)-NFκB signaling and production of ROS (165). Significant increases in ER stress response genes, including CHOP, GADD34, and GRP78, were seen when palmitic acid and stearic acid were incubated with a rat hepatoma cell line followed by mitochondria-dependent apoptosis (166, 167). ER stress may have a role in human NAFLD; Puri et al. (163) showed variable degrees of UPR activation in biopsies from patients with NAFLD and NASH compared with normal biopsies from patients with metabolic syndrome.

Innate immunity and NASH pathogenesis. Endotoxin (LPS), one of the key components of the outer wall of gram-negative bacteria, plays a central role in innate immune responses and has been a focus of research in the promotion of NASH. Further discussion on the role of gut-derived signals and the microbiome in NAFLD progression is covered elsewhere in this review.

LPS is delivered directly to the liver via the portal vein and is recognized by TLR4 located on Kupffer cells, the resident hepatic macrophages; activation of TLR4 by LPS requires co-receptors CD14 and MD-2 and results in activation of myeloid differentiation factor 88 (MyD88)–dependent and Toll/interleukin-1 receptor domain–containing adaptor-inducing interferon β (TRIF)–dependent signaling pathways, finally resulting in upregulation of the NF-κB pathway and JNK (168). Murine models have demonstrated enhanced TLR4 expression and portal endotoxemia in wild-type mice fed an MCD diet; hepatic lipid accumulation and hepatic mRNA levels of key fibrogenic markers were attenuated in TLR4 mutant mice (169). A link between gut flora and liver injury was further demonstrated by Cani et al. (170), as feeding mice a 4-week high-fat diet altered the content of gut microflora and increased levels of LPS; mice with metabolic endotoxemia induced by subcutaneous infusions of LPS also developed a steatohepatitic phenotype. In genetically overweight mice (leptin-deficient or leptin-resistant), sterilization of the gut of endotoxin-bearing, gram-negative organisms with probiotics prevents the development of NASH and IR; they are also more prone to developing steatohepatitis when exposed to low-dose LPS (171). The exact downstream pathway of TLR-4 that contributes to the pathogenesis of NASH is currently unknown but may include chaperone proteins, transcription factors, and ROS (172).

TLRs comprise a family of pattern recognition receptors that play a key role in the innate immune system and recognize specific invariant motifs on pathogens, including LPS (TLR4), peptidoglycan (TLR2), and unmethylated CpG motifs (TLR9); NASH research has generally focused on TLR4, as detailed above. However, the roles of TLR9 and more recently TLR2 are being investigated.

TLR9. TLR9 was recently identified as a key player in the pathogenesis of NASH; using several strains of knockout mice, Miura et al. showed that consumption of a CDAA diet activates TLR9 signaling on Kupffer cells thereby inducing Il-1β production via a MyD88-dependent pathway (173). Indeed, blockage of Il-1 signaling in mice leads to a reduction of TLR-9–mediated liver injury (174). In vivo models of hepatic fibrogenesis suggest a role for TLR9 in hepatic stellate cell (HSC) activation and fibrogenesis; a direct role of TLR9 in NAFLD fibrogenesis is yet to be

defined (173). However, TLR9-MyD88 signaling can mediate Il-1β production in Kupffer cells, leading to NASH progression via stimulation of HSCs and hepatocytes (174).

TLR2. Our knowledge of the role of TLR2 in NAFLD is in its early stages, partly owing to the complexity associated with TLR2 activation and ligand specificity. However, in 2013, Miura et al. (175) showed that TLR2 knockout mice have less inflammation after having been fed a CDAA diet to induce NASH; however, other groups have shown increased TLR4 expression and enhancement of NASH in TLR2 knockout mice fed an MCD diet (176).

Hepatocyte Injury and Death in NASH

Ultimately, multiple distinct insults combine to induce cellular damage and activate cell death pathways, marking the transition to NASH. Hepatocyte death occurs in the form of either programmed (apoptotic, necroptotic) or accidental (necrotic) cell death; recent evidence suggests cross talk between other types of cell death. Terminal events include breakdown of cellular components with intact plasma membranes (apoptosis) or lytic processes (necrosis).

Apoptosis is a highly regulated programmed cell death and a prominent morphologic and pathogenic feature of NASH (177). Apoptosis is executed by two distinct pathways: the extrinsic pathway mediated by death receptors at the cell surface and the intrinsic pathway activated by mechanisms of cell and membrane stress (ER-based, mitochondrial).

Death receptors that can initiate the extrinsic pathway include Fas, tumor necrosis factor receptor 1 (TNF-R1), and tumor necrosis factor–related apoptosis–inducing ligand receptors (TRAIL-Rs). FFAs induce upregulation of Fas and TRAIL-R5 (DR5); both Fas and DR5 expression were higher in patients with NASH than in patients with steatosis (177, 178). Similarly, both TNF expression and TNF-R1 expression in liver tissue were increased in NASH patients (179).

In hepatocytes, FFAs can induce lysosomal permeabilization and mitochondrial dysfunction, which consequently lead to apoptosis (155, 180). Cathepsin B–deficient mice are unable to induce lysosomal-driven apoptosis and are thus protected against diet-induced steatohepatitis (181, 182). Also, silencing of Cathepsin B prevented FFA-induced mitochondrial dysfunction (182). Redistribution of Cathepsin B into the cytoplasm is present in livers of patients with NAFLD (183). Cleaved cytokeratin 18 fragments, markers of apoptotic activity, have been correlated with disease severity and could potentially be used as markers of disease progression in NAFLD (184).

Experimental studies using pan-caspase inhibitors lend further proof of apoptosis as a critical pathogenic mechanism in NASH. VX-166 is an irreversible pan-caspase inhibitor that can reduce apoptosis, improve inflammation, and reduce fibrogenesis in mice fed an MCD diet, prior to the development of NASH (185); VX-166 can reduce apoptosis and improve liver injury and oxidative stress in even established steatohepatitis (MCD-fed *db/db* mice) (111). Inflammation was significantly reduced, with an overall improvement in NAS and reduction in TNFα and MCP-1 expression (111). Caspase inhibitors have recently been trialed in humans; in a phase 2 study, 124 patients with biopsy-proven NASH were randomized to placebo or different strengths of GS-9450, a selective caspase inhibitor of caspases 1, 8, and 9. After 4 weeks of treatment, significant reductions in ALT and smaller, nonsignificant reductions in AST and cytokeratin 18 were reported (6).

Necrosis (or oncosis) leads to cell swelling, karyolysis, and cell membrane rupture; an unprogrammed form of cell death, it occurs in ATP depletion and is commonly associated with an inflammatory cell reaction. Both apoptosis and necrosis can occur as a consequence of the same triggering event, possibly representing different ends of a spectrum of cell death (186).

A third form of programmed cell death, incorporating features of both apoptosis and necrosis, termed necroptosis may also play a role in NASH. Necroptosis shares upstream mediators with apoptosis (receptor-induced) but results in caspase-independent organelle and cellular swelling; this may function as a pathway to enable cell death where apoptosis is inhibited. A recent study showed that NASH livers express high levels of receptor-interacting protein (RIP)-3, which along with RIP-1 forms a complex known as the necrosome, a critical transducer of the necroptotic signal (187).

Finally, a caspase-1 dependent form of programmed cell death known as pyroptosis has been shown to contribute to NASH development in mouse models. Hepatic caspase 1 activation occurs in hepatocytes and tissue macrophages and is mediated by NLRP3 [nucleotide oligomerization domain (NOD)-like receptor family, pyrin domain–containing 3] inflammasomes; mice deficient in the NLRP3 inflammasome develop less severe liver disease in dietary-induced NASH (188). Conversely, mice with a constitutively activated NLRP3 inflammasome showed hepatocyte pyroptotic cell death (189).

Autophagy is a phylogenetically conserved pathway of autodigestion of cellular proteins and organelles within cells; in times of nutrient deficiency, autophagy provides energy through degrading cellular constituents and clearing damaged cellular components, enabling the continuous cell cycle (190). Inhibition of autophagy in hepatocytes cultured in MCD medium leads to increased levels of TGs (191). Autophagy may protect against cell death by clearance of dysfunctional mitochondria via mitophagy (192). In vitro data suggest that certain FFAs such as palmitic acid suppress autophagy whereas other SFAs such as oleic acid promote autophagy (193). More studies are needed to fully elucidate the role of autophagy in NAFLD pathogenesis.

Fibrosis

Persistence of the combined effects of the fundamental biochemical and immunological mechanisms detailed above ultimately leads to activation of HSCs, collagen deposition, and hepatic fibrogenesis (scar formation) (112). Initial collagen deposition occurs in the perisinusoidal space of zone 3 of the hepatic acinus, commonly referred to as the chicken-wire pattern of fibrosis. With progression, periportal collagen deposition occurs with activation of the hepatic progenitor cells (the ductular reaction) (46); the extent of both the ductular reaction and fibrogenesis, in conjunction with evidence of hepatocyte senescence and decreased hepatocellular proliferation, correlates with calculated scores of IR (46). Eventually, vascular architectural and parenchymal remodeling occurs whereby portal tracts and hepatic veins are connected by collagen bundles and cirrhosis develops that may then lead to liver failure and HCC (46). Discussion of the basic mechanisms of hepatic fibrogenesis is not within the scope of this article, and this topic is reviewed elsewhere (194). Profibrogenic mechanisms in NASH are also shared with other forms of chronic liver disease such as induction of cytochrome P450 2E1 and oxidative stress, apoptosis, cytokine levels and inflammation, but there are clearly specific mechanisms related to NASH that are associated with adipose tissue expansion, IR, and metabolic syndrome. Indeed, it has recently been suggested that the degree of dysfunctional adipose tissue is more important than visceral adiposity; adipose tissue IR, a marker of dysfunctional adipose tissue, has been linked to liver fibrosis severity in NASH patients (195).

Adipokines. Adipokines can contribute to fibrogenesis, the most well-studied being leptin and adiponectin. Leptin-deficient mice (*ob/ob*) have reduced fibrogenesis, which is reverted with leptin (196). HSCs express functional leptin receptors and are thus responsive to increased circulating

levels of leptin; the activated phenotype of stellate cells is modulated by leptin, including stimulation of proliferation, suppression of apoptosis, increase in collagen 1 and tissue inhibitor of metalloproteinase 1 expression (197), activation of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase and ROS generation, and increase in phagocytosis of apoptotic bodies (198). Interestingly, low levels of leptin are seen in quiescent stellate cells and increase on activation (199). Leptin also targets Kupffer cells and sinusoidal endothelial cells, increasing transforming growth factor β (TGFβ) expression (200). The compelling experimental evidence for a relationship between leptin and fibrogenesis does not seem to mirror data from human studies; no study has convincingly correlated leptin levels independently with fibrosis progression.

Adiponectin has been shown to have antifibrotic effects, as adiponectin-deficient mice have more fibrosis when challenged by chronic carbon tetrachloride (CCl_4) administration (201). Adiponectin modulates stellate cells by inhibition of proinflammatory pathways (NF-κB), reduced TGFβ-induced profibrogenic gene expression, and increased caspase-mediated apoptosis. HSCs express adiponectin receptors and its antifibrotic action is mediated through AMPK-mediated pathways (199, 202). As is the case with leptin, no clear evidence exists linking low levels of adiponectin with fibrosis in human NAFLD, independent of severe IR.

Renin-angiotensin system. The renin-angiotensin system is a key modifier of IR and is activated locally in adipose tissue where it facilitates metabolic syndrome (203). Effector cytokines of the renin-angiotensin system are key in fibrogenesis in NASH. NF-κB promotes survival of hepatic myofibroblasts and hepatic fibrogenesis; it is recognized that angiotensin II mediates this effect through activation of IKK phosphorylation of the NF-κB subunit RelA at Ser536 (204).

Insulin resistance. Altered glucose metabolism and the presence of IR can affect HSCs; they express receptors for both insulin and insulin-like growth factor 1 and advanced glycation end products promoting fibrogenesis, upregulating TGFβ and connective tissue growth factor (205).

Nuclear receptors. Other molecular mechanisms involved in fibrosis include PPAR isoforms; experimental work concluded that suppression of PPARγ is required for HSC activation (206), with ligands for PPARγ reverting the activated phenotype of HSCs in vitro and abrogating fibrosis in vivo (207). Interestingly, the protection conferred by PPARγ from fibrosis may be from its expression in nonparenchymal cells, as when PPARγ was specifically deleted from discrete cells types, necroinflammation, oxidative stress, and fibrosis worsened in response to chronic injury (208). GFT505, a dual PPARα/PPARδ agonist, inhibited proinflammatory and profibrogenic gene expression in a murine model of NASH fibrosis (209).

BAs act as metabolic signaling molecules, aiding dietary lipid absorption, and are involved in cholesterol homeostasis; they are reabsorbed into the enterohepatic circulation and direct hepatic TG and glucose metabolism. They activate nuclear hormone receptor FXR and the G protein–coupled cell surface receptor TGR5, which inhibit hepatic de novo lipogenesis, hepatic gluconeogenesis, and glycogenolysis and improve insulin sensitivity. In animal studies, FXR activation has anti-inflammatory actions, partly through inhibiting NF-κB (210, 211). In vivo evidence exists for a protective effect of FXR agonists against liver inflammation and fibrosis (212). Thus, obeticholic acid, an FXR agonist, improved insulin sensitivity and reduced markers of fibrosis in NAFLD patients with diabetes (213).

Free cholesterol. Interestingly, dietary cholesterol may accelerate liver fibrosis in NASH via the accumulation of FC in HSCs, which results in high expression levels of TLR4, thus sensitizing

HSCs to the profibrogenic stimulus of TGFβ (214). foz/foz mice develop NASH with fibrosis, with cholesterol-lowering agents reversing steatohepatitis and limiting fibrosis (215).

Cannabinoid system. The endogenous cannabinoid (CB) system has also been implicated in fibrogenesis in NAFLD; HSCs express both CB1 and CB2 receptors, and inactivation of CB1 receptors results in reduced fibrogenesis in vivo, with reduced liver levels of TGFβ and reduced accumulation of liver myofibroblasts after growth inhibition and apoptosis (216). In contrast, enhanced activation of CB2 receptors may be antifibrogenic and anti-inflammatory, via a number of distinct mechanisms, including a reduction of the proinflammatory effects of Il-17 (217). Rimonabant, a CB1 antagonist, was withdrawn from the market due to central nervous system side effects, but CB1 antagonists unable to traverse the blood-brain barrier are currently in development.

Senescence. Cellular senescence refers to the irreversible growth arrest occurring when cells are exposed to potentially oncogenic insults (218). Senescence inducers include repeated cell division and strong mitogenic signals, telomere shortening, DNA damage, protein aggregation, and increased ROS (219–221); the abundance of senescent cells increases in tissues with chronological aging (222). Senescent cells are metabolically active, resistant to apoptosis, and removed by the immune system (218). Interestingly, senescent cells secrete proinflammatory cytokines and proteases termed the senescence-associated secretory phenotype (SASP). Recent experimental evidence suggest that this may be important in fibrosis progression; mice chronically treated with CCl_4 showed senescence-activated HSC accumulation in cirrhotic liver (223). Furthermore, SASP matrix metalloproteinases limit fibrosis following liver injury. In addition, p53 knockout mice whose senescence pathways are disrupted had worsened fibrosis compared with wild type (223). Interestingly, senescence of hepatocytes has been linked to fibrosis progression in NAFLD; hepatocyte p21 expression, the universal cell cycle inhibitor, independently correlated with fibrosis stage in liver sections from 70 NAFLD patients (224). Future work may involve investigating the role of cell-cell interactions or how release of soluble factors from each cellular compartment influences the other.

Hedgehog pathway. Hedgehog pathway signaling modulates myofibroblast transdifferentiation within the space of Disse; hedgehog pathway–stimulated immature ductular cells secrete chemokines resulting in accumulation of natural killer T (NKT) cells in the liver (225). These cells enhance the growth of myofibroblasts by secreting profibrogenic cytokines, osteopontin, and hedgehog (226); the level of hedgehog pathway activity correlates with fibrosis stage in NASH patients (227).

Autophagy. As mentioned above, although autophagy appears to ameliorate hepatic lipid accumulation, recent data suggest that autophagy in HSCs may promote fibrogenesis. Liver fibrosis was attenuated in two models (CCl_4 and thioacetamide) in mice with a Cre recombinase–controlled deletion of Atg7, an apoptosis related protein (228).

Chemokine ligand 2. Links between inflammation and fibrosis in NASH arise from the TLR-dependent stimulation of Kupffer cells and HSCs to produce chemokine (C-C motif) ligand 2 (CCL2); this induces the recruitment of circulating chemokine receptor 2 (CCR2)+ Ly6C+ monocytes into the liver (229). Indeed, CCR2 or CCL2 inhibitors prevent the progression of NASH and fibrosis (229, 230) and also enhance the clearance of scar tissue in the fibrosis resolution phase (231).

MODIFIERS OF NAFLD

Genetics

Obesity, CVD, T2DM, and NAFLD display a heritable component to susceptibility accounting for approximately 30–50% of relative risk (232). In contrast to Mendelian inheritance patterns, which are characterized by a single, uncommon, highly penetrant mutation in a specific gene causing disease, complex traits such as NAFLD result from the interaction between environmental exposure and a susceptible polygenic background and comprise multiple independent modifiers (232). Evidence from twin studies, familial aggregation, and different ethnic susceptibility, combined with clear variability in prognosis, suggests a heritable component to NAFLD (233–235).

Only a handful of genes associated with NAFLD either by genome-wide association studies (GWASs) or through candidate gene analyses have been independently validated; a detailed discussion of both methodologies is beyond the scope of this article, and this topic has been reviewed elsewhere (236). The number of robustly validated genes in large, independent cohorts with mechanistic studies reduces further to include only patatin-like phospholipase domain–containing protein 3 (*PNPLA3*), glucokinase regulator (*GCKR*), and the recently discovered transmembrane 6 superfamily 2 (*TM6SF2*) (106). Each of these genes has been associated with not only variations in hepatic TG content but also hepatic fibrogenesis.

PNPLA3. The first GWAS reported *PNPLA3* as a modifier of NAFLD pathogenesis (237); it is the only gene that has been consistently identified as a potential modifier across multiple studies using radiologically determined TAG accumulation and biochemical indices (237–241). The association with NAFLD has been independently replicated in candidate-gene studies addressing both adult (242, 243) and pediatric (243) cohorts, as has the association with raised ALT/AST levels (244). Histological studies also show that the rs738409 (Ile148Met) variant is associated with severity of inflammation and increased fibrosis (242); recent data suggest that *PNPLA3* might also increase the risk of NAFLD-associated HCC (245). It is noteworthy that the differing prevalence and severity of NAFLD across ethnic groups can, in part, be explained by carriage of the Ile148Met variant, with data demonstrating that the frequencies of the variant allele matched the increased prevalence of NAFLD in Hispanics (49%) as compared with European Americans (23%) and African Americans (17%) (237).

Determining the mechanistic effects of *PNPLA3* has been challenging and a focus of intense research activity. The *PNPLA3* gene on chromosome 22 encodes a 481-amino-acid protein closely related to adipose triglyceride lipase, the predominant TAG hydrolase in adipose tissue (246). *PNPLA3* differs from classic lipases by the use of a catalytic dyad (S47/D166) instead of the more common triad (247). The index single nucleotide polymorphism (SNP; rs738409 encoding Ile148Met) is a nonsynonymous cytosine-to-guanine nucleotide transversion mutation that results in an isoleucine-to-methionine amino acid change at residue 148 (106); this substitution does not appear to impinge on the highly conserved catalytic site, but it alters the hydrophobic substrate–binding groove, possibly preventing substrate entry to the catalytic site (248).

Consistent with NAFLD as a complex disease trait, *PNPLA3* variation sensitizes the liver to environmental stressors; in a southern European cohort, rs738409 carriage was found to associate only with elevations of ALT/AST in obese patients with BMI > 30 kg/m^2 (249). The modifying effect of *PNPLA3* on TAG accumulation appears to be independent of any influence on the severity of broader features of metabolic syndrome, and as assessed by hyperinsulinemic, euglycemic clamp, of direct alterations in insulin sensitivity (249).

Defining both the physiological role of *PNPLA3* and the effect of the rs738409 variant has so far remained elusive. Studies examining recombinant adiponutrin expressed in HuH-7 cells and the in vitro biochemical properties of purified *PNPLA3* demonstrate that *PNPLA3* hydrolyzes acylglycerols with maximal activity against the three major glycerolipids (tri-, di-, and monoacylglycerol), with a preference for oleic acid as the acyl moiety; the rs738409 variant is associated with substantially decreased enzymatic activity without decreasing substrate affinity (248). Additionally, carriers of the variant I148M *PNPLA3* allele have lower levels of VLDL secretion for the same amount of liver fat content; in vitro studies using rat McA-RH7777 cells that secrete apoB-containing VLDL-like particles show that carriage of the rs738409 variant reduces VLDL secretion, which may be due to a failure to shift TAG from intracellular lipid droplets because of loss of TAG hydrolase activity (250).

In vivo studies have been hindered primarily by differences in the gene and its expression pattern among species. *PNPLA3* is expressed principally in adipose tissue and the liver, where it compartmentalizes to membranes and lipid droplets (248). In mice adipose tissue expression is predominant with low hepatic expression (251), whereas in humans expression is primarily in the liver (252). However, in both species fasting reduces *PNPLA3* expression (251) and feeding increases such expression. Indeed, it is further increased in obese humans (253) and *ob/ob* mice (251). Postprandial *PNPLA3* expression is controlled by insulin via liver X receptor–retinoid X receptor and SREBP-1c; specific FAs (such as palmitate, C16:0, oleate, C18:1, linoleic acid, and C18:2) provide additional posttranslational control by increasing *PNPLA3* expression (254).

Initial in vitro data principally point to a loss of function for the rs738409 variant, but deletion of *pnpla3* in mice did not initiate hepatic steatosis, even when the mice were fed a high-sucrose diet (255). Similarly, adenovirus-mediated overexpression of human wild-type *PNPLA3* in mice did not provoke hepatic TAG accumulation, but hepatic steatosis was induced when *PNPLA3* Ile148Met was overexpressed (248). Further studies have confirmed that targeted overexpression of wild-type *PNPLA3* in adipose tissue or liver is ineffectual at increasing TAG accumulation in hepatocytes (106), whereas *PNPLA3* Ile148Met when expressed exclusively by the liver led to increased hepatic steatosis (256). Three notable metabolic effects were seen: increased synthesis of FAs and TAG, reduced TAG hydrolysis, and relative reduction of TAG long-chain PUFAs (256). Taken together, these results suggest that the rs738409 variant affects multiple aspects of TAG remodeling in lipid droplets, as they accumulate in the fed state. Indeed, the mixed enzymatic actions combined with the postprandial transcriptional regulation of *PNPLA3* and the specific FA profile suggest that tissue and metabolic milieu influence the action of *PNPLA3*.

Further research should aim to clarify how PNPLA3 influences progression to steatohepatitis and fibrosis, and the molecular mechanism underpinning this; it is likely that *PNPLA3* has different effects in different cell types it is expressed in. Indeed, Li et al. (256) found that although *PNPLA3* Ile148Met drove lipid accumulation, no change in markers of fibrosing steatohepatitis (TNFα, α smooth muscle actin, or collagen type 1a mRNA expression) was observed in mice at 12 weeks of age. Studies aimed at clarifying the role of PNPLA3 are summarized in **Table 2**.

It has recently been proposed that *PNPLA3* is involved in retinol metabolism in primary human HSCs, its expression regulated by retinol availability and insulin, acting to reduce lipid droplet content (260). It has been demonstrated that purified wild-type *PNPLA3* hydrolyzes retinyl palmitate into retinol and palmitic acid, with the rs738409 variant markedly reducing this activity; there also appeared to be no retinyl esterase activity in hepatocytes (260). A link among PNPLA3, retinol metabolism, and progressive NAFLD has recently been suggested; patients with the rs738409 variant have lower levels of retinol binding protein 4 (RBP4) indicating an intracellular retention of intracellular retinol due to a loss of function in this *PNPLA3* variant (260).

Table 2 Functional studies in PNPLA3 and rs738409 (Ile148Met) variant

Study	Type	Experimental design	Result
Chen et al. (255)	In vivo	*Pnpla3* knockout mice by gene targeting	Loss of *Pnpla3* had no direct effect on liver TAG accumulation or on aminotransferase levels after dietary challenge.
Basantani et al. (257)	In vivo	*Pnpla3* knockout mice by gene targeting	Loss of *Pnpla3* had no effect on adipose tissue TAG hydrolysis or on liver TAG accumulation. Data argue against loss of function for Ile148Met substitution.
Huang et al. (254)	In vitro	Purified human PNPLA3 and Ile148Met	PNPLA3 catalyzes TAG hydrolysis. Ile148Met reduces TAG hydrolysis.
Pertilla et al. (258)	In vitro	Immortalized human hepatocytes and HuH7 hepatoma cells	Ile148Met inhibits hydrolysis of hepatic TAGs.
He et al. (248)	Both	In vitro: (*a*) recombinant PNPLA3 purified from Sf-9 cells; (*b*) overexpression of wild-type and Ile148Met mutant in HuH-7 cells In vivo: adenovirus-mediated overexpression of PNPLA3 in C57BL/6J mice	PNPLA3 Ile148Met does not catalyze TAG hydrolysis; wild type does catalyze TAG hydrolysis. PNPLA3 Ile148Met overexpression in liver or cultured hepatocytes causes TAG accumulation.
Kumari et al. (259)	Both	In vitro: (*a*) enzymatic activity of purified murine and human wild-type and PNPLA3-Ile148Met protein; (*b*) overexpression of wild-type and Ile148Met Pnpla3 in HepG2, CHO, and Cos-7 cells In vivo: wild-type and *Pnpla3* knockout mice fed chow or high-sucrose diets	Ile148Met exhibits elevated LPAAT activity compared with the wild-type protein, promoting hepatic lipid synthesis. Data argue for a gain of function. Pnpla3 knockout had no effect on hepatic TAG accumulation.
Pirazzi et al. (250)	Both	In vivo: stable isotope study of VLDL kinetics in nondiabetic, obese, PNPLA3-genotyped men In vitro: overexpression of wild-type and Ile148Met in McA-RH7777 cells	In humans, carriers of the PNPLA3 Ile148Met allele have relatively lower hepatic VLDL1 secretion; Ile148Met variant is associated with lower apoB secretion in vitro. Reduced TAG hydrolysis leading to decreased incorporation into VLDLs is proposed.
Li et al. (256)	In vivo	Transgenic wild-type or Ile148Met-overexpressing mice in adipose or liver tissue	No effect of wild-type Pnpla3 in adipose or liver tissue. No effect of Ile148Met AT. Ile148Met overexpressed in liver tissue caused increased TAG accumulation via increased formation of fatty acids and TAG, impaired hydrolysis of TAG, and relative depletion of TAG long-chain PUFAs. PNPLA3 may act by remodeling TAG droplets.
Pirazzi et al. (260)	Both	In vitro: overexpression of wild-type and Ile148Met PNPLA3 in primary HSCs In vivo: levels of RBP4 in human NAFLD cohort	PNPLA3 has no triglyceride hydrolase activity in HSCs. PNPLA3 148I has retinyl palmitate lipase activity in HSCs; Ile148Met results in loss of function. Lower levels of RBP4 observed in Ile148Met carriers and impaired release of retinol in homozygote HSCs are proposed.

Abbreviations: apoB, apolipoprotein B; HSCs, hepatic stellate cells; LPAAT, lysophosphatidic acid acyltransferase; NAFLD, nonalcoholic fatty liver disease; PNPLA3, patatin-like phospholipase domain–containing protein 3; RBP4, retinol binding protein 4; TAG, triacylglycerol; VLDL, very low density lipoprotein.

PNPLA2 [adipose triglyceride lipase (ATGL)] shares close homology to PNPLA3 and is a key enzyme hydrolyzing intracellular TAGs and FA signaling; recent evidence suggests a role for this lipase in NASH progression. When challenged with MCD diet or LPS, mice lacking ATGL had worse hepatic steatosis and inflammation, which was rescued with PPARα ligands (261). The role of SNPs in PNPLA2 is yet to be clarified.

GCKR. *GCKR* controls glucose metabolism by regulating glucokinase activity (106). The *GCKR* SNP (rs780094) has been frequently linked [strong linkage disequilibrium (LD)] with a functional nonsynonymous SNP encoding Pro446Leu (rs1260326) (239, 240). This variant results in a constant increase in hepatic glucokinase activity and glucose uptake by the liver, mediated by a reduced ability of GCKR to inhibit glucokinase in response to fructose-6-phosphate (262). Thus, unrelenting hepatic glycolysis associated with rs1260326 suppresses glucose and insulin levels, thereby increasing production of intracellular malonyl-CoA. Hepatic lipid accumulation occurs as malonyl-CoA serves as substrate for DNL and impairs mitochondrial FA β-oxidation by blocking CPT-1–mediated FFA transport into the mitochondria (106). Significantly, up to one third of variability in hepatic steatosis among obese European children may be explained by the coupled effects of PNPLA3 rs738409 Ile148Met and GCKR rs1260326 Pro446Leu SNPs (263); GCKR has been validated across a number of ethnic groups (264).

TM6SF2. Previously, the largest GWAS totaling 2.4 million SNPs (imputed or assayed) used a meta-analysis of GWAS data from several cohorts and identified loci associated with computerized tomography–evaluated TAG levels. These loci were then studied using a candidate gene approach in a histologically characterized NAFLD cohort of 529 patients. Aside from known variants in *PNPLA3*, a region on chromosome 19 (19p13) was found to be associated with hepatic TAG content as well as plasma cholesterol, TG, and low-density lipoprotein levels (239). This region contained a variant in *NCAN* (rs2228603 C > T), which encodes the cell adhesion molecule neurocan. However, closer examination of the LD patterns around the rs2228603 variant shows that the *NCAN* SNP is in strong LD with a cluster of other SNPs ∼400 kb upstream in the region of *GMIP* and *PBX4*, and also in strong LD with *TM6SF2* (rs58542926, c.499 A > G; p.Glu167Lys) ($D' = 0.926$, $r^2 = 0.798$). Thus, until recently, there was uncertainty as to which gene signal was ascribed to.

Kozlitina et al. (265) showed that a nonsynonymous SNP in *TM6SF2*, a gene of unknown function on chromosome 19, was associated with ^{1}H-MRS quantified liver fat content based on a genome-wide exome chip of >80,000 patients. The *TM6SF2* variant encoding p.Glu167Lys was also associated with higher ALT levels, and lower serum levels of low-density lipoprotein–cholesterol, TGs, and alkaline phosphatase (265); this variant has also been associated with dyslipidemia and cardiovascular risk (266). *TM6SF2* is more strongly associated with an increased liver fat content phenotype than is an *NCAN* gene variant (rs2228603 c.274 C > T). In vitro studies showed that 50% less Glu167Lys *TM6SF2* protein was produced relative to wild-type *TM6SF2*, when recombinant protein was expressed in cultured hepatocytes (265). Adenovirus-mediated short hairpin RNA knockdown of *tm6sf2* in mice was shown to reduce VLDL secretion and increase liver fat (265).

We have recently shown in two large histologically characterized cohorts (combined n = 1,074 patients) that when adjusted for age, sex, BMI, T2DM status, and *PNPLA3* genotype, *TM6SF2* rs58542926 and *NCAN* rs2228603 were both significantly associated with stage of fibrosis; the association with *NCAN* rs2228603 was lost when the analysis was conditioned on the rs58542926 *TM6SF2* variant, suggesting that the association is driven by that variant (267). Univariate analysis showed associated increased risk of NAFLD-HCC, but this significance was lost on multivariate analysis (267). Further validation of the effect of *TM6SF2* rs58542926 on progressive NAFLD

was confirmed by Dongiovanni et al. (268): In 1,021 biopsied patients, the rs58542926 variant conferred lower serum lipid levels than found in noncarriers ($p < 0.05$), more severe steatosis, necroinflammation, ballooning, and fibrosis ($p < 0.05$), and these patients were more likely to have NASH (OR 1.84, 95% CI 1.23–2.79) and advanced fibrosis (OR 2.08, 95% CI 1.20–3.55), after adjustment for age, sex, BMI, fasting hyperglycemia, and *PNPLA3* genotype. However, one study failed to show the associations of *TM6SF2* with progressive NAFLD reported in the other studies; this was unsurprising, given that only 130 biopsy-proven NASH subjects were analyzed with a mean fibrosis stage of 1.4+/−1.3 (269); unfortunately the study was likely underpowered and incorrectly designed to detect an association with fibrosis. The currently published GWASs suggesting a role for TM6SF2 are summarized in **Table 3**.

Further mechanistic study is needed to determine the pathophysiological role of *TM6SF2* as a modifier of hepatic fibrogenesis. This gene seems to be involved with perturbed cholesterol metabolism and modifies cardiovascular risk, suggesting that *TM6SF2* is an important determinant of multiple aspects of metabolic syndrome–related end organ damage. Given recent evidence suggesting that cholesterol accumulation in HSCs promotes NAFLD fibrosis (214, 271), an appealing hypothesis is that *TM6SF2* may act as a master regulator of metabolic syndrome outcome, with the rs58542926 T allele mediating hepatic retention of TG and cholesterol promoting hepatic fibrosis susceptibility but the C allele promoting VLDL secretion to the detriment of cardiovascular risk, although the liver is protected (**Figure 8**).

Epigenetic Modifiers of Disease Progression in NAFLD

Epigenetics refers to a panoply of cross-talking mechanisms that orchestrate gene expression and cellular phenotype, which are sensitive to environmental changes and heritable. The three most commonly described constituent mechanisms are DNA (CpG) methylation, posttranslational histone modifications, and microRNAs (miRNAs); these mechanisms and their relevance to liver disease have been reviewed recently (272). Briefly, DNA methylation is a common DNA modification that represses gene expression by either direct binding with DNA or recruitment of Methyl-DNA Binding Proteins.

Studies investigating DNA methylation and NASH are beginning to emerge. Repression of PPARγ is key to myofibroblast transdifferentiation of quiescent stellate cells; this is, in part, regulated epigenetically by DNA methylation, and methyl CpG binding protein (MeCP-2)-dependent chromatin remodeling (273). A recent translational study in a small cohort of NAFLD patients demonstrated that greater DNA methylation at the PPARγ promoter is associated with advanced (F3 and F4) disease, suggesting that this epigenetic signature may favor stellate cell activation and subsequent hepatic fibrogenesis (274). A landmark study showed differential DNA methylation in mild (F1 and F2) versus severe (F3 and F4) NAFLD of genes that were hypomethylated and thus overexpressed in advanced NAFLD (275). Another recently reported study showed that key antifibrogenic genes were hypomethylated in mild NAFLD, whereas profibrogenic genes were hypomethylated in severe NAFLD (276).

Of the noncoding RNAs, only miRNAs have been investigated in NAFLD; miR-122 is abundantly expressed in the healthy liver and is reduced in NASH. miR-122 has been shown to be involved in lipid and cholesterol metabolism (277).

Thus, further work is needed to fully elucidate the interplay of epigenetic drivers of complex disease traits.

Dietary Factors

Specific dietary factors may influence the progression of NAFLD to inflammation and fibrosis; PUFAs can have an anti-inflammatory or proinflammatory influence depending on their structure.

Table 3 Summary of genetic association studies and functional studies on TM6SF2 in NAFLD

Study	Phenotype	Population	Number of participants	Association with NAFLD	Association with severity of steatosis	Association with NASH	Association with fibrosis
Kozlitina et al. (265)	^{1}H-MRS measured steatosis	United States (mixed ethnicity)	2,736	Not assessed	Not assessed	Not assessed	Not assessed
Liu et al. (267)	Histologically characterized NAFLD	European Caucasian	1,074	$p = 0.0008$[a]	Not significant	Not significant	$p = 6.36 \times 10^6$
Wong et al. (270)	^{1}H-MRS measured steatosis	Chinese	922	Not significant	Not assessed	Not assessed	Not significant (as assessed by liver elastography)
Dongiovanni et al. (268)	Histologically characterized NAFLD	European Caucasian	1,201	Not assessed	$p = 0.001$	$p = 0.003$ (OR 1.84, 95% CI 1.23–2.79)	$p = 0.008$ (OR 2.08, 95% CI 1.20–3.55), abolished when NASH conditioned for
Sookoian et al. (269)	Histologically characterized NAFLD	Argentinean	226	$p = 0.038$	$p = 0.0299$	$p = 0.027$ (OR 1.66, 95% CI 1.08–2.55)	$p =$ not significant (OR 0.85, 95% CI 0.60–1.20)

[a] 349 patients from NAFLD discovery cohort versus 379 participants from 1,000 Genomes European Caucasian population sample.

Abbreviations: ^{1}H-MRS, proton magnetic resonance spectroscopy; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

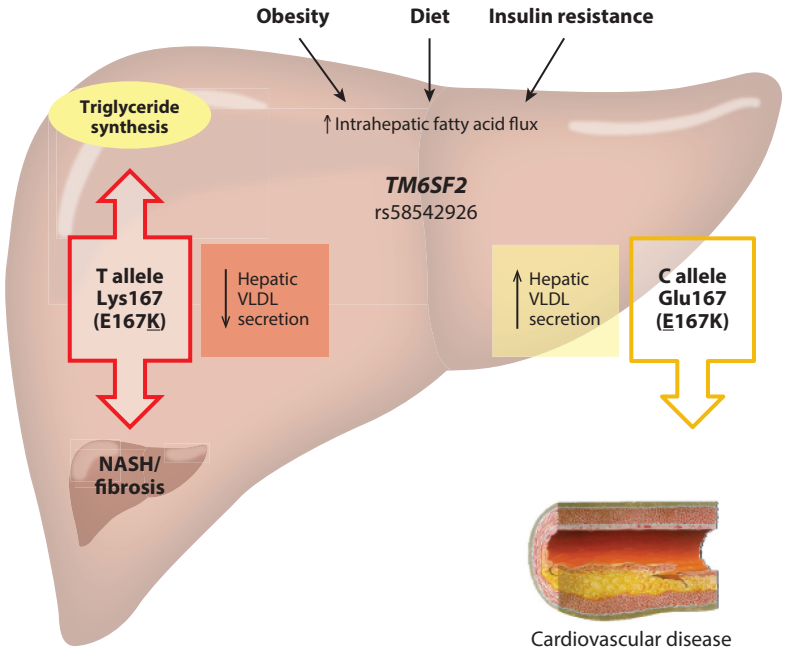

Figure 8

TM6SF2 as the master regulator of end organ function. Increased hepatic fatty acid flux due to obesity, diet, and insulin resistance. The *TM6SF2* T allele causes decreased very low density lipoprotein (VLDL) secretion. Fatty acids are retained in the liver, leading to hepatic steatosis and progression of nonalcoholic steatohepatitis (NASH). The *TM6SF2* C allele causes increased hepatic VLDL secretion and export from the liver, ultimately leading to atherogenesis.

n-3 PUFAs such as α-linoleic acid have an anti-inflammatory role, decreasing lipogenesis via SREBP-1 suppression and increasing FA oxidation, thus leading to a reduction in liver fat (278, 279). A recent meta-analysis in 2012 with a total of 355 patients suggested that ω-3 PUFA supplementation had a beneficial effect on reduction of liver fat and improving liver enzymes (280). Indeed, a study in NAFLD patients showed a high ratio of long-chain n-6 PUFAs (a proinflammatory PUFA) compared with n-3 PUFAs (281).

Fructose consumption may be a key contributor to NAFLD pathogenesis, with recent attention shifting to this highly lipogenic sugar. Fructose is a monosaccharide commonly found in corn syrup, or as part of sucrose. Recently, studies have shown increased consumption of fructose in NAFLD patients, with soft drink consumption being a strong predictor of fatty liver (282). A large clinical study conducted by the NASH Clinical Research Network showed that dietary fructose consumption was associated with more severe fibrosis stage (283). Preclinical data have shown that hepatic lipid accumulation was greater in mice fed fructose compared with any other sugar; furthermore, both endotoxin and TNFα levels were greater in these mice. Antibiotic treatment in mice fed fructose reduced levels of lipid accumulation, leading to the conclusion that fructose, as well as increasing DNL, may have direct proinflammatory effects (284).

The aryl hydrocarbon receptor (AhR) is a transcription factor activated by ligands such as dioxin; the human diet comprises AhR agonists such as flavonoids. Spontaneous hepatic steatosis and oxidative stress develop in transgenic mice with a constitutively activated AhR (285).

Microbiome

The relationship between gut microbiota and chronic liver disease is now well established. Recent technological advances have shed light on the role of gut microbiota disruption (dysbiosis) in the pathophysiology of NAFLD.

Functional effects of microbiota composition in NAFLD/NASH. The ability of the gut microbiota to modulate NAFLD progression in humans has been known for more than 30 years. "Perhaps the feature of most interest in the post-jejuno-ileal bypass patient is the creation of a model for alcoholic liver disease without the necessity of alcohol" (286, p. 57). Thus, the fatal steatohepatitis associated with jejunoileal bypass that commonly complicated surgery for morbid obesity has provided an unexpected model for NASH research. These observations have also implied a role for small intestinal bacterial overgrowth (SIBO) in NASH pathogenesis; indeed, the liver injury associated with bariatric surgery could be reversed by metronidazole (287). A key paper showed that SIBO was present in 50% of patients with NASH, and only 22% of control subjects, as assessed by the 14C-D-xylose-lactulose breath test (288); TNFα levels were also significantly increased compared with controls (288). Moreover, SIBO has a prevalence of 34% in diabetic patients (289). Another study in 40 morbidly obese patients found increased levels of a surrogate marker of endotoxin, LPS protein, in NASH (290). Patients with NAFLD also have significantly increased intestinal permeability and alterations in tight junctions, thus allowing microbial products to enter the portal circulation and cause inflammation; a study of 35 biopsy-proven patients with NASH showed that these patients had increased gut permeability compared with controls as measured by 51Cr-ethylenediaminetetraacetate (51Cr-EDTA) testing, although the association was with severity of steatosis rather than degree of inflammation (291). Evidence from murine models of a role for endotoxin, TNFα, and TLR4 in NASH has been discussed above.

Dysbiosis and inflammasomes. Gut microbiota disruption (dysbiosis)-induced inflammation may contribute to altered barrier function of the intestine and bacterial translocation. Recent evidence suggests that the cytosolic multiprotein complex, known as the inflammasome, is central to this process, by regulating the gut microbiota and thus a microbiota-driven phenotype of NAFLD. Inflammasomes are sensors of exogenous PAMPs and DAMPs (pathogen- and damage-associated molecular patterns) that regulate caspase-1–mediated cleavage of precursors to the inflammatory cytokines pro-Il1β and pro-Il18. Studies in mice deficient in components of the NLRP3 and NLRP6 inflammasomes have overrepresentation of the strict anaerobes Prevotellaceae and members of the uncultivated bacterial division TM (292); lactobacillus was also reduced. Importantly, these mice had an increased susceptibility to liver injury when exposed to an MCD diet; this phenotype was transmitted to wild-type mice when cohoused with inflammasome-deficient mice (293). Antibiotic treatment both reduced the severity of NASH in inflammasome-deficient mice and abrogated the transmissible phenotype effect on wild-type mice (293). Thus, dysbiosis can induce colonic inflammation and bacterial translocation, influencing progression from hepatic steatosis to NASH.

Metagenome. The bacterial genome (metagenome) may provide a direct connection between hepatic steatosis and the intestinal microbiome; obesity is associated with an increased capacity of the metagenome to collect energy from the host by digesting "indigestible" dietary polysaccharide (294). Indeed, germ-free mice are resistant to obesity induced by a high-fat diet (295, 296). Enteric bacteria can suppress the synthesis and secretion of fasting-induced adipocyte factor (Fiaf)

from the small intestine resulting in increased TG synthesis in the liver via lipoprotein lipase (295, 296).

Furthermore, the enteric bacteria's capacity to digest dietary fiber may influence systemic immune responses; short-chain fatty acids, such as proprionate and acetate, are anti-inflammatory via their interaction with G-protein-coupled receptor 43 (Gpr43) (297). Gpr43 −/− mice show systemic inflammation, similar to germ-free wild-type mice lacking bacterial fermentation capacity (298). Other pathways such as Fiaf and Gpr41 have also been implicated in regulation of inflammation (295, 299). Such studies integrate diet, microbiota, and the intestine as a nutrient sensor for determining systemic metabolic function.

Diet-induced changes to the microbiome have been implicated in NAFLD progression. Choline deficiency has been linked to liver disease for a long time (300), but recently high-fat diets have been linked to a microbiota that converts dietary choline into methylamines producing a choline deficiency and NASH (301).

Intestinal microbiome composition in NAFLD and HCC. A few cross-sectional human studies have provided evidence that dysbiosis may have a modifying effect on the development of NASH and fibrosis albeit in small cohorts. A study of 50 patients (22 patients with NASH) showed that patients with NASH had decreased Bacteroidetes and increased *Clostridium coccoides* (302). However, another study showed an increase in Bacteroidetes (303), although the differing sample population, method, and small numbers must be considered.

Recent evidence showed that changes in the microbiota induced by a high-fat diet aid the development of inflammation and fibrosis in a bile duct ligation (BDL) model (304). This microbiome was characterized by an increased amount of gram-negative proteobacteria, with a virtual disappearance of bifidobacteria, and caused more severe liver fibrosis in control mice when subjected to BDL, their having received the gut microbiota from high-fat-diet fed mice prior to injury (304).

Some studies have reported that dysbiosis may drive the development of HCC. In a diethylnitrosamine model of liver cancer, the microbiota was shown to aid the development of HCC via TLR4, with replication with chronic injections of LPS (305).

CONCLUSIONS

The burden of NAFLD is increasing at an alarming rate, and NAFLD is already the most common cause of liver dysfunction in the Western world. Despite the clear advances in understanding the events that lead to NASH, there are no reliable biomarkers of early disease, nor are there any licensed therapies. As presented in this review, the pathogenesis of NAFLD is impeccably complex and likely involves a delicate interplay between genetics and environmental factors, of which much has been elucidated recently. Further work in identifying patients at risk of advanced disease by genetic and/or epigenetic profiling combined with metabolomics and proteomics is needed; this would allow a truly stratified approach to managing patients with NAFLD and identifying those who would benefit most from future drug trials.

DISCLOSURE STATEMENT

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