Nonalcoholic Fatty Liver Disease: Pathology and Pathogenesis

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is recognized as the leading cause of chronic liver disease in adults and children. NAFLD encompasses a spectrum of liver injuries ranging from steatosis to steatohepatitis with or without fibrosis. Fibrosis may progress to cirrhosis and complications including hepatocellular carcinoma. Histologic findings represent the complexity of pathophysiology. NAFLD is closely associated with obesity and is most closely linked with insulin resistance; the current Western diet, high in saturated fats and fructose, plays a significant role. There are several mechanisms by which excess triglycerides are acquired and accumulate in hepatocytes. Formation of steatotic droplets may be disordered in NAFLD. Visceral adipose tissue dysfunction in obesity and insulin resistance results in aberrant cytokine expression; many cytokines have a role in liver injury in NAFLD. Cellular stress and immune reactions, as well as the endocannabinoid system, have been implicated in animal models and in some human studies.

NAFLD: nonalcoholic fatty liver disease

NASH: nonalcoholic steatohepatitis

T2DM: type 2 diabetes mellitus

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a clinico-pathologically defined entity most commonly associated with characteristics associated with increased cardiovascular risk, referred to collectively as metabolic syndrome. Fatty liver itself is independently associated with increased cardiovascular risk (1) and biomarkers of cardiovascular disease (2), and, in obese individuals, it is a marker of insulin resistance (3) and diabetes (4). Population studies have shown that NAFLD itself is probably a cause of increased mortality (5). By definition, NAFLD occurs in individuals whose alcohol consumption is insignificant (<10 g per day for women, <20 g per day for men) and is characterized histologically by at least 5% steatosis and other parenchymal changes, ranging from inflammation to hepatocyte apoptosis/necrosis to fibrosis. The most worrisome form of the disease is nonalcoholic steatohepatitis (NASH) because of the risk of progressive liver disease (6).

PREVALENCE AND RISK FACTORS

The prevalence of NAFLD is increasing in parallel with the prevalence of obesity; both processes are closely linked to insulin resistance. The worldwide epidemic of obesity and the prevalence of NAFLD are most certainly heavily influenced by, if not directly related to, the diet and relative lack of exercise of the Western lifestyle. The most recent reports from the Centers for Disease Control and Prevention (CDC) indicate that 66% of adults in the United States are overweight, and that of those, half are obese.

It is projected that by 2025 up to 45% of Americans will be obese (7). As a reflection of the geographical and ethnic spread of obesity, the projected percent increase in type 2 diabetes mellitus (T2DM) by 2030 is 32% in Europe, 72% in the United States, and ≥150% in sub-Saharan Africa, India, and the Middle East (7). Children and adolescents, who are significantly less likely to have confounding processes such as alcohol use and/or viral hepatitis, are likewise affected by the increasing prevalence of overweight, obesity, and the features of metabolic syndrome, and these factors are important associations with the comorbidity of NAFLD (8, 9).

As with other complex disease processes, underlying environmental, genetic, and hormonal factors that result in phenotypic disease expression have to be considered in prevalence estimates. Notable differences in predilection for NAFLD are found related to age, gender, and ethnicity. To date, none of the varying methods for estimating prevalence can distinguish steatosis and steatohepatitis resulting from nonalcoholic sources from those resulting from alcohol abuse. Although it is recognized that at present the only unequivocal means of diagnosis for NAFLD is liver tissue evaluation, this method clearly cannot be employed for population screening, and outside the setting of a study, liver biopsy is only undertaken for clinically detected abnormalities. Serologic assays based on liver tests are fraught with difficulties, not the least of which is agreeing on what constitutes the upper limits of "normal" for the common liver tests alanine amino transferase (ALT) and aspartate amino transferase (AST), as levels of these enzymes are frequently elevated in the obese population (10). Also, NAFLD and even NASH with fibrosis and/or cirrhosis may be present histologically in the setting of normal ALT levels; 79% of subjects with >5.5% steatosis by quantitative imaging had normal ALT in a large multiethnic population study (11).

Finally, imaging may indicate hepatic steatosis, but no modality detects disease activity. Further, ultrasound evaluation is only accurate when >33% of the liver is affected (12), and computed tomography is limited by radiation exposure. Quantitative steatosis measurements by proton magnetic resonance spectroscopy are challenging to perform, but the technique is evolving (13).

Increased age has an unexplained impact on NALFD. In adult studies, the older individuals (40–60 years old) had hepatic steatosis

ranging from 24% to 54%, compared with 18% in ≤20 year olds (14). An autopsy study of 742 unselected subjects 2–19 years old showed that 9.6% overall had steatosis (15); when further analyzed, the data showed that 17% of adolescents, compared with 0.7% in the 2-4-year-old range, were affected by NAFLD (16). Increased age is also associated with prevalence of significant fibrosis in NAFLD/NASH (17).

The role of gender in NAFLD and NASH is under evaluation. Early biopsy-based studies indicated that NASH more commonly affected women than men; current studies of NAFLD indicate that men are more commonly affected (18). The latter finding is in keeping with the stronger association of NAFLD with increased abdominal adiposity (the apple-shaped body habitus) than with total or subcutaneous adiposity. The majority of studies in children have also shown a higher incidence of NASH in boys than in girls (16, 19).

In all studies, compared with Caucasians, African Americans are significantly underrepresented and Hispanic and Asian individuals are overrepresented (18, 20). An apparent paradox in African Americans of high insulin resistance but decreased visceral adiposity and hepatic steatosis has been documented in a large population study by imaging techniques for quantitative steatosis and body fat distribution (8).

Given these caveats, the literature continues to support the idea that NAFLD is the most common cause of chronic liver disease in American adults (20), adolescents, and children (16). It is estimated that approximately 3–36% of the general American population has NAFLD, with increased risks to 95% in patients with obesity and 70% for patients with T2DM (21). Of these patients, it has been reported that 20–30% have NASH and that a subgroup will have the increased risk(s) associated with it for cirrhosis and its long-term complications, including liver failure and hepatocellular carcinoma (HCC) (22).

Genetic predisposition studies are suggestive, but to date they remain too small for firm conclusions (23). Studies have documented familial fatty liver disease and cirrhosis within kindreds (24). Another recent small study showed a trend toward familial clustering and maternal linkage for insulin resistance in patients with NAFLD (25). Krüppel-like factor 6 (*KLF6*), proposed to control several components of NASH as well as hepatic stellate cell (HSC) activation in response to inflammation and hepatocyte growth, has recently been implicated. Possession and transmission of alleles with a polymorphism in *KLF6* in NAFLD were shown, then confirmed to be associated with advanced NASH in two large multiethnic pediatric and adult cohorts; a protective functional polymorphism was also identified (26). A genomic and proteomic study of 98 bariatric patients noted downregulation of genes involved in defense against oxidative stress in NAFLD and upregulation of genes associated with fibrogenesis and apoptosis in steatohepatitis (27). An in-depth review of current published work in gene studies has summarized nonexclusive categories and potential genes under investigation (23): (*a*) amount and location of body fat deposits [e.g., peroxisome proliferators–activated receptor gamma (PPARγ)], (*b*) insulin sensitivity (e.g., adiponectin, resistin), (*c*) hepatic steatosis [free fatty acid (FFA) delivery, de novo lipogenesis (DNL), processing, and egress, e.g., leptin, adiponectin, MTP, stearol-CoA desaturase 1 (SCD1)], (*d*) mitochondrial, peroxisomal, and microsomal fatty acid oxidation (e.g., adiponectin, PPARα, *CYP2E1*, *CYP4-A*), (*e*) hepatocellular oxidative stress [e.g., tumor necrosis factor alpha (TNF-α), *HFE*], (*f*) response to oxidant stress (e.g., UCP2, SOD2), (*g*) cytokines and receptors related to adiposity [e.g., interleukin-10 (IL-10), TNF-α], (*h*) endotoxin receptors (e.g., *CD14*, *NOD2*, *TLR4*), (*i*) immune response (e.g., *CTLA-4*, IL-4, IL-10), (*j*) determinants of the complex factors involved in fibrogenesis in NAFLD [e.g., connective tissue growth factor (CTGF), leptin, adiponectin, angiotensin], and (*k*) general fibrosis/fibrinolysis [e.g., transforming growth factor beta (TGF-β), matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs)].

FFA: free fatty acid

TNF-α: tumor necrosis factor alpha

TGF-β: transforming growth factor beta

TG: triacylglycerol (triglycerides)

MDB: Mallory–Denk bodies

HISTOPATHOLOGIC FINDINGS

Although a plethora of noninvasive tests for diagnosing NASH have emerged in recent years (28), the literature continues to support the concept that liver biopsy is the gold standard for confirming or excluding the diagnosis as well as documenting amounts of activity and fibrosis and architectural integrity (14, 28). The diagnosis of NASH relies not only on the presence of the lesions discussed below, but also on the pattern(s) of these lesions within the hepatic parenchyma. Thus, a simple listing or scoring of lesions is not considered adequate for diagnosis.

Steatosis

The unequivocal histological hallmark of NAFLD in both adults and children is steatosis, the histologic manifestation of intracytoplasmic lipid in the form of triglycerides (TG) within hepatocytes (29); by definition, steatosis is always a component of NAFLD. The intracytoplasmic fat in NAFLD can take several forms: (*a*) large droplets of macrovesicular fat that fill the cytoplasm, displacing the remaining contents of the cell and nucleus peripherally, (*b*) mixed large and small droplets, which can easily be delineated, or (*c*) rarely, in addition to macrovesicular steatosis, foci of hepatocytes with true microvesicular steatosis. Semiquantitative methods for the histological assessment of steatosis are based on routine stains, not on those that specifically identify lipids, such as Oil Red O. The most reproducible method follows the acinar architecture and refers to the percentage of liver parenchyma occupied by steatotic hepatocytes: 0–33% (or 0–5% and 5–33%), 33–66%, or >66% (14).

In adult patients, steatosis is most commonly observed in the perivenular, acinar zone 3 hepatocytes; in some adult and pediatric cases, steatosis may occupy the entire acinus. In children, another pattern of steatosis is observed in periportal, zone 1 hepatocytes. Finally, steatosis in children is more commonly moderate or severe (19, 30, 31) than in adults.

Steatohepatitis

Adult pattern. The minimal histologic criteria for the diagnosis of adult NASH include steatosis, hepatocellular injury (usually in the form of ballooning), and lobular inflammation, typically occurring with a zone 3 predominance. As in other forms of chronic liver disease, fibrosis is not required for the diagnosis of steatohepatitis.

Hepatocellular injury in NASH usually occurs in the form of ballooning, but it may also present as apoptotic (acidophilic) bodies and lytic necrosis. Several studies have shown positive correlations with both histologic and serum markers of apoptosis in NASH (32), which we discuss further below. Hepatocellular ballooning refers to enlarged hepatocytes, with rarefied cytoplasm, that may have a reticulated appearance or contain Mallory–Denk bodies (MDB; also discussed below). The ballooned hepatocytes are predominantly located among steatotic hepatocytes in acinar zone 3 and are frequently, but not always, associated with perisinusoidal fibrosis. Loss of the normal intracytoplasmic deposition of keratin 8/18 (K8/18) immunostaining has recently been proposed as an objective marker for their histological recognition (33).

Lobular inflammation, usually mild, consists of small foci of inflammatory cell infiltrates composed of lymphocytes, eosinophils, and occasionally polymorphonuclear leukocytes. In addition, Kupffer cell aggregates (microgranulomas) and lipogranulomas may be seen in the lobules.

Portal inflammation in NAFLD most often takes the form of chronic inflammatory infiltrates. Varying degrees of portal inflammation occur in NASH, and increased severity may correlate with increased severity of activity and laboratory features of insulin resistance, but not with serologic markers in a large series of pediatric and adult biopsies (34). However, when marked and disproportionate to the lobular findings or to fibrosis, consideration of a possible concurrent liver disease such as hepatitis C is given (14).

The characteristic "chicken wire" pattern of fibrosis in adult patients with noncirrhotic NASH is perisinusoidal/pericellular and usually is first observed in acinar zone 3. With progression, portal and periportal fibrosis may develop in conjunction with the zone 3 perisinusoidal fibrosis, followed, in some cases, by bridging (central-portal, portal-portal, or central-central) fibrosis and cirrhosis (35). NASH cirrhosis is most commonly macronodular or mixed (14); studies from biopsy series have proven that steatosis and other lesions may not persist in cirrhosis. The term cryptogenic cirrhosis has been applied to many such cases; however, if prior specimens show evidence of NASH, the resultant cirrhosis is, by definition, not cryptogenic. Clinical studies had related cases with true cryptogenic cirrhosis to underlying clinical correlates for NAFLD, as well as occurrence of NASH in posttransplant liver specimens (reviewed in Reference 14).

Other histological lesions include the following:

1. MDB (previously known as Mallory's hyaline) are dense, ropy, eosinophilic, intracytoplasmic perinuclear inclusions often found in ballooned hepatocytes. In untreated adult NASH, they are most commonly found in zone 3 and in areas of perisinusoidal fibrosis. MDB alone, however, are not unique to and may occur in steatohepatitis of other etiology (alcoholic or drug induced), chronic cholestasis, copper storage diseases, glycogenesis, proliferative lesions, and hepatocellular neoplasms (36). When present, they are considered by most pathologists to be a useful morphologic finding (37) and are related to higher necroinflammatory activity (35).
2. Variable degrees and distributions of iron deposition within hepatocytes and/or the cells of the reticulo-endothelial system may be seen in NAFLD and NASH. Studies on the significance of iron deposition and abnormal iron metabolism genetics in the pathogenesis and/or perpetuation of injury and fibrosis in NASH have given conflicting results (38, 39). The hormonal iron regulator, hepcidin, is under investigation in obesity and NASH (40).
3. Ductular reaction, 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. In NASH, ductular reaction is related to portal and advanced fibrosis and may provide a pathway for progressive fibrosis, as discussed below (41).
4. Megamitochondria (giant mitochondria) are intracellular, round or cigar-shaped eosinophilic structures and are commonly observed in hepatocytes with microvesicular steatosis. Electron microscopy reveals that mitochondria in NAFLD may show paracrystalline inclusions, loss of cristae, and multilamellar membranes (42). Unlike in alcoholic liver disease, in which megamitochondria are associated with progression (43), the significance of megamitochondria in NAFLD is not known.
5. Glycogenated hepatocyte nuclei are vacuolated and may be seen in clusters within the parenchyma. When located within periportal hepatocytes, they are considered by some investigators to be characteristic of NASH (44) or diabetes (45).

Pediatric pattern(s). Pediatric patients (2–19 years old) have been reported to have differing histologic findings in clinically characterized NAFLD/NASH. Instead of the zone 3 accentuation of the adult biopsies, the former findings are largely portal based. This may include the deposition of macrovesicular steatosis, the predominance of chronic inflammation, and the first stage of fibrosis. In addition, many biopsies are characterized by (*a*) extensive, panacinar steatosis, the uncommon occurrence of definitive hepatocellular ballooning and MDB and (*b*) the absence of lobular inflammation. This portal (zone 1) accentuation

Table 1 Brunt scoring system for NASH[a]

Grade of activity	Steatosis[b]	Ballooning[c]	Inflammation
Mild, grade 1	1–2 (up to 66%)	Minimal	L = 1–2; P = 0–1
Moderate, grade 2	2–3	Present	L = 1–2; P = 1–2
Severe, grade 3	2–3	Marked	L = 3; P = 1–2

[a]Adapted from Reference 35.
[b]Steatosis: grade 1 ≤ 33%; grade 2 = 33%–66%; grade 3 = ≥66%.
[c]Ballooning: zonal location noted. Abbreviations: NASH, nonalcoholic steatohepatitis; L, lobular inflammation; P, portal inflammation (see **Table 2** for descriptions).

pattern was identified as type 2 pediatric NASH, and the less commonly observed pattern, which resembles that of adult NAFLD, was denoted type 1 (31). The initial study showed that type 2 NASH is more commonly encountered in liver biopsies of younger, obese boys of Asian, Hispanic, and Native American origin (31). Subsequent studies have shown that most cases of pediatric NAFLD/NASH, in fact, have features of both type 1 and type 2 and that the purely type 2 pattern is less common (19, 46). The switch from the portal-predominant pattern to the zone 3 pattern, which is more common in older children, may be related to hormonal or endocrine changes caused by puberty. Cirrhosis has been observed in rare cases in children with NAFLD/NASH, and this condition carries significant concern for future health-related personal and societal consequences (47).

SCORING SYSTEMS FOR PATHOLOGIC EVALUATION

Semiquantitative histological scoring systems have been developed for grading necroinflammatory lesions and staging fibrosis in NAFLD in both adults and children. A method of deriving a global disease activity grade based on the major histological lesions of NASH, namely steatosis, hepatocellular ballooning, and lobular and portal inflammation, was proposed in 1999 (35); the method noted that the significant differential in activity is not the steatosis amount, but rather ballooning and inflammation. The score also proposed a five-tier (0–4) staging method for the characteristic pattern of fibrosis observed in NASH from zone 3 perisinusoidal to cirrhosis (**Tables 1–3**).

Recently, the National Institute of Diabetes and Digestive and Kidney Diseases–sponsored NASH Clinical Research Network (CRN) proposed and validated a scoring system for the clinical trials of the CRN. The system, a modification of the 1999 NASH scoring method, covers the spectrum of NAFLD. For disease activity, the NAFLD activity score (NAS) is feature based and derived from addition of individual scores for steatosis, lobular inflammation, and hepatocellular ballooning. The staging system for fibrosis was expanded to include the pediatric pattern of portal fibrosis (stage 1c), and the initial perisinusoidal fibrosis in zone 3 was further divided into delicate and dense (stages 1a and 1b, respectively) (**Table 4**) (48).

Sampling variability may be problematic in the histological diagnosis of NAFLD/NASH, as in other chronic liver diseases, but it may be overcome using biopsies of adequate length and diameter (14). Sampling error may be an important consideration in the design and interpretation of trials and natural history studies (reviewed in Reference 14).

Table 2 Brunt scoring system for lobular and portal inflammation[a]

Lobular inflammation (L)		Portal inflammation (P)	
Degree	Description	Degree	Description
0	None	0	None
1	<2 foci/20 × field	1	Mild
2	2–4/20 × field	2	Moderate
3	>4/20 × field	3	Marked

[a]Adapted from Reference 35.

PATHOGENIC MECHANISMS PROPOSED IN NONALCOHOLIC STEATOHEPATITIS

Scientific investigations into pathogenesis of NAFLD/NASH have increased exponentially in the past 10 years. **Figure 1** highlights current concepts of the complex systems that ultimately result in liver disease. **Table 5** lists examples of the various types of rodent models with genetic and/or dietary manipulations, as well as the pros and cons of each model.

Table 3 Brunt scoring system for fibrosis[a]

Stage of fibrosis	Zone 3 perisinusoidal fibrosis	Periportal fibrosis	Bridging fibrosis	Cirrhosis
1	Focal or extensive	0	0	0
2	As above	Focal or extensive	0	0
3	±	±	+	0
4	±	±	Extensive	+

[a]Adapted from Reference 35.

The commonly quoted "normal" amount of hepatocellular lipids is <5% lipid by weight (49). In the normal hepatocyte, energy received from circulation of glucose, fructose, and lipids is stored as glycogen, and increased lipids are redistributed to peripheral storage in adipocytes or used for combustion (50); there is little capacity for storage of lipids. In NAFLD, the processes of lipid trafficking are dysregulated with resultant increased intrahepatocellular lipids (51), particularly as saturated lipids (52). In adipose tissue, which is specialized for storage of TG, extensive overload with lipids results in metabolic incompetence and subsequent macrophage infiltration (50). In hepatocytes, excessive lipid storage may directly contribute to organelle failure, including mitochondrial dysfunction and endoplasmic reticulum (ER) and other organelle stress, and may play a role in hepatic insulin resistance (53, 54).

In cell cultures, exposure of hepatocytes directly to saturated FFA leads to mitochondrial depolarization and to increased production of reactive oxygen species (ROS) (55). However, studies also show that lipid droplets and formation of TG (a stable fat) safely sequester and release nonesterified fatty acids, thus protecting the cells from the lipotoxic effects of ectopic fats (54, 56). Thus, the increased flux of FFA from the diet and from DNL is proposed as a toxic "hit" to the liver in NAFLD (57). It may not be TG per se in the hepatocytes that results in lipotoxicity, but rather the accumulation of TG may serve as a biomarker of increased hepatic exposure to potentially toxic FFA (57, 58) and the pathogenic consequences thereof.

ER: endoplasmic reticulum

ROS: reactive oxygen species

Table 4 NASH Clinical Research Network scoring system for NAFLD[a]

NAFLD activity score							
Steatosis grade		Lobular inflammation		Hepatocellular ballooning		Fibrosis score	
Degree	Description	Degree	Description	Degree	Description	Degree	Description
0	<5%	0	None	0	None	0	None
1	5–33%	1	<2 foci/20 × optical field	1	Mild; few	1a 1b 1c	Mild (delicate) zone 3 perisinusoidal fibrosis Moderate (dense) zone 3 perisinusoidal fibrosis Portal/periportal fibrosis only
2	34–66%	2	2–4 foci/20 × optical field	2	Moderate/ marked; many	2	Zone 3 perisinusoidal fibrosis with portal/periportal fibrosis
3	>66%	3	>4 foci/20 × optical field	–	–	3	Bridging fibrosis
–	–	–	–	–	–	4	Cirrhosis

[a]Adapted from Reference 48. Abbreviations: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

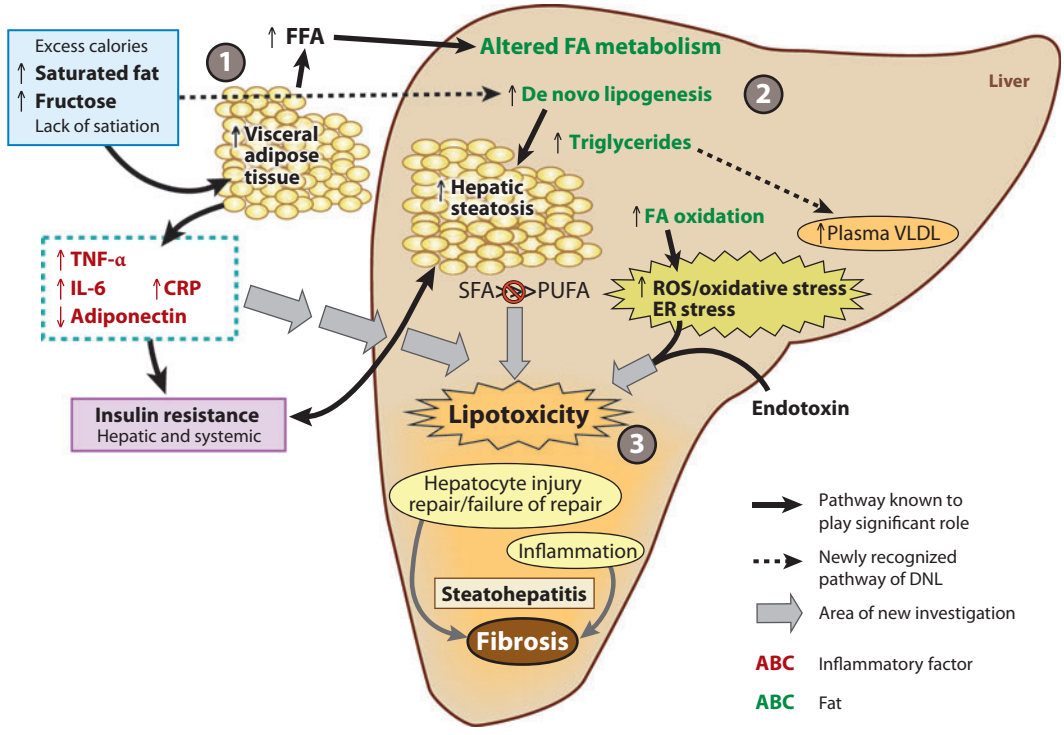

Figure 1

Current concepts in the pathways of steatosis and steatohepatitis. Many of the current concepts of nonalcoholic fatty liver disease are highlighted. There are three major intersecting components: (*1*) increased visceral adipose tissue (VAT) and altered systemic and hepatic response to increased insulin (insulin resistance); (*2*) altered hepatic fatty acid (FA) export, oxidation, and desaturation within the liver; and (*3*) the initiation and subsequent effects of lipotoxicity. Black arrows highlight the pathways that are known to play significant roles. Gray arrows indicate areas of newer investigation and highlight developing work showing that steatosis and steatohepatitis are not necessarily interdependent processes. Increased caloric intake, in combination with increased saturated fat and fructose consumption, leads to increased VAT; fructose consumption also stimulates de novo lipogenesis (DNL) and does not cause the satiety signaling that occurs with glucose. VAT is a metabolically active tissue that produces numerous proinflammatory cytokines [tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP)] and is associated with decreased adiponectin, an adipokine that is anti-inflammatory. Both VAT and hepatic steatosis cause and are caused by hyperinsulinemia and insulin resistance in a feed-forward relationship. VAT also increases delivery of free fatty acids (FFA) to the liver via the portal circulation, resulting in an increased load of FA metabolism within the liver. DNL, increased reesterification of triglycerides, and increased oxidation are all affected. In some patients, compensatory mechanisms to prevent lipotoxicity from the altered FA metabolism fail, and steatohepatitis and fibrosis result. The circulating proinflammatory cytokines from VAT, lack of appropriate desaturation of saturated fatty acids (SFA) to polyunsaturated fatty acids (PUFA), increased reactive oxygen species (ROS) from oxidative stress and endoplasmic reticulum (ER) stress, and increased portal endotoxin have all been speculated to play a role in lipotoxicity. Failure of hepatocyte repair and inflammation perpetuate the process(es) that initiate and promote fibrosis. Abbreviation: VLDL, very low density lipoprotein.

Failure of Lipid Formation and Storage in Nonalcoholic Fatty Liver Disease: PAT and CIDES

PAT [perilipin, adipophilin, and tail-interacting proteins of 47kd (TIP47)] are a family of lipid droplet–associated proteins that are actively involved in droplet formation and turnover (50, 54). Sophisticated techniques have shown the localizations and complex associations of PAT with intracellular lipid droplets (59). PAT have been shown to play a role in insulin resistance and obesity; they may also play a role in NAFLD. Dysfunction of PAT can result in increased size and decreased numbers of lipid droplets, as well as increased lipolysis with resultant insulin pathway dysregulation

(54). Studies have shown differential expression of PAT in human and mouse steatotic livers (54, 60). This remains an area of ongoing investigation.

The cell death–inducing DNA fragmentation factor-α-like effector (CIDE) proteins (designated CIDEA, CIDEB, etc.) were initially characterized as mitochondrial activators of apoptosis, but they are now being investigated for their roles in cellular energy balance (61–63). CIDEA is thought to control lipid metabolism; CIDEA knockout mice are lean and obesity resistant (64). In a human genetic study, an association was found between a polymorphism of CIDEA and obesity (61). CIDEB knockout mice had increased adiponectin, increased insulin sensitivity, and improved plasma and liver TG (63). CIDEC (or fat-specific protein of 27 kDa) is a lipid droplet protein that plays an important role in droplet formation. In vitro, CIDEC is sufficient to stimulate formation of lipid droplets and necessary for lipid droplet expansion during adipogenesis (62). Expression of exogenous CIDEC results in spontaneous accumulation of TG-containing lipid droplets (62). Although PAT proteins seem to play overlapping roles in droplet formation, such that the absence of one has a minimal effect on droplet size, the absence of CIDEC inhibited large lipid droplet formation (but not formation of small droplets) in vivo (62).

Hepatic Steatosis: Lipid Disequilibrium

TG in the liver can be utilized as metabolic fuel through oxidation, exported out of the hepatocyte as very low density lipoprotein (VLDL), or stored. The primary sources of increased TG in hepatocytes are (*a*) fatty acids in the diet delivered to the liver, the amount of which increases in systemic insulin resistance, (*b*) DNL within hepatocytes, (*c*) recirculation of nonesterified fatty acids from the peripheral tissues (some from adipose tissues and some from skeletal muscle), and (*d*) inadequate removal via VLDL production and secretion. The imbalance between these inputs and outputs results in steatosis and possibly contributes to inflammation and the subsequent downstream effects.

Increased free fatty acids: dietary sources. Although "fat" is a generic term, it should not inappropriately imply uniformity of structure, function, and pathogenic propensity. Investigations in humans (29) and in animals (65) show there are significant differences in liver injury related to the types of fatty acids. Alterations in the type(s) of fat both in the diet and stored in the liver likely contribute to NAFLD. This has been studied in a variety of animal models, most commonly in those with various dietary manipulations. Even though a high-fat diet is frequently used to induce hepatic steatosis, in principle it is difficult to alter one nutrient without also altering others. Thus, by definition a high-fat diet (60% of calories or more) is lower in carbohydrates and/or protein, making results more difficult to decipher. Another challenge with animal studies using high-fat diets is the common practice of simply referring to the diet as high fat without providing information regarding the balance of specific fats, such as (*a*) polyunsaturated fatty acids with several double bonds on the carbon backbone (PUFA), (*b*) monounsaturated fatty acids (MUFA), (*c*) saturated fats (SF), (*d*) hydrogenated fats with no double bonds, and (*e*) various sugars. Buettner et al. (65) compared high-fat diets (consisting of 42% of energy from fat) utilizing coconut oil (primarily SF), olive oil (primarily MUFA), lard (mixture of SF and MUFA), and fish oil (primarily PUFA) in rats. After 12 weeks, the first three diets led to significant hepatic steatosis as well as to increased plasma TG, whereas the fish oil (PUFA)-based diet did not. A final challenge arising from animal models is histologic evaluation; several studies require use of special stains to demonstrate the tiny lipid droplets. This is not characteristic of human NAFLD, as described above, and raises

Table 5 Animal models of fatty liver disease[a]

Model	Description of model	Metabolic features	NAFLD features	Pros	Cons	Reference(s)
ob/ob mice	Naturally occuring mutation preventing synthesis of leptin	Hyperphagia, obesity, hyperglycemia, hyperinsulinemia, hyperlipidemia	Steatosis (addition of LPS can cause NASH-like features)	Includes metabolic syndrome features	Leptin deficiency rarely occurs in humans; leptin may be important for fibrogenesis	110, 122
db/db mice	Inherited mutation in the leptin receptor gene	Hyperphagia, obesity, hyperglycemia, hyperinsulinemia, hyperlipidemia	Steatosis	Includes metabolic syndrome features	No spontaneous fibrosis	154, 155
fa/fa mice	Inherited mutation in the leptin receptor genes	Hyperphagia, obesity, hyperglycemia, hyperinsulinemia, hyperlipidemia	Steatosis	Includes features of the metabolic syndrome similar to human NAFLD	No spontaneous fibrosis	146, 154, 155
SREBP-1c transgenic mice	Genetic overexpression of SREBP-1c in adipose tissue	Congenital lipodystrophy with severe insulin resistance, diabetes	Steatosis, lobular inflammation, fibrosis	Features of NASH with appropriate insulin resistance	Low leptin levels; weight loss is contrary to human model	154, 155
Lieber-deCarli diet fed Sprague-Dawley rats	Liquid high-fat diet (71% calories from fat, corn oil = 13% saturated fat)	Hyperinsulinemia	Steatosis, mononuclear inflammation, oxidative stress (abnormal mitochondria)	Features of NASH	Not associated with obesity; fat level is atypical of human consumption	154, 155, 156
High-fat diet (lard) fed C57Bl mice, male Wistar rats, Sprague-Dawley rats	High-fat chow (45–60% calories from fat; lard = 40% saturated fat) 10–50 weeks	Obesity, hyperinsulinemia, insulin resistance, increased cholesterol	Steatosis, slight inflammation seen in mice, slight fibrosis	Imitates human diet–induced NAFLD	Features of inflammation and fibrosis are minimal	65, 124, 154, 155

High-fat diet (mostly saturated fat) fed male Sprague-Dawley rats or C57BL/6 mice	High-fat chow (58% calories from fat; 85% saturated); high ratio of n-6 to n-3 PUFA	Obesity, hyperinsulinemia, hyperglycemia	Steatosis, lobular inflammation, some hepatocyte necrosis and apoptosis	Imitates human diet–induced NAFLD	Percent carbohydrate from fructose is often unspecified and varies	122, 155
Intragastric overfeeding in C57BL/6 mice	High-fat liquid chow (37% calories from corn oil; 13% saturated) fed at up to 185% of needs via implanted gastric tube for 9 weeks	Obesity, hyperinsulinemia, hyperglycemia	Steatosis, neutrophilic inflammation, pericellular and sinusoidal inflammation	Imitates hypercaloric state	Increased stress from intragastric tubes may contribute to diet effects; technically challenging	155, 157
"Western lifestyle" in C57BL/6 mice	High-fat chow (45% calories from fat; 30% trans fat); fructose and glucose in drinking water; decreased activity for up to 16 weeks	Obesity, hyperinsulinemia, hyperglycemia	Steatosis, necroinflammation	Imitates combination of increased fat and sucrose	Inflammation in model primarily driven by trans fat, which has decreased in consumption due to Food and Drug Administration regulations	71
High-fructose/-sucrose diet	Addition of fructose to water or chow to replace typical dextrose or starch	Obesity, hyperinsulinemia, hyperglycemia, hyperlipidemia, hypertension	Steatosis	Imitates human metabolic syndrome	No spontaneous inflammation or fibrosis in wild-type animals	78, 118
Methionine-/choline-deficient diet	Chow deficient in methionine and choline leads to decreased hepatic lipid export	Weight loss, low insulin, no dyslipidemia	Steatosis, rapid necroinflammation and fibrosis	Histopathology appears similar to NASH	Does not reflect the human model without features of metabolic syndrome; dietary deficiency of methionine/choline in humans unlikely	125, 154, 155

[a]Abbreviations: *db/db*, diabetic; *fa/fa*, obese Zucker; LPS, lipopolysaccharides; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; *ob/ob*, obese; PUFA, polyunsaturated fatty acids; SREBP, sterol response element binding protein.

concerns about the overall applicability of many models to the human situation.

Reduced availability of PUFA in the diet may also contribute to steatosis by favoring lipid synthesis over oxidation and export. The underlying mechanisms are incompletely understood, but PUFA have been demonstrated to decrease expression of prolipogenic nuclear receptors such as sterol response element binding protein (SREBP)-1 and carbohydrate responsive element binding protein (ChREBP) while enhancing expression of genes related to lipid oxidation (65). In cell culture and animal models, SF cause steatosis as well as increased insulin resistance (66). In NAFLD patients, a relative depletion of PUFA, particularly long-chain PUFA of the n-3 and n-6 class, has been observed (67). Another research group found decreased levels of PUFA in the hepatic lipids in NASH patients compared with patients with steatosis only, or patients with normal livers, despite the fact that the reported diets of the patients did not differ: All patients studied reported that approximately 5–6% of their calories came from PUFA (68). Several factors may be responsible for the lower hepatic PUFA content. Fatty acid distribution in adipose tissue reflects diet (69); however, this may not necessarily be applicable to fatty acid distribution in the liver (68). Further, defective desaturation processes, which result in decreased cellular transition of SF to MUFA, have been shown to increase steatosis and hepatocyte injury both in vivo and in vitro (70).

Trans fats (partially hydrogenated fats whose double bonds are located in *trans* rather than *cis*, resulting in a less flexible backbone) occur both naturally from bacterial conversion and artificially from partial hydrogenation. Trans fats are common in modern diets, especially fast foods and baked goods, as they extend food's shelf life. However, trans fats have been shown to cause NASH in sedentary mice, both alone and when combined with a high-fructose diet (71). Tetri et al. (71) speculated that one possible result of increased trans fats was reduction in TG production, as plasma TG were not elevated. With increased recognition of the dangers of trans fats, these fats are being removed from many foods, but it should be noted that even foods labeled "zero trans fat" are allowed to contain up to 0.5 g of trans fat.

Increased free fatty acids: de novo lipogenesis and carbohydrates. DNL typically waxes and wanes in response to feeding and fasting, with increased synthesis occurring after consumption of a meal. DNL is increased in NAFLD patients compared to healthy subjects (72) and fails to increase in the postprandial period (73). It is unclear how this contributes to steatosis overall, as Donnelly et al. (73) found that despite increased DNL in NAFLD patients only 26% of the TG stored in the liver were from this source. This defect in postprandial DNL flux can be induced in normal individuals as well as in hyperinsulinemic individuals via a high-carbohydrate meal (74). Parks et al. (75) tested simple sugars to determine the lipogenic response in healthy controls and found that acute fructose (but not glucose) ingestion doubles DNL, thus augmenting postprandial lipemia.

Fructose consumption may be an important dietary contributor to NAFLD pathogenesis. Fructose is a monosaccharide that is commonly found in the diet as a component of high-fructose corn syrup or as part of the disaccharide sucrose (cane sugar). It is well documented that fructose consumption has markedly increased over the past several decades, especially in the form of high-fructose corn syrup, a primary component in most soft drinks and "juice" drinks (76). For reasons that are not well understood, fructose (but not glucose) fails to stimulate postprandial ghrelin or insulin secretion, thus failing to initiate the central satiety response (77). Thus, high fructose consumption may result in overeating and weight gain, as well as in prolonged aberrant fasting hyperinsulinemia (71). Animal models with increased dietary fructose have shown increased hepatic steatosis (78). More recently, studies of NAFLD patients have shown that increased consumption of fructose (79, 80), soft drink

consumption in particular (even of the so-called diet drinks), is associated with NAFLD (81).

Endocannabinoids in Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis

The endocannabinoids are endogenous lipid mediators that form part of the complex neural circuitry controlling energy intake and regulation and that elicit a broad range of effects similar to those of marijuana (82). Interest in this system is increasing due to the demonstration of effects in the liver and the development of a chemical mediator, rimonabant, that antagonizes the effects. The actions of endogenous endocannabinoids are mediated via cannabinoid (CB1) receptors. These receptors are predominantly expressed in the central nervous system but are also found on adipocytes and hepatocytes. Stimulation of CB1 has a broad range of effects, including increased lipogenesis, decreased adiponectin, increased leptin and insulin resistance, and decreased fatty acid oxidation (82). CB1 blockade improves increased adiposity and hepatic steatosis in rat models. In a two-year randomized, placebo-controlled human trial, the CB1 antagonizer rimonabant resulted in significant weight loss, improved waist circumference, and improved plasma TG and insulin sensitivity (83).

Effects of Steatosis in the Liver

Accumulation of lipids, especially FFA, causes detrimental effects in the hepatocytes, including oxidative stress, induction of ER stress, and the uncoupled protein response and subsequent expression of proinflammatory cytokines (reviewed in Reference 50). Exposure to fatty acids in vivo induces an inflammatory response, as well as steatosis (66).

Fatty acid oxidation. In normal hepatic lipid metabolism, fatty acids are disposed of via either synthesis of TG or oxidation. Fatty acid oxidation in the liver begins with the conversion to acyl-CoA and results in the production of energy (ATP). Several classes of transcription factors, including PPARs and SREBPs, control lipid metabolism in the liver. SREBP-1c is the predominant isoform in the liver, and it regulates other crucial downstream target genes involved in fatty acid and TG synthesis such as fatty acid synthase (FAS), SCD1, and acetyl-CoA carboxylase (84). The crucial role of SCD1 in conversion of toxic SF to nontoxic MUFA, and in subsequent protection from development of steatohepatitis, has been shown both in cell culture and in animal models (70).

Adiponectin is a key factor in FFA metabolism. Adiponectin is decreased in obesity; thus, its effects of controlling FFA entry and oxidation in the mitochondria are subsequently decreased, allowing FFA to accumulate in the cytoplasm (85). Mitochondria are the primary organelles for glucose and FA oxidation. Although structural mitochondrial abnormalities have been observed in NAFLD, fatty acid oxidation in this disease seems to increase, not decrease (86). This results in the production of ROS and in the accompanying downstream effects of oxidative stress.

Oxidative stess, insulin resistance, and inflammation. Oxidative stress (defined as an imbalance in the production of ROS and protective antioxidants) has often been linked to NAFLD (86, 87), and may be a final common mechanistic occurrence in the lipotoxicity of FFA (57, 56). Sanyal et al. (86) demonstrated that immunohistochemical staining for 3-nitrotyrosine, a marker for oxidative stress, was elevated in liver biopsies from subjects with NAFLD and was even higher in NASH than in steatosis alone. Allard et al. (68) recently showed that oxidative stress in the liver tissue of NAFLD patients (as measured by total lipid peroxides) significantly correlated with inflammation and that steatosis negatively correlated with a composite measurement of antioxidant levels in the tissue. Potential sources of oxidative stress include mitochondrial dysfunction (88) and depleted antioxidants (87).

Li et al. (55) demonstrated that exposure of hepatocytes to SF leads to mitochondrial

IL-6: interleukin-6

depolarization, cytochrome *c* release, and increased ROS. In cell culture studies examining TNF-α-induced hepatocyte cell death, mitochondrial permeability, cytochrome *c* release, and procaspase-3 activation occurred only after mitochondrial glutathione was depleted, suggesting that oxidative stress is required for TNF-α toxicity (89). Human studies that used vitamin E or ursodeoxycholic acid to replete antioxidants have had positive results in children (90, 91) but, to date, none in adults (92).

Insulin resistance, defined as the lack of appropriate downstream effects of insulin signaling, is almost universally found in NAFLD (93). The association between steatosis and insulin resistance is clear, but the causal relationship(s) is under investigation. An early-phase rodent study based on differential fat loading in hepatic tissue, but not muscle, allowed investigation of hepatic insulin resistance in the setting of normal peripheral insulin sensitivity. This study showed a dose-response relationship between an increase in hepatic insulin resistance and the degree of hepatic steatosis. Samuel et al. (94) proposed that the effect was mediated via blunting of steps in the insulin-signaling cascade, including (*a*) insulin receptor substrate (IRS)-1 and -2 tyrosine phosphorylation, (*b*) impaired activation of AKT2, and (*c*) inactivation of glycogen synthase kinase 3 (GSK3). Thus, lipid accumulation itself may increase insulin resistance, which results in ineffective lipid handling and further accumulation in a feed-forward cycle. Human treatment trials focused on improvement of insulin resistance have included various insulin-sensitizing agents (thiazolidinediones), gluconeogenic agents (metformin), and weight-loss regimens (reviewed in Reference 95).

Pro- and Anti-Inflammatory Conditions

Obesity is considered a proinflammatory condition. However, not all obese and diabetic subjects have steatosis or steatosis with inflammation. It is the latter condition that more often progresses to fibrosis and end-stage liver disease; thus, inflammation has been recognized as a key pathophysiologic feature of steatosis. In 1998, Day & James (96) proposed the "two-hit" hypothesis in NAFLD: Inflammation, caused by a "second hit" combined with the primary insult (the "first hit," which led to steatosis) leads to progressive disease. Variations of the two-hit hypothesis exist, and more recently Jou et al. (58) proposed that a more important step is a putative "third hit": hepatocyte death and lack of repair. They suggest that as combined oxidative/metabolic stresses and dysregulated cytokine production continue, hepatocyte compensatory mechanisms are eventually overwhelmed, leading to an increased rate of hepatocyte death. Necrosis can result in the production of chemoattractants that recruit various types of immune cells into the liver, and a fibroinflammatory repair response is generated. As in other forms of liver disease, if this response is unregulated, it progressively distorts the normal liver parenchyma. Because both steatosis and inflammation are intertwined in a circular feed-forward relationship, NASH probably develops and/or progresses when multiple compensatory mechanisms carried out by liver cells are repeatedly overwhelmed. An example of an otherwise potentially protective mechanism that when missing or depleted may lead to increased hepatocyte cell death is the adipokine adiponectin.

Role of Visceral Adipose Tissue

Adipose tissue, rather than serving simply as a passive organ for excess energy storage, is a metabolically active organ with endocrine and inflammatory functions. This is especially true of visceral fat, which is an important source of a variety of cytokines, collectively referred to as adipocytokines or adipokines. This role may be the mechanism by which visceral adiposity contributes to NAFLD.

Visceral fat is more metabolically active than subcutaneous fat, and it secretes more TNF-α and IL-6 and less adiponectin (97). Ethnic differences in visceral fat are under evaluation as potential links to understanding differences

in NAFLD. In a magnetic resonance imaging study of hepatic TG content in over 2000 Hispanic, Caucasian, and African American individuals, Guerrero et al. (18) found that African Americans had lower levels of hepatic TG, despite similar total body adiposity and insulin resistance. Interestingly, controlling for amounts of intraperitoneal fat almost completely eliminated the ethnic variation in hepatic TG. In addition, women had higher subcutaneous fat and overall adiposity than did men, but they had lower hepatic TG levels. Asians have increased amounts of visceral fat depots in relation to their body mass index (BMI), and as such, are also at equivalent risks of diabetes and NAFLD/NASH as Caucasians (20), despite lower BMI.

In a comprehensive analysis of both fat distribution and circulating adipocytokines, Van Der Poorten et al. (98) found that visceral fat was independently associated with both inflammation and increasing fibrosis on biopsy in NALFD patients. For every 1% increase in visceral fat, odds ratios—independent of other features of the metabolic syndrome—were two to one for increasing necroinflammation and two to eight for increasing fibrosis stage; this finding suggests that visceral fat depots are primary factors in NAFLD. The study also showed that insulin resistance itself was not an independent predictor, but that IL-6 levels were independently associated with histologic inflammation, thus supporting the key role of inflammation generated by visceral fat in NAFLD. Overall, abdominal visceral fat is most consistently associated with increased IL-6 levels and C-reactive protein (CRP), a systemic marker of inflammation (99), and may be an important variable determining which overweight individuals develop NAFLD and NASH.

Stefan et al. (100) characterized metabolically benign obesity by studying normal, overweight, and obese adults. Despite finding similar amounts of visceral fat, the authors found increased skeletal muscle TG and hepatic steatosis in the obese insulin-resistant group compared with the obese insulin-sensitive group. One conclusion was that hepatic fat, rather than visceral fat, is more important in determining the metabolic consequences of obesity. However, as only 56% of the subjects in the obese insulin-resistant group had hepatic steatosis, it is difficult to draw firm conclusions.

Adipokines/cytokines. Abnormal cytokine metabolism is a major feature of all models of NALFD/NASH, and the most extensively investigated adipocytokines in NAFLD/NASH are TNF-α, IL-6, adiponectin, and resistin. Obesity-related hepatic steatosis is directly associated with increased production of inflammatory cytokines and decreased production of anti-inflammatory cytokines (101, 102). Several interventional trials in humans have shown a beneficial link between weight loss and alterations of cytokines (95).

Cytokines are involved in the recruitment and activation of Kupffer cells (resident hepatic macrophages) and are responsible for the transformation and perpetuation of hepatic stellate cells to the myofibroblastic phenotype (103). Kupffer cells reside along the luminal side of the sinusoids and can be activated by exposure to portal and systemic circulatory products, including gut-derived bacteria, microbial debris, and bacterial endotoxins. Lipid accumulation in the liver is also associated with Kupffer cell activation (104).

TNF-α. TNF-α, secreted by adipocytes and by hepatocytes, was the first cytokine known to be elevated in obesity and NAFLD (105). TNF-α regulates inflammation, cell viability, metabolism, and other cytokines (106) and plays a central role in the response to endotoxin by generating both inflammation and apoptosis. As currently understood, TNF-α both promotes and is activated by insulin resistance via activation of IKK-β (inhibitor of nuclear factor kappa B (NF-κB). Activation of IKK-β results in activation of NF-κB, a proinflammatory "master switch" that regulates inflammatory mediators including CRP, plasminogen activator inhibitor (PAI-1), TNF-α, IL-6, and IL-1β (104). Importantly, TNF-α antagonizes adiponectin, an anti-inflammatory adipocytokine, so as TNF-α

levels increase, the balance shifts in the direction of inflammation.

TNF-α has been shown to be elevated (107, 108) in relation to the anti-inflammatory adipokine adiponectin (109) in human NAFLD. Specifically, TNF-α is elevated in patients with NASH and increases stepwise from obesity to simple steatosis to NASH (108). In the liver tissue of NAFLD patients, Crespo et al. (107) identified increased levels of mRNA and a TNF-α receptor, p55, and showed that circulating levels correlate with significant fibrosis.

In obese (*ob/ob*) mice fed a high-fat diet, dietary manipulation to favor anti-TNF pathways and anti-TNF antibody improved insulin sensitivity, hepatic steatosis, and visible inflammation in the liver (110). Pentoxifylline, a phosphodiesterase inhibitor, was shown to prevent production of TNF-α, as well as other cytokines, and has had a modest positive effect in small studies of human NAFLD (95).

Adiponectin and resistin. Effects of visceral adiposity may be at least partially mediated via altered levels of circulating adiponectin, as increased visceral adiposity is a strong predictor of decreased adiponectin (111). Adiponectin is produced by visceral adipocytes and demonstrates insulin-sensitizing properties via (*a*) activation of AMPK, (*b*) stimulation of glucose utilization, and (*c*) promotion of fatty acid oxidation (112). Adiponectin and TNF-α are mutually antagonizing adipokines (113). Unlike TNF-α, adiponectin is decreased in obesity, particularly with increased visceral adiposity (111), and has likewise been shown to be decreased in pediatric and adult NAFLD (108, 114) and to have a negative correlation for hepatic steatosis (115). This deficiency may be critical in the progression to NASH, as adiponectin may protect the liver from inflammation via direct antagonism of TNF-α. In two mouse models of hepatic steatosis, recombinant adiponectin inhibited TNF-α, improved insulin sensitivity, and improved steatohepatitis (113). In contrast to TNF-α, adiponectin has antilipogenic and anti-inflammatory effects; thus, imbalance in the TNF-α/adiponectin ratio may be important for NASH development (109). Many therapies associated with improvement in NAFLD have also resulted in increased plasma adiponectin levels including weight loss, decreasing visceral adiposity, dietary PUFA, and PPARγ ligands [thiazolidinediones (TZDs)] (112).

Resistin is a proinflammatory cytokine also associated with impairment of insulin action (108). In contrast to adiponectin, increased resistin levels have been correlated with NAFLD severity and NASH development (116).

Interleukin-6. IL-6 is an adipocytokine associated with NALFD and obesity that is secreted in larger amounts by visceral fat compared to subcutaneous fat in obese adults (117). Increased systemic IL-6 was associated with increased inflammation and fibrosis in NAFLD patients (98). Using a transgenic model with twofold increased IKK-β to stimulate NF-κB, Cai et al. (104) produced similar insulin resistance as a high fat diet. They demonstrated that both liver mRNA and plasma levels of IL-6 were increased and that an anti-IL-6 antibody significantly improved insulin resistance. These findings suggest a sequence of cellular events: Steatosis activates IKK-β and NF-κB, which then induces IL-6. IL-6 causes insulin resistance locally and could be the link to systemic insulin resistance. High-fat diets in mice lead to increases in hepatic NF-κB activity as well as to increased hepatic steatosis and mRNA levels of IL-6, IL-1β, and TNF-α. Feldstein et al. (118) clarified the role of steatosis in this mechanism by demonstrating that FFA activated IKK-β/NF-κB via a cathepsin B–dependent mechanism that subsequently increased TNF-α with further liver injury. IL-6 levels decreased with therapy in a small pilot study of vitamin E in NAFLD, whereas levels of TNF-α remained constant (119).

Gut: Endotoxin

Endotoxin is another focus of investigation in the promotion of inflammation in NAFLD.

Endotoxin, one of the components of the outer wall of gram-negative bacteria, is released by the microbiota in the gut and is directly introduced into the liver via the portal blood. There, via Toll-like receptor 4 (TLR4), endotoxin stimulates an inflammatory response, including increasing levels of TNF-α. Kupffer cells, the first line of defense, are activated by endotoxin (120). Marked activation of Kupffer cells in human NASH has been observed (121). Genetically obese (122) and diet-induced obese mice (123) are sensitized to endotoxin hepatotoxicity, and *ob/ob* mice (who are prone to steatosis) have increased small intestinal bacterial overgrowth (110). Alterations in gut microbial flora could presumably decrease endotoxin; in animal models, antibiotics and probiotics have led to variable improvement in steatosis and inflammation (78, 110, 124, 125).

Problems with examining endotoxin in human NAFLD include the short half-life of endotoxin and the fact that systemic levels may not reflect portal blood levels. Ruiz et al. (126) examined 40 morbidly obese NAFLD patients and found elevated circulating lipopolysaccharide-binding protein (a surrogate marker of endotoxin) in NASH. Other researchers have found elevated plasma endotoxin in NAFLD patients compared to healthy controls (79) as well as increased small bowel overgrowth. Studies in obese rodents and twin studies in humans have elegantly shown metabolic alterations related to loss of diversity of the gut microbiota related to energy use and storage in obesity (127).

Innate Immunity in Nonalcoholic Steatohepatitis

Recently, several lines of investigation have focused on the interacting role(s) of the innate immune system in obesity, insulin resistance, and liver pathology (128). Fatty acids can directly activate inflammatory signaling and hepatic insulin resistance as ligands for TLRs with downstream, intracellular activation of c-Jun N-terminal kinase (JNK) and IKK. Liver-related innate immune responses to excess fat can directly result in hepatocyte apoptosis (via increased FAS expression). All resident cells of the liver express TLRs. Their exact functions have not been fully explored, but the specific TLRs demonstrated in NASH (in animal models) include TLR2 and TLR4, and coreceptors for TLR4, CD14, and MD2 have also been identified in these models. Intracellular lipid accumulation may sensitize hepatocytes to ligand binding effects, and inhibitors of the TLRs have been shown to decrease inflammation and fibrosis in dietary animal models.

PROGRESSION OF NONALCOHOLIC FATTY LIVER DISEASE AND NONALCOHOLIC STEATOHEPATITIS

Hepatocyte Injury in Nonalcoholic Steatohepatitis

Hepatocyte injury in NASH results from the above-described mechanisms related to FFA lipotoxicity, oxidative stress, unfolded protein response, adipokine/cytokine effects, mitochondrial injury, and inflammation.

Histological forms of hepatocellular injury in nonalcoholic steatohepatitis. Two forms of hepatocyte death are recognized, although not clearly separated. One is necrotic death, in which hepatocytes become swollen and lose metabolic functions; the other, a metabolically active form of cell death is apoptosis. Apoptosis results in nuclear and cytoplasmic fragmentation.

Hepatocyte ballooning. Ballooned hepatocytes occur in many forms of hepatitic and cholestatic liver diseases. They result from microtubular disruption and from alterations to the intermediate filament cytoskeleton in severe cell injury preceding lytic necrosis (129). Their large cell volume may be related to increased fluid in the cytosol (130), accumulated fat (131), or other factors. Displacement of cytoskeletal filaments K8/18 to the periphery can

be observed via immunohistochemistry (33). Hepatocyte ballooning is a common form of cell injury in NAFLD.

Hepatocyte death. Hepatocyte death occurs in the form of lytic (oncotic) necrosis, apoptosis, or a combination of the two (necroapoptosis). Necrosis and apoptosis are commonly the result of the same triggering factors and signaling pathways, and they likely represent extremes in the spectrum of cell death (132). Necrosis leads to cell swelling, karyolysis, and rupture of the cytoplasmic membrane, whereas necrosis is a metabolically inactive event associated with ATP depletion and is commonly associated with an inflammatory cell reaction. Histologically, the presence of cell lysis is inferred from foci of Kupffer cells in the sinusoids (spotty necrosis) or from foci or regions of hepatocyte "drop-out." Apoptosis is a highly regulated, metabolically active form of cell death. Pathways for apoptosis have been characterized as extrinsic and intrinsic; both pathways converge on the final effector caspases to mediate cell death. The extrinsic pathway is activated by death ligands and their receptors, such as FAS and FASL, and by TNF-α-related apoptosis-inducing ligand (TRAIL); the intrinsic pathway is activated by mechanisms of cell and membrane stress (lysosomal, ER, and mitochondrial injury) (133).

In NASH, apoptosis correlates with other above-described components of disease inflammatory activity and fibrosis (32, 134). Serum levels and immunohistochemical detection of apoptotic markers, such as the M30 neoantigen, correlate with disease severity in NAFLD (32), confirming the role of apoptosis in NASH. Markers of apoptosis are included in noninvasive, diagnostic biomarker panels to predict the presence of NASH in obese patients (135).

In NASH, apoptotic hepatocytes may be the result of lipotoxicity, mainly induced by saturated FFA, that can modulate both intrinsic and extrinsic pathways (133). The latter is a TNF-α pathway (118), and the former is a mitochondrial or a lysosomal pathway involving either JNK-dependent activation of the proapoptotic protein Bax or direct Bax activation, respectively (133). Genetic polymorphisms of transcription factor 7-like 2, which predisposes to diabetes, may impact liver injury and modulate a fat-induced increase of circulating markers of apoptosis in NASH.

Interestingly, the antiapoptotic B cell lymphoma 2 (BCL-2) protein appears strongly expressed in human steatohepatitis, probably representing an adaptive response (134). Thus, on the basis of the recognized significance of hepatocyte apoptosis in NAFLD pathogenesis, it has been proposed that the development of progressive NAFLD in some patients, but not in others, may be the result of increased susceptibility of steatotic hepatocytes to apoptosis arising from abnormal regulation of BCL-2 proteins, alteration in JNK activation, or preferential activation of ER stress (132, 133).

Other Histologic Findings Related to Hepatocyte Injury

Histologic alterations within hepatocytes and the surrounding parenchyma constitute the constellation of lesions of NAFLD.

Mallory–Denk bodies. Hepatocytes contain K8/18. MDB, which are perinuclear, "ropy" eosinophilic aggregates, are composed of misfolded, ubiquitinated K8/18 decorated by the stress-induced and ubiquitin-binding protein p62, heat-shock proteins 70 and 25, and αB-crystallin (136). A high K8/18 ratio and keratin cross-linking by transglutaminase 2 play important roles in MDB formation (137). Studies in transgenic animals have shown that background genetic susceptibility alters susceptibility to MDB formation and to liver injury (138). Both the association of p62 (an immediate early-response gene product) with ubiquitinated keratins and the protective role played by K8/18 in guarding hepatocytes from apoptosis (137) suggest that MDB represent the end product of the sequestration process of abnormal, possibly deleterious proteins and that they may

actually be hepatoprotective (136). During NASH development, the recognized aldehyde by-products of lipid peroxidation, HNE and MDA, can cross-link keratin filaments to form MDB; MDB can stimulate neutrophil chemotaxis (139). Furthermore, TGF-β may participate in MDB formation by induction of tissue transglutaminase (140). Definitive MDB have been reported in only a few animal models of NASH.

Megamitochondria. For a description of megamitochondria, see the subsection entitled Steatohepatitis, above. In NASH, mitochondrial dysfunction results from reactive lipid peroxidation products, ROS, reactive nitrogen species, and TNF-α, all of which can block respiratory chain components, alter mitochondrial DNA, and lead to increased mitochondrial ROS formation. The additional finding of functional abnormalities in mitochondria of skeletal muscle in some cases of NASH has led to the suggestion that NASH may be a mitochondrial disease (140). An alternative interpretation of the structural alterations of NASH is that they represent a generalized response to oxidative stress, rather than being a result of cell injury, and that they may be a form of adaptation (141). Ultrastructural mitochondrial abnormalities were found to increase following otherwise histologically successful therapy for NASH with a TZD (142).

Pigmented Kupffer cells/microgranulomas. Kupffer cells are resident macrophages within the liver parenchyma with close physical proximity to hepatocytes, stellate cells, endothelial cells, and circulatory products within the sinusoids. As macrophages, Kupffer cells engulf apoptotic and necrotic hepatocytes, and their digestion products may be visualized with histochemical stains (pigmented Kupffer cells). Phagocytosis of apoptotic hepatocytes results in activation of Kupffer cells (143). Other activation pathways in NAFLD include proinflammatory cytokine production, locally by hepatocytes or distantly by adipocytes, and activation of scavenger receptors for the clearance of oxidized lipids and gut-derived endotoxin (102). Activated Kupffer cells play a central role in pathogenesis and the progression of liver disease by contributing to parenchymal inflammation, hepatocyte injury, and initiation of fibrosis via cytokine secretion (TNF-α, TGF-β). The loss of the normal periportal accentuation of Kupffer cells in normal liver to the zone 3 distribution in NASH accentuates the zonal injury in this process (121).

Fibrosis

Injury to the hepatic parenchyma and/or the biliary tree results in well-characterized, stereotypic responses of the macrophages (Kupffer cells and portal macrophages) and of the fibrogenic cells of the liver, the hepatic stellate cells, and portal myofibroblasts that lead to deposition of matrix materials with varying degrees of fibrosis (scar), and consequently, vascular architectural parenchymal remodeling. The end-stage form of scarring and remodeling, cirrhosis, may lead to liver failure and carries a risk of HCC.

Fibrosis and progression to the remodeled parenchyma of cirrhosis may be viewed as a failure of appropriate adaptive and repair mechanisms. There is an increasing appreciation for the loss of hepatocytes' normal ability to regenerate and the subsequent activation of hepatic progenitor cells (41, 144). These complex processes ultimately involve activation of the fibrogenic cells of the liver, primarily hepatic stellate cells, but also portal myofibroblasts (103). Epithelial-mesenchymal transition is another possible source of matrix deposition under investigation (discussed below). Clinical tests to predict fibrosis in NASH are based on the presence of serum factors reflecting fibrogenesis, matrix deposition, and resultant liver dysfunction (145). Any mechanistic understanding of fibrogenesis and fibrosis in NAFLD/NASH must account for several recognized features of the disease: (*a*) the patterns of fibrosis noted in both adults

and pediatric patients (discussed above), (*b*) the fact that in many cases of cirrhosis the active lesions of NASH and even steatosis may no longer be present, (*c*) the fact that fibrosis may regress, along with the other features of activity, and (*d*) the fact that there are correlations with well-recognized clinical risk factors of increased age, presence of diabetes, and increased BMI (17). Investigators have found increased CTGF protein and mRNA both in liver biopsies with increased fibrosis in patients with NAFLD and in cultured stellate cells exposed to high levels of glucose and insulin (146). Lipotoxicity, oxidative stress, and inflammation play recognized roles in the initiation and progression of fibrosis in NASH (147). In addition, secreted adipokines, including adiponectin, leptin, and the effector cytokines of the renin-angiotensin system, are central to fibrogenesis in NAFLD/NASH (148). The role or roles of PPARγ and other nuclear receptors in treatment of lipid disorders, inflammation, and fibrosis are also under investigation (50).

The recently proposed paradigm of concurrent periportal ductular proliferation and portal/periportal fibrosis (the ductular reaction) for the portal-based fibrosis in chronic hepatitis C has also been shown in studies of biopsies from patients who have NASH with portal fibrosis (41). The amounts of both ductular reaction and fibrosis, as well as direct evidence of hepatocyte senescence and decreased hepatocellular proliferation, were shown to correlate with elevated serum levels of insulin and calculated levels of insulin resistance (41).

Epithelial-mesenchymal transition (EMT), a (patho)physiologic process by which local environmental alterations induce differentiation of mature epithelial cells into cells with mesenchymal phenotypic markers and functions, is being demonstrated in a variety of inflammatory conditions of solid organs and organs with endocrine and/or exocrine function, as well as in cancers of solid organs. EMT has recently been shown to occur in diseases of the biliary system (149), but to date no studies of EMT as a mechanism of fibrosis in NAFLD/NASH have been published.

HEPATOCELLULAR CARCINOMA IN NONALCOHOLIC FATTY LIVER DISEASE AND NONALCOHOLIC STEATOHEPATITIS

The risk of HCC in NAFLD-related liver disease is commonly proposed but less well established compared to ALD and other chronic liver diseases that result in cirrhosis (93). Two comparative studies have shown HCC secondary to NASH cirrhosis to be less common than that for hepatitis C virus–related cirrhosis (150, 151). Published rates of HCC in NASH cirrhosis and/or cryptogenic cirrhosis vary from 0% to 47% (14). Recently, attention has been drawn to noncirrhotic patients with NAFLD/NASH (14, 152) or metabolic syndrome (153) with HCC.

CONCLUSIONS AND CONTROVERSIES

As we have shown in this review, the mechanisms by which obesity and insulin resistance result in fatty liver, steatohepatitis, and fibrosis are quite complex and are likely to differ in humans compared to cultured cells and the various animal models utilized to study NASH. The complex nature of the underlying ethnic differences and genetics of humans is challenging to dissect, yet it is known to factor into predisposition and severity of metabolic diseases, including NASH. The role(s) of dietary components, energy use, and the central and peripheral nervous systems in liver diseases related to overweight conditions cannot be discounted. One of the fundamental controversies of NAFLD/NASH remains: What is the actual potential cytotoxicity of steatosis without inflammation and fibrosis? Is this a hepatoprotective mechanism, or is it simply a marker of disease, or is steatosis the first stimulus ("hit") to the cascade of pathologic processes? The fact that not every phenotypically disposed

individual develops NASH and fibrosis raises another fundamental question: Does NASH necessarily progress, or could it begin de novo as an inflammatory condition? And finally, why is NAFLD an underlying predisposition to the development of HCC?

DISCLOSURE STATEMENT

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