Nonalcoholic Fatty Liver Disease: Clinical Features and Pathogenesis

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Abstract: Nonalcoholic fatty liver disease (NAFLD) describes a spectrum of liver abnormalities from benign steatosis to nonalcoholic steatohepatitis (NASH). NASH is characterized by chronic and progressive liver pathology and can cause advanced fibrosis, cirrhosis, hepatocellular carcinoma, end-stage liver disease, and liver-related death. Unlike other forms of chronic liver disease, NAFLD is usually associated with insulin resistance and often at least one feature of the metabolic syndrome (obesity, impaired fasting glucose, hypertriglyceridemia, low high-density lipoprotein cholesterol, and hypertension). Although its progression rate may be slower than that of other types of liver disease, the incidence of both NAFLD and its sequelae is increasing throughout the world in parallel with the obesity epidemic. One of the important and unresolved problems is the pathogenesis of hepatocyte injury in NASH. The natural history of NAFLD remains unclear because of the paucity of histologic follow-up studies. Although there have been recent attempts to identify treatments for NAFLD, currently there is no well-established and approved therapy. Lifestyle modifications that include increased exercise and weight reduction address the underlying insulin resistance and may be the best advice for patients.

Keywords
Nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, obesity, insulin resistance, ballooning of hepatocytes

Nonalcoholic fatty liver disease (NAFLD) is a term used to describe a spectrum of histologic abnormalities, from benign steatosis to nonalcoholic steatohepatitis (NASH), in a person consuming little or no alcohol (Table 1). Although the natural history of NAFLD is not fully understood, currently available data indicate that NAFLD has the potential to progress to cirrhosis, hepatocellular carcinoma (HCC), end-stage liver disease, liver-related death, and recurrence after transplantation.[1-9] A different spectrum of the disease, namely NAFLD-associated subacute liver failure, has also been reported.[10]

As the prevalence and severity of both obesity and diabetes continue to increase throughout the world, the incidence of NAFLD and its sequelae will also likely increase,[11-13] though perhaps not as quickly as some other chronic liver diseases.[9,14-17] Thus, NAFLD could develop into a large disease and health budget burden throughout the world.

Table 1. Terminology

Benign steatosis: the generally nonprogressive form of NAFLD
HCC: hepatocellular carcinoma
NAFLD: nonalcoholic fatty liver disease; is characterized by predominantly macrovesicular steatosis in which hepatocytes contain vacuoles of triglycerides
NASH: nonalcoholic steatohepatitis; the progressive form of NAFLD that includes steatosis, necroinflammation, and variable degrees of fibrosis
NASH-associated cirrhosis: may lose the histologic features of NASH
NASH-associated HCC: HCC arising in the setting of cirrhosis caused by NASH
NASH-associated subacute liver failure: unexplained rapid and severe decompensation of liver function in the setting of NASH

Table 2. Criteria for Metabolic Syndrome*

• Abdominal obesity
- Men: waist circumference >40 inches
- Women: waist circumference >35 inches
• Fasting glucose ≥110 and <126 mg/dL
• Blood pressure ≥130/80 mm Hg
• Triglycerides ≥150 mg/dL
• HDL cholesterol: Men <40 mg/dL; women <50 mg/dL

* The metabolic syndrome is present when three or more of the five criteria are met.

Adapted from Reaven.[25]

HDL = high-density lipoprotein.

Although recent studies support the hypothesis that NAFLD is a part of the metabolic syndrome, this remains an area of active investigation.[18-25] For example, although insulin resistance seems to play an important role in the pathogenesis of NAFLD, not all patients with insulin resistance develop NAFLD and not all patients with NAFLD have insulin resistance.

An important unresolved problem is determination of the pathogenesis of hepatocyte injury in NASH. The natural history of NAFLD also remains unclear because there have been few histologic follow-up studies.[9,16,26,27] The aim of this article is to provide a brief overview of the current knowledge about NAFLD and to raise awareness about this growing problem.

Definition and Prevalence of NAFLD

NAFLD is defined by the presence of excessive fat in the liver, detected either by imaging or liver biopsy.[28] NAFLD used to be a diagnosis of exclusion in patients without other ongoing liver disease; however, as histologic criteria have evolved, the presence of NAFLD and NASH coexisting with other forms of liver disease has been described.[29] To establish the diagnosis, patients must abstain from alcohol or drink infrequently. Recent studies suggested that the maximal safe level of ethanol consumption may be 30 g/day,[30] although more stringent criteria such as 20 g/day for men and 10 g/day for women are often used in studies of patients with NAFLD.

It is estimated that approximately 10–40% of the adult population in the United States has some degree of NAFLD, and about 2–5% has NASH.[31,32] Investigators from other developed nations report similarly high figures.[33-37] NASH is the third most common liver disease in North America and the most common liver disease in both Australia and New Zealand.[38] The prevalence of advanced disease varies somewhat with the population studied; for example, hispanic patients with NAFLD seem to progress to both NASH and cirrhosis more frequently than either blacks or whites.[39,40] NAFLD is the most common histologic abnormality in patients with unexplained elevated liver enzymes in industrialized countries (4.5% of the US population is reported to have unexplained elevated alanine aminotransferase [ALT] levels).[12,13,41-44] Obesity and diabetes are also important risk factors for advanced disease. An early autopsy study found NASH in 18.5% and 2.7% of markedly obese and lean subjects, respectively.[45]

NAFLD as Part of the Metabolic Syndrome

NAFLD is considered to be the hepatic manifestation of the metabolic syndrome (Table 2).[18,19,25,46,47] Marchesini and colleagues reported that of 120 NASH patients, 88% met at least three criteria of the metabolic syndrome.[23] The prevalence and severity of liver disease progressively increased with the number and severity of features of the metabolic syndrome. The prevalence of obesity, type-2 diabetes, and hyperlipidemia was 30–100%, 10–75%, and 20–92%, respectively, in different NAFLD case series.[48]

Obesity and NAFLD

Obesity can be considered a low-grade chronic inflammatory condition and obesity-related cytokines such as interleukin-6 (IL-6), adiponectin, leptin, and tumor necrosis factor (TNF)α may play important roles in the development of NAFLD. The World Health Organization estimated that there were 200 million obese people in the world in 1995 and 300 million in 2002.[49] Epidemiologically, the prevalence of elevated aminotransferases increases with increasing body mass index and a correlation between degree of obesity and the prevalence and severity of NAFLD has been found in several studies.[50-52]

For a given patient, the degree of ALT elevation does not predict the severity of disease and the full spectrum of NAFLD has been found in morbidly obese patients undergoing bariatric surgery.[53-55] The prevalence of NASH is 3% and 20% in nonobese and obese subjects, respectively. Obesity is increasely common in children, and both fibrosis and cirrhosis have been seen in obese children with NAFLD. There is currently, however, no reliable estimate of the prevalence of NAFLD or its consequences in children.[56-58]

Type-2 Diabetes, Hyperlipidemia, and NAFLD

The prevalence of NASH associated with both cirrhosis and HCC was reported to be high among patients with type-2 diabetes with or without obesity.[2,3,7,59] One study found that half of the hyperlipidemic patients had evidence of NAFLD by both ultrasound and abnormal liver enzymes.[60] Even in the absence of obesity, hypertriglyceridemia is associated with insulin resistance.[61]

Pathogenesis of NAFLD

One hypothesis for the pathogenesis of NAFLD is the "two-hit" hypothesis proposed by Day and James in 1998.[62] According to this paradigm, the primary abnormality is a metabolic disturbance—most frequently insulin resistance—which causes NAFLD, then a second hit induces injury and inflammation, or NASH and its sequelae (Figure 1).

First Hit

The presence of excess fat is a prerequisite for the subsequent events of NASH. The main characteristic of NAFLD is the accumulation of triglycerides (TG) as fat droplets within the cytoplasm of hepatocytes. It is defined practically as more than 10% of hepatocytes having fat droplets evident on liver biopsy.[63] Increased delivery of both free fatty acids (FFA) and TG to the liver, diminished hepatic utilization of FFA, diminished export of TG from the liver, and impaired beta-oxidation of FFA within hepatocytes cause TG accumulation within the cytoplasm of hepatocytes.[64-66] Excess carbohydrates, either from dietary sources or de novo gluconeogenesis in the liver, is also a major stimulus for de novo fatty acid synthesis in the liver. Paradoxically, direct uptake of dietary fat as chylomicron remnants or FFA constitutes a relatively minor contribution to liver fat accumulation.[67]

Insulin resistance is a common cause of fat accumulation in the liver. However, we also know that a small subgroup of NAFLD patients do not exhibit detectable features of impaired insulin sensitivity. This suggests that possibilities other than insulin resistance are important in a subset of patients. Furthermore, a growing body of evidence suggests that insulin resistance does not act only as a first hit, but also may play an important role in the inflammation and hepatocellular injury that characterize NASH (Figure 2).[63,66,68]

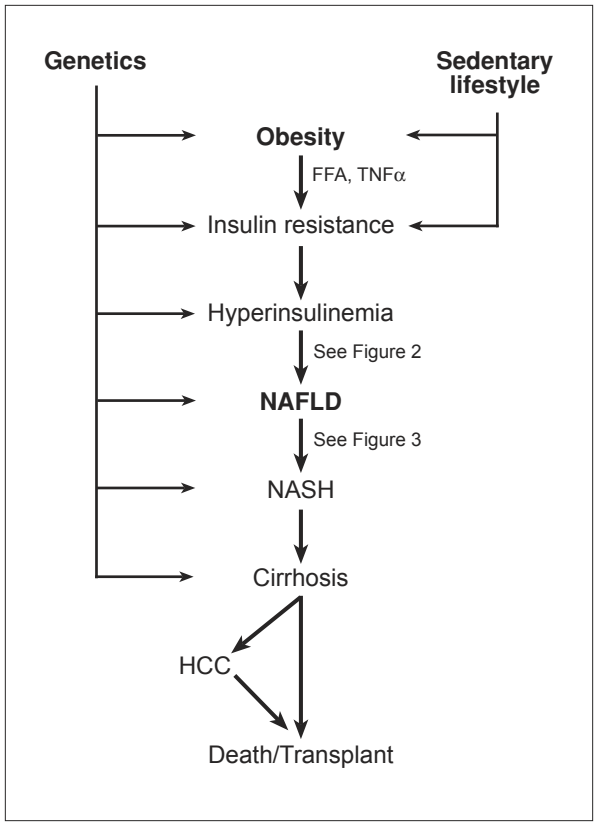

Figure 1. Genetics and obesity contribute to the development of insulin resistance and these three factors together lead to the development of nonalcoholic fatty liver disease (NAFLD). The presence of NAFLD puts certain individuals at risk for developing nonalcoholic steatohepatitis (NASH), cirrhosis, hepatocellular carcinoma (HCC), and death from end-stage liver disease or liver transplantation.

FFA = free fatty acids; TNF = tumor necrosis factor.

Second Hit

A liver with excess fat may be more vulnerable to stressors such as reactive oxygen species (ROS), adipokines, and cytokines than a normal liver. The regenerative capacity of a fatty liver is also impaired. Yang and colleagues demonstrated that obese mice with fatty livers clear endotoxins less than nonobese controls.[69] Nonetheless, the factors that play key roles in the development of NASH from NAFLD remain uncertain. Some possibilities include the duration of fatty infiltration of the liver and the duration and severity of hyperinsulinemia. Other possible second hits are oxidative stress (increased ROS and decreased antioxidants), lipid peroxidation and reactive metabo-

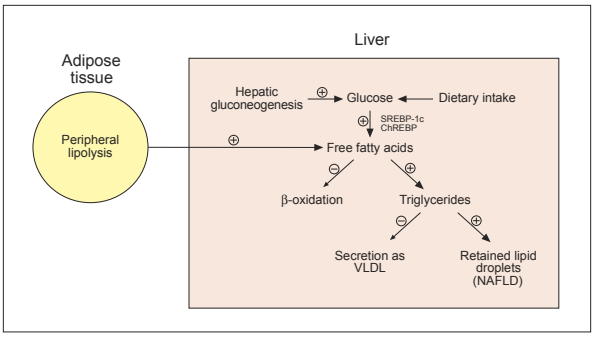

Figure 2. Metabolic pathways altered by insulin resistance and hyperinsulinemia. Pathways marked by "+" signs are increased and those marked by "-" signs are inhibited. Hyperinsulinemia and insulin resistance increase both hepatic glucose production and adipose tissue free fatty acid (FFA) release, respectively, and circulating FFA are taken up by the liver. In adipocytes, insulin resistance impairs glucose uptake, further contributing to the need for increased insulin levels. In the liver, hyperinsulinemia induces sterol regulatory element–binding protein-1c (SREBP-1c) expression while hyperglycemia activates carbohydrate response element–binding protein (ChREBP). The activation of both SREBP-1c and ChREBP induces lipogenic enzymes that convert excess glucose to fatty acids in the liver. Excess fatty acids undergo mitochondrial β-oxidation or are esterified to produce triglycerides. Excess triglycerides that are not secreted as very-low-density lipoproteins (VLDL) are stored as fat droplets within the cytoplasm of hepatocytes to cause nonalcoholic fatty liver disease (NAFLD).

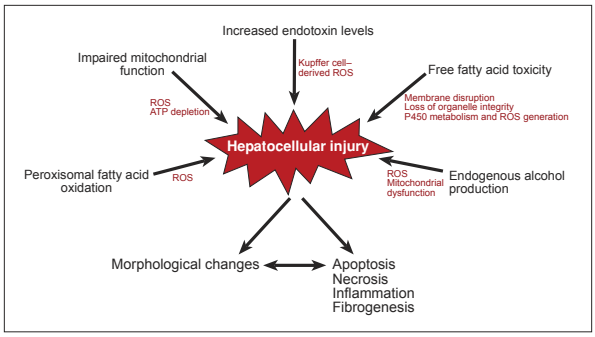

Figure 3. Multiple mechanisms may play a role in causing hepatocellular injury in the setting of nonalcoholic fatty liver disease, many of which generate reactive oxygen species (ROS). For example, peroxisomal fatty acid oxidation generates hydrogen peroxide, "electron leak" from the mitochondrial respiratory chain produces superoxide, sinusoidal Kupffer cells release superoxide when stimulated by endotoxin, cytochrome P450 metabolism of fatty acids leads to electron leak from downstream redox factors to release superoxide and gut-derived ethanol may cause ROS production during its hepatocellular metabolism. Mechanisms of injury not requiring ROS may also be important and include adenosine triphosphate (ATP) depletion and disrupted organelle membranes. Hepatocytes respond with morphological changes such as ballooning, cytoskeletal protein aggregation, apoptosis, and necrosis. Individually or in sum, these processes stimulate inflammation and fibrogenesis, the latter leading to cirrhosis in some patients.

lites such as malondialdehyde and 4-hydroxynonenal, adipose tissue products, transforming growth factor-β,[1] Fas ligand, mitochondrial dysfunction and respiratory chain deficiency, and small intestinal bacterial overgrowth (endotoxins and TNF-α) (Figure 3).[63,66,70]

Adipose tissue is now recognized as a source of important metabolic and inflammatory mediators. These adipokines have both proinflammatory (leptin, TNF-α, and IL-6) and anti-inflammatory (adiponectin) effects.[68,71-75] Adiponectin also has antilipogenic effects. Adipokines regulate both hepatic and peripheral glucose and lipid metabolism. Although these cytokines and hormones normally work in a balance, this homeostasis may be disturbed in NASH patients and studies are now focusing on these changes. NASH patients have reduced adiponectin levels and increased TNF-α levels.[75,76]

Hepatocyte Injury and NASH Pathology

Hepatocyte ballooning is one of the features of injury observed on NASH liver biopsies.[28] Whether ballooning of hepatocytes is an adaptive (physiologic) or degenerative (pathologic) change of hepatocytes is unknown. The initial response of hepatocytes to a stressor is to increase in volume,[77-82] and mild volume changes (up to 5–10%) without biochemical evidence of free radicals may be physiologic or adaptive. However, greater swelling of hepatocytes (≥30% volume increase) is usually degenerative and can cause stress protein expression, macromolecular overcrowding, disturbed cellular architecture, Mallory hyaline formation, hepatocyte apoptosis, necrosis, and cell death.

Clinical Features, Biochemical Abnormalities, and Radiological Evidence of NAFLD

NAFLD occurs at equal rates among males and females.[63] The majority of cases are found within the fourth or fifth decade of life, although the full spectrum has also been described in children. Patients with NAFLD are often obese and may have hypertension. The most frequent symptoms are fatigue and right upper quadrant pain or dullness, although many patients have no symptoms. Mild or moderate hepatomegaly is one of the most common physical examination findings. Biochemically, patients with NAFLD may have hyperlipidemia, hyperglycemia, hyperinsulinemia, and reduced insulin sensitivity.[63]

Aminotransferases are often mildly or moderately increased, with an aspartate aminotransferase (AST)/ALT ratio less than 1, although the prevalence of NAFLD

Table 3. Proposed Grading and Staging System of NASH[94]

Histologic Feature	Definition	Score
Steatosis		
Grade	The evaluation of parenchymal involvement by steatosis	
	<5%	0
	5–33%	1
	>33–66%	2
	>66%	3
Location/predominant distribution pattern	Zone 3	0
	Zone 1	1
	Azonal	2
	Panacinar	3
Microvesicular steatosis	Presence of contiguous patches	1
Fibrosis		
Stage	None	0
	Perisinusoidal or periportal	1
	Mild, zone 3, perisinusoidal	1A
	Moderate, zone 3, perisinusoidal	1B
	Portal/periportal	1C
	Perisinusoidal and portal/periportal	2
	Bridging fibrosis	3
	Cirrhosis	4
Inflammation		
Lobular inflammation	Overall assessment of all inflammatory foci	
	No foci	0
	<2 foci per 200X field	1
	2–4 foci per 200X field	2
	>4 foci per 200X field	3
Microgranulomas	Presence of small aggregates of macrophages	1
Large lipogranulomas	Present, usually in portal areas or adjacent to central veins	1
Portal inflammation	Greater than minimal when assessed from low magnification	1
Liver Cell Injury		
Ballooning	None	0
	Few balloon cells	1
	Many cells/prominent ballooning	2
Acidophil bodies	Many	1
Pigmented macrophages	Many	1
Megamitochondria	Many	1
Other findings		
Mallory hyaline	Many visible on routine stains	1
Glycogenated nuclei	Many contiguous patches	1

in patients with normal aminotransferases is difficult to estimate. Inversion of the AST/ALT ratio to greater than 1 suggests progression to cirrhosis[83] or covert alcohol consumption; however, serum aminotransferase values fluctuate during the course of the disease.[63] Moreover, NAFLD patients with normal aminotransferases can exhibit the full spectrum of histopathologic abnormalities from benign steatosis to cirrhosis.[84] Gamma-glutamyltranspeptidase can also be elevated in patients with NAFLD but is not a reliable test for the presence of NASH. Unfortunately, there is also no good correlation between serum liver tests and necroinflammatory activity or stages.[63] Ferritin is usually elevated and may indicate increased steatosis and inflammation or the severity of fibrosis.[85,86] Abdominal ultrasonography, computed tomography, and magnetic resonance imaging (MRI) all can identify NAFLD, but cannot separate benign steatosis from NASH.[87-90] MRI, particularly localized proton magnetic resonance spectroscopy, has the capability of accurately measuring hepatic TG content but is costly.[32,91,92]

Histopathologic Features of NAFLD

Grading, Staging, and Individual Histopathologic Features of NAFLD

Liver biopsy is the gold standard diagnostic tool not only to reach a correct diagnosis and to document grading of necroinflammation and staging of fibrosis, but also to predict prognosis in patients with clinical or radiologic evidence of NAFLD. A commonly used method for the pathologic evaluation of NASH is the system established by Brunt and colleagues[93] and its recent revision.[94] The newly revised system includes grading of necroinflammatory activity and staging of fibrosis (Table 3). This system requires only routine histochemical stains and includes 14 histologic features. A NALFD activity score was proposed based on the unweighted sum of the steatosis, lobular inflammation, and ballooning scores. Scores of 0–2 were usually not considered to be NASH, 3–4 possibly NASH, and 5–8 usually indicative of NASH. Although the scoring system covers the range of histologic features of pediatric NAFLD, some difficulties remain in the application of this system to pediatric subjects.

Both steatosis and necroinflammation predominantly involve zone 3. The staging of NASH using this system begins with perisinusoidal fibrosis and progresses to portal fibrosis, bridging fibrosis, and cirrhosis. Ballooning of hepatocytes and Mallory hyaline are the two hallmarks of ongoing injury and inflammation and have been associated with hepatic fibrosis (Table 4).[95] A pathologic typing system of NAFLD has also been described as a predictor of outcomes (Table 5).[1]

Table 4. Histopathologic Abnormalities in NASH

- Steatosis
- Mixed lobular inflammation
- Hepatocyte ballooning with or without Mallory hyaline
- Perisinusoidal fibrosis

Table 5. Matteoni Typing System for NAFLD Associated with Outcomes[1]

Type 1:	Fatty liver alone
Type 2:	Fat and inflammation
Type 3:	Fat and ballooned hepatocytes
Type 4:	Fat and ballooned hepatocytes and either Mallory bodies or fibrosis

Table 6. Histopathologic Criteria of the Proposed Typing System for NASH-Associated Cirrhosis[98]

1. Definite: Steatosis plus intralobular mixed inflammatory foci
2. Probable: Steatosis plus intralobular mononuclear inflammation

3a. Possible: Intralobular mixed inflammation without steatosis

3b. Possible: Steatosis without any inflammation

4. Cryptogenic cirrhosis (burned-out NASH): No steatosis and no inflammation

NASH-associated Cirrhosis

Initial studies defined NAFLD-associated cirrhosis on the basis of a history of metabolic abnormalities. Powell and colleagues reported that the features of steatohepatitis may disappear with fibrosis progression[96] and Caldwell and coauthors reported that some cryptogenic cirrhosis cases do not show any characteristic pathologic features of NAFLD (burned-out NASH).[2] However, these patients typically had NAFLD-associated metabolic abnormalities and subsequent studies have shown that most of the cryptogenic cirrhosis cases had prior NASH, particularly within obese and diabetic groups.[3-5,39,40,97,98] Recently, Hui and colleagues proposed a definition for NAFLD-associated cirrhosis using strict clinicopathologic criteria.[98] Their case definition requires clinical risk factors for more than 5 years such as obesity, diabetes, and hyperlipidemia and a pathologic description (Table 6). HCC may also be a late complication of the metabolic syndrome and NASH, typically in the setting of cirrhosis.[6-8]

When to perform a liver biopsy in a patient with imaging evidence of NAFLD or risk factors for NASH remains an unresolved issue. A biopsy is generally war-

Table 7. Summary of Longitudinal Natural History Studies

Source	Number of Patients	Follow-up Period (mean, years)	Fibrosis Progression (% of patients)	Fibrosis Regression (% of patients)	Rate of Fibrosis Progression (stages/year)
Evans[26]*	7	8.2	57%	None	0.088†
Harrison[27]*	22	5.7	32%	18%	Not reported
Fassio[16]‡	22	4.3	32%	18%	0.059†
Adams[9]*	103	3.2	37%	29%	0.09†

* Changes in fibrosis were analyzed using the classification developed by Brunt and colleagues.[93,94]
† In noncirrhotic nonalcoholic steatohepatitis patients.
‡ Changes in fibrosis were analyzed using the Ishak classification.

ranted to further evaluate persistently elevated aminotransferases even when NAFLD is suspected on clinical grounds because a biopsy can identify another diagnosis or lead to changes in therapy up to a third of the time.[99] Data from several series of patients undergoing bariatric surgery that show a high prevalence of advanced liver disease despite normal aminotransferases suggest that a liver biopsy should be considered in patients with risk factors for advanced disease such as extreme obesity, diabetes, or an elevated AST/ALT ratio despite normal enzymes.[53,54,100] By comparison, deferring a liver biopsy while lifestyle modifications are pursued in earnest may be a reasonable alternative in a young person found to have elevated aminotransferases after weight gain if imaging demonstrates NAFLD and other causes of chronic liver disease are excluded. The inherent risk of this approach is the possibility of missing significant treatable liver diseases such as Wilson disease and autoimmune hepatitis.

Natural History of NAFLD

Knowledge about the natural course of NAFLD is still limited to relatively few small studies. Cross-sectional studies reported 30–40% of NASH patients had advanced fibrosis and 10–15% had cirrhosis at the time of biopsy.[63]

Natural History of NAFLD in Longitudinal Studies

An early follow-up study of 26 patients with NAFLD for a median of 11 years showed that steatosis alone is usually a benign, nonprogressive condition and its prognosis is favorable,[101] although a study of 59 patients with benign steatosis reported that 2 patients (3.4%) did progress to cirrhosis.[1] A recently published study investigating patients with fatty livers alone in both nonalcoholic and alcoholic groups (median follow-up 19.9 years in the nonalcoholic group) showed that 1 nonalcoholic patient (0.6%) developed cirrhosis during the follow-up period,[102] whereas a larger population-based study in Minnesota over 20 years found that a diagnosis of NAFLD was associated with increased mortality, especially in those with impaired glucose tolerance or cirrhosis.[103]

Natural History of NASH in Longitudinal Studies

Matteoni and colleagues reported that 25% of patients with fibrosis develop cirrhosis within 10–25 years.[1] Four histologic follow-up studies have given some important clues about the natural history of NASH (Table 7).[9,16,26,27] The reported fibrosis progression rates in these studies are slower than those of patients with alcoholic liver disease and chronic hepatitis C infection, which measure at 0.12 units of fibrosis per year.[9,14-16]

Therapy of NAFLD

For the patient with comorbidities such as obesity, hyperlipidemia, or type-2 diabetes, lifestyle modifications that include exercise and changing dietary habits to achieve gradual and sustained weight loss are the primary recommendations. It was reported that both hepatic steatosis and inflammation respond rapidly to changed environmental conditions such as weight loss, although the response of fibrosis is slow.[104] If the patient's condition does not improve despite these interventions, medications may be considered. Goals for the pharmacologic therapy of NAFLD should target both the accumulation of fat and the consequent injury and fibrosis (Table 8).[63,105,106] One pharmacologic agent, ursodiol acid, was evaluated over a 2-year period in a randomized, placebo-controlled study and showed no more benefit than placebo.[107] Interventions for the treatment of NAFLD raise the important question of how these patients should be monitored for therapy effectiveness. To evaluate and compare histopathologic features of the initial and final biopsies is the best method but the decision to obtain liver biopsies must weigh the risks and benefits. In this regard, noninvasive follow-up markers would be beneficial. Potentially useful serum

Table 8. Potential Future Pharmacologic Therapy Modalities in NAFLD[63,105,111]

1. Insulin sensitizers such as metformin and thiazolidinediones
2. Antilipidemic agents such as fibrates and statins
3. Anticytokines such as anti-TNF antibodies and TNF-receptor antagonists
4. Cytoprotectives and antioxidants such as ursodeoxycholic acid, vitamin E, S-adenosylmethionine, N-acetylcysteine, selenium, carnitine, and silymarin
5. Antibiotics and probiotics to reduce gut-derived endotoxins
6. Phlebotomy, choline, and betaine
7. Antifibrotic agents

TNF = tumor necrosis factor.

markers such as hyaluronic acid or the measurement of insulin sensitivity or serum cytokines levels such as adiponectin, leptin, and TNF-α may be promising for future applications.

Conclusion

There has been growing concern and interest in NAFLD in the last decade, and each month approximately five new papers about NAFLD are published. With its increasing prevalence, it is estimated that NAFLD will eventually become the most frequently seen liver disease. Understanding the underlying causes of NAFLD and designing rational treatments will require continued research with collaboration among investigators in fields such as endocrinology, pathology, biochemistry, and biophysics.

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