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## Role of liver biopsy in nonalcoholic fatty liver disease

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### Abstract

Nonalcoholic fatty liver disease (NAFLD), defined as abnormal accumulation (> 5%) of hepatic triglyceride without excess alcohol intake, is the most common form of chronic liver disease in adults and children in the United States. NAFLD encompasses a spectrum of histologic findings including uncomplicated steatosis, steatosis with inflammation and steatohepatitis [nonalcoholic steatohepatitis (NASH)]; the latter can advance to cirrhosis and hepatocellular carcinoma. NASH is currently accepted as the hepatic manifestation of the set of cardiovascular risk factors collectively known as metabolic syndrome. In 1999 a system for histologic grading and staging for NASH was proposed; this was revised by the NASH Clinical Research Network in 2005 for the entire spectrum of lesions in NAFLD, including the lesions and patterns of pediatric NAFLD, and for application in clinical research trials. Diagnosis remains distinct from grade and stage. A recent European proposal separates steatosis from activity to derive a numeric diagnosis of NASH. Even though there have been promising advancements in non-invasive testing, these tests are not yet detailed enough to replace the full range of findings provided by liver biopsy evaluation. Limitations of biopsy are acknowledged, but liver biopsy remains the "gold standard" for diagnosis and determination of amounts of necroinflammatory activ-

ity, and location of fibrosis, as well as remodeling of the parenchyma in NASH. This review focuses on the specific histologic lesions of NAFLD and NASH, grading and staging, differential diagnoses to be considered, and the continuing role of the liver biopsy in this important liver disease.

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**Key words:** Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Liver biopsy; Histopathology; Grading and staging

**Core tip:** Nonalcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease; no clinical or serologic tests have yet replaced liver biopsy for definitive diagnosis. The histologic spectrum includes steatosis, steatohepatitis, and cirrhosis with or without active steatohepatitis. Hepatocellular carcinoma may occur in cirrhosis, or prior to cirrhosis. Liver biopsy provides vital data for patient care, clinical trials, and for ongoing research into nuances of the disease process. The histologic spectrum of NAFLD, features with co-existent diseases, differential diagnoses, grading and staging methods and the role of liver biopsy, as well as a brief description of non-invasive alternatives, are discussed.

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### INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), defined as abnormal accumulation of triglycerides in the liver (*i.e.*, > 5%) in the absence of significant alcohol intake, is recog-

nized as the most common cause of chronic liver disease, and is estimated to affect 30% of adults and 10% of children in United States<sup>[1]</sup>. The prevalence of NAFLD parallels that of metabolic syndrome, insulin resistance, type 2 diabetes and central obesity<sup>[2]</sup>. It is currently recognized that mortality in the majority of individuals with NAFLD is more likely from cardiovascular diseases than from liver disease<sup>[3]</sup>, thus, even though recent studies have documented similar epidemiologic and histologic features of NAFLD and nonalcoholic steatohepatitis (NASH) in the geriatric population<sup>[4,5]</sup>, this age group will not be further discussed in this review. The prevalence is highest in the ethnic Hispanic population, followed by non-Hispanic whites, Asians and African Americans<sup>[6,7]</sup>. The prevalence increases significantly up to 80%-90% in obese adults, 60% in patients with hyperlipidemia, and 30%-50% in diabetics<sup>[2]</sup>. Only a minority of subjects with steatohepatitis actually progress to fibrosis or cirrhosis<sup>[8]</sup>. Currently, however, NAFLD is the third most common cause of liver transplantation in United States<sup>[9]</sup>, and is projected to be the leading cause of liver transplantation in the United States by 2020<sup>[1]</sup>. In addition, approximately 4%-22% of hepatocellular carcinomas in the Western world are attributed to NAFLD<sup>[10]</sup>. Hepatocellular carcinoma may develop in either cirrhosis or non-cirrhotic NAFLD, as recently reviewed<sup>[10,11]</sup>.

NAFLD is a spectrum of histologic lesions of steatosis at one end, and steatohepatitis and cirrhosis at the other<sup>[12]</sup>. However, whether in a given patient there is actual "progression" from steatosis to steatohepatitis without concurrent clinical complications such as weight gain, or new onset diabetes, has not been adequately studied. What is accepted is that steatosis itself is considered "non-progressive" whereas steatohepatitis is the constellation of lesions with potential to progress; this was first shown in a seminal series of Matteoni *et al.*<sup>[13]</sup>. Thus, the ability of the liver biopsy is to separate individuals with steatohepatitis from those with "only" steatosis (which includes steatosis with inflammation); this is an important goal that any diagnostic test should meet. NASH is defined histopathologically by the presence of a constellation of features: steatosis, lobular and portal inflammation and liver cell injury in the form of hepatocyte ballooning. Initially, in adults, the ballooning and fibrosis are in a zone 3 distribution; once abnormal matrix is deposited (*i.e.*, fibrosis), and architectural remodeling occurs, the zonality of injury is less apparent.

Many advances have occurred since the initial recognition that fatty liver disease could occur in overweight and/or diabetic subjects who weren't over-exposed to alcohol by several researchers including Thaler<sup>[14]</sup>, Klatskin *et al.*<sup>[15]</sup>, Schaffner *et al.*<sup>[16]</sup> and Ludwig *et al.*<sup>[17]</sup> in the late 1970's and early 1980's. These include histopathologic work in humans with careful clinical correlations discerning the prevalence of NAFLD and NASH<sup>[18]</sup>, the roles of progenitor cells and the ductular reaction in fibrogenesis<sup>[18-21]</sup>, and the role of innate and adaptive immune-mediated mechanisms in steatohepatitis<sup>[22]</sup>, progression

of steatohepatitis to cirrhosis and hepatocellular carcinoma<sup>[10,23,24]</sup> and genetic and genomic underpinnings of disease susceptibility and progression<sup>[25,26]</sup>.

## LIVER BIOPSY: ROLE, INDICATIONS, AND DRAWBACKS

The general indications for performing a liver biopsy in patients with NAFLD are to confirm or exclude the diagnosis, diagnose other liver diseases, and to determine amounts of damage to the liver for treatment and prognosis. The last includes necroinflammatory activity, which is potentially reversible, and collagen deposition with varying degrees of remodeling, which is potentially less reversible. More specific indications have been recently stated. According to the 2012 guidelines from American Association for the Study of Liver Disease (AASLD), liver biopsy should be reserved for subjects who will "benefit", for subjects with potentially competing diagnoses, and for children with either an unclear diagnosis or in whom consideration is being given for medication<sup>[27]</sup>. The European Association for the Study of Liver Disease position statement on liver biopsy differed slightly and recommended liver biopsy in all bariatric surgery subjects, and as an endpoint in all clinical trials<sup>[28]</sup>. Liver biopsy remains the standard against which noninvasive (serologic and imaging) methods are judged in order to assess these features. By histologic evaluation, one is able to distinguish between NASH, a lesion with progressive potential, and no NASH, lesions without potential to progress<sup>[28-33]</sup>.

Performing a liver biopsy on every patient with suspected NAFLD remains a controversial subject in daily practice, and clearly is not a practical consideration as a "screening" tool. There are studies, however, that support the value of liver biopsy. A frequently cited albeit older study by Skelly *et al.*<sup>[34]</sup> showed that of 354 biopsied patients with otherwise unexplained abnormal liver tests 66% had fatty liver, 50% of those had steatohepatitis, approximately 19% of the remaining biopsies had other treatable causes diagnosed by the pathology evaluation including autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), hemochromatosis and alcoholic liver disease (ALD).

A more recent study used a modeling system to show that obtaining a liver biopsy could provide survival benefit to patients with NAFLD. Gaidos *et al.*<sup>[35]</sup> evaluated the long-term benefit of biopsy *vs* no biopsy. The study showed that performing an early liver biopsy resulted in a higher percentage of having mild liver disease ultimately in NAFLD patients. Early diagnosis and treatment resulted in decrease in progression to severe disease or transplant and a predicted survival advantage in NAFLD patients. In addition, the risk of death related to liver biopsy did not offset the survival advantage. Others have demonstrated the benefits of liver biopsy in NAFLD by demonstrating the presence of NAFLD or NASH in "atypical settings" such as concurrent disease processes such as Hepatitis C<sup>[36-38]</sup>, AIH, PBC, hereditary hemochromatosis (HH)<sup>[36]</sup>, as well as drugs and occupational

exposures<sup>[39-41]</sup>. Liver biopsy studies proved the validity of the concept of the presence of all ranges of necro-inflammatory activity and fibrosis, including cirrhosis, in the presence of normal alanine aminotransferase values in adults<sup>[42-44]</sup>, and children<sup>[45]</sup>. The current state of investigation for pathologists is to evaluate which subjects will develop fibrosis and why, and which findings on early biopsies, if any, may be predictive of future outcomes.

Liver biopsy studies have given insight into natural history of NASH, albeit in a selected group of primarily adult subjects. The prevalence of NASH, 12.2% of over 300 multi-ethnic adult subjects, was established in a Texas city by liver biopsy in an unbiased community who were willing to undergo ultrasound and subsequent liver biopsy. Nearly 30% had evidence of NAFLD by ultrasound<sup>[18]</sup>. This was the first study of its kind in adults, as all prior studies had been done either in living donors, who did not have evidence of steatohepatitis, or in autopsies. Liver pathology evaluation was the first to establish the remarkable incidence of fatty liver of up to 13% in an autopsy study of children who died from accidental deaths<sup>[46]</sup>. Liver biopsy further characterized the association of cardiovascular disease and biopsy proven NAFLD in 150 overweight children compared with 150 overweight children without NAFLD<sup>[47]</sup>.

At the current time, it remains common practice to attribute cases of cryptogenic cirrhosis to burned out “NASH”. This is not altogether appropriate unless there is prior biopsy proof of NASH because clinically it is known that other forms of liver injury can “burn out”, in particular alcoholic liver disease and autoimmune liver disease. It was liver biopsy studies that indicated that NASH may be one of the underlying causes of cryptogenic cirrhosis, which, by definition, has no serologic markers for determination of cause of liver disease. This was shown in a small case series<sup>[48]</sup> then larger case studies comparing NAFLD-related cirrhosis to cirrhosis related to other forms of serologically diagnosed chronic liver disease<sup>[49]</sup>, and finally in kindred studies<sup>[50]</sup>. Liver biopsy in cirrhosis of otherwise unknown etiology can show evidence of ongoing steatohepatitis, or burned out disease without any activity or steatosis in subjects with clinical features of metabolic syndrome. Additionally, not only will NASH progress to advanced fibrosis and cirrhosis in a subset of individuals if the underlying metabolic conditions are unchanged, but several studies have verified that NASH may also regress spontaneously, as reviewed<sup>[51]</sup>. This phenomenon has largely been observed in either placebo arms or in the non-responders of treatment trials<sup>[52-54]</sup>.

In 2009, an AASLD sponsored workshop raised important questions for advancement in the field of NAFLD, providing guidance for investigators for clinical design and end points including pathologic and laboratory data<sup>[55]</sup>. As evidenced by published recommendations, liver biopsy is an important tool in clinical trials. Outcomes remain based on histologic features which provide information regarding the effects of intervention on extent and severity of hepatocellular injury, inflammation, and architectural alterations as well as the ultimate diagnosis.

To date, histologic evaluation remains the “hard endpoint” that can be measured with the most reliability<sup>[12]</sup>. The most commonly used tool for histologic evaluation is the nonalcoholic fatty liver disease activity score (NAS) (Details will be discussed in later in the text). The NAS was not intended to replace the diagnosis but to provide a sensitive tool to assess the changes that might occur with treatment<sup>[56]</sup>. However, unintended use of this score has been noted by the investigators<sup>[56]</sup>. A study by NASH clinical research network (CRN), including 976 adult liver biopsies, challenged this concept and demonstrated that while the NAS correlated with the diagnosis, it was in the lower and higher ends of the spectrum. Therefore, if NAS were to reflect the diagnosis, a significant portion of these patients would be misclassified<sup>[56]</sup>. Most importantly diagnosis of NASH was strongly associated with the presence of diabetes, quantitative insulin sensitivity check index (QUICKi) and homeostasis model assessment-estimated insulin resistance (HOMA-IR)<sup>[56]</sup> while the diagnosis and the NAS both correlated with aminotransferases. This data further supported the concept that scoring lesions and diagnosing patterns of injury are different processes for a pathologist<sup>[29,56]</sup>.

## LIVER BIOPSY LIMITATIONS

Liver biopsy, as useful as it is, however, does have limitations. The major limitation of liver biopsy is the invasive nature of the procedure. Though considered “minimal”, liver biopsy is an invasive procedure and can have complications even in the ideal clinical conditions, including pain, minor and major bleeding (0.3%). Organ perforation is uncommon, but more likely in blind biopsy. Death albeit rare, has been reported at 0.01%<sup>[57]</sup>. Currently, the majority of liver biopsies are performed under ultrasound guidance. As with liver biopsy interpretation, operator experience is an important factor in success<sup>[58]</sup>.

As in other chronic liver diseases, biopsy size is an important, but often unrecognized consideration in diagnostic accuracy<sup>[12,59,60]</sup>. A biopsy, at least 1.6 cm in length with 1.2-1.8 mm diameter, containing approximately 10 portal tracts is considered adequate<sup>[61,62]</sup>. Even then an adequate liver biopsy represents approximately 1:50,000 of the entire organ<sup>[63]</sup>.

Some limitations of liver biopsy are due to variability of the disease process itself, as with all other forms of chronic liver disease. NAFLD, while a diffuse process of the liver, can have differences particularly in fibrosis due to the location of the samples under evaluation. The subcapsular liver tissue is generally more fibrotic, and the left lobe has larger portal areas near the capsule than the right. Thus, it is important, particularly for studies, that both pre and post study biopsies are done in a similar fashion and from the same region of the liver<sup>[12]</sup>. Authors have not always agreed on the amounts of histologic sampling variability in NAFLD. Larson *et al*<sup>[62]</sup> found minimal variability in steatosis,  $NAS \geq 5$  and fibrosis between two samples in bariatric subjects. The study also emphasized the need for not only appropriate length, but also width of liver bi-



opsy needle. Another study in morbidly obese individuals undergoing bariatric surgery found moderate histologic variability between lobes<sup>[64]</sup>. In two additional studies, one in bariatrics and one in non-morbidly obese subjects, two separate biopsies from the same location in the same lobe were graded and staged independently and showed one or more points in discordance in fibrosis stage<sup>[59,60]</sup>. In addition hepatocyte ballooning, one of the diagnostic requirements of NASH, was not present in 24% of patients in one study in one set of the biopsies<sup>[60]</sup>. The implications for clinical studies for evaluating pre and post treatment biopsies are apparent.

The experience of the pathologist also plays a significant role in making the diagnosis of NAFLD; this is similar to other liver diseases<sup>[58]</sup>. The interobserver agreements on steatosis, ballooning and fibrosis were good amongst pathologists in the study of Kleiner *et al.*<sup>[65]</sup> that included 32 adult biopsies and 9 pathologists but not so strong for location of steatosis and for inflammation<sup>[66]</sup>. Similar observations were reported in a study of 21 liver biopsies read by eight experienced Japanese hepatopathologists with good agreement on fibrosis and extent of steatosis. Younossi *et al.*<sup>[67]</sup>, showed good concordance for extent of steatosis and degree of fibrosis along with ballooned hepatocytes in 53 liver biopsies interpreted by 4 experienced liver pathologists.

The high prevalence of NAFLD in the population and the limitations, risks and cost of liver biopsy have led investigators to seek for non-invasive methods to diagnose, and stage NAFLD. The ideal test would be cheap, reproducible, and would be able to diagnose the full spectrum of NAFLD, predict fibrosis, and also reflect changes that occur with treatment<sup>[31,32]</sup>. Several different methodologies including imaging modalities, serum markers and combined tests are currently being investigated. Even though advancements are being made in these fields, none of these can provide detailed and accurate enough information to replace the liver biopsy. For a more comprehensive summary of recent non-invasive tests, the reader is referred to current reviews<sup>[31,32]</sup>.

In summary an adequate liver biopsy, with appropriate clinical history, interpreted by a trained liver pathologist, is not only pivotal for an accurate and complete diagnosis (or exclusion) of NAFLD (or NASH), but also is optimal for obtaining detailed information regarding disease pattern, severity and fibrosis. It provides important information with respect to subtypes, potential future risks, possible etiology, and natural history of disease, and sets the ground work for future molecular studies and clinical trials, assisting clinical colleagues and patients with treatments and follow-up.

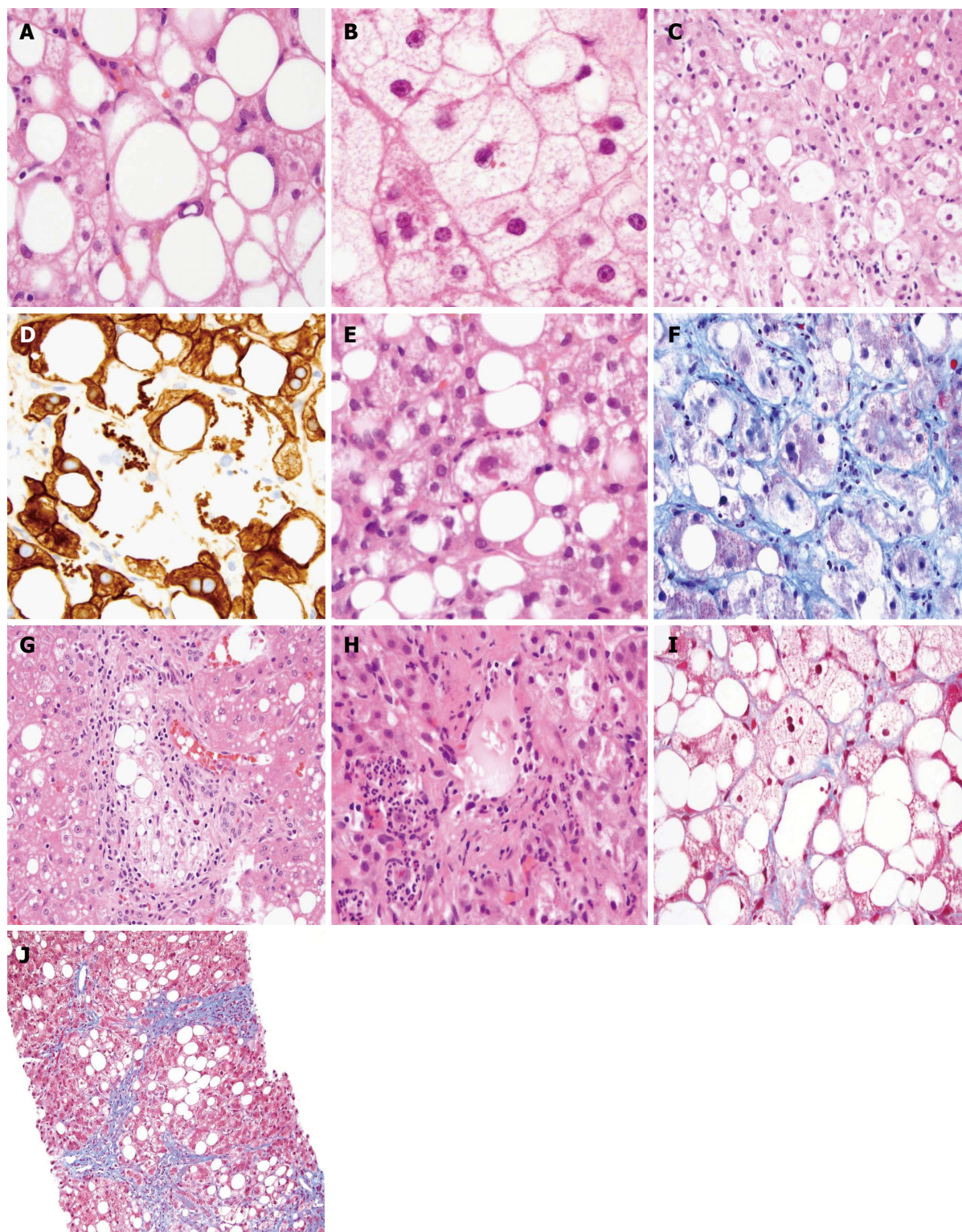
## HISTOLOGIC FEATURES, GRADING, AND STAGING OF NAFLD: ADULTS

In NAFLD, 5% or more macrovesicular steatosis is required for the diagnosis. This may occur with or without other findings, but without it, the diagnosis cannot be

entertained. Steatosis initially is found predominantly in hepatocytes around the terminal hepatic venule (zone 3); when abundant, steatosis can be panacinar, and when resolving, may be irregular, or azonal. The type of triglyceride accumulation that is observed in NAFLD is predominantly macrovesicular and is typically characterized by a single or a few lipid droplets within the affected hepatocytes, displacing the nucleus peripherally within the cell (Figure 1A). When the droplets are smaller, *i.e.*, “small droplet” steatosis, they are well-defined and easily delineated from one another. Small droplets often form droplets around the larger droplets and may coalesce to form the macrodroplet. Albeit small, these types of droplets should not be confused for true “microvesicular” steatosis, as the pathophysiology and thus implications for liver function differ (Figure 1B). Microvesicular steatosis may occur in a patchy distribution in up to 10 % of NASH cases<sup>[68,69]</sup>, and has been shown in a large study to be associated with ballooned hepatocytes, and advanced fibrosis<sup>[69]</sup>.

There are a constellation of histopathologic lesions required for the diagnosis of steatohepatitis. These include steatosis, inflammation (lobular and portal) and hepatocyte injury, commonly in the form of ballooning. Ballooned hepatocytes appear as enlarged hepatocytes with a flocculent, cleared appearance of the cytoplasm with irregular cytoplasmic borders<sup>[30,70]</sup> (Figure 1C). Often the nucleus is hyperchromatic. Hepatocyte ballooning has been shown to correlate with oxidative damage<sup>[71]</sup> and microtubule disruption<sup>[30,70]</sup>, and loss of normal structure of the cytoskeleton<sup>[71,72]</sup>. In adult NASH, ballooned hepatocytes are most commonly seen in zone 3 and if fibrosis is present, they are intermixed with perisinusoidal collagen fibers. Keratins 8/18, present throughout the cytoplasm of all normal hepatocytes<sup>[72]</sup>, are damaged in ballooned hepatocytes; this is manifest with loss of cytoplasmic immunoreexpression (Figure 1D), movement to submembranous location, and highlighting of Mallory-Denk bodies (MDB)<sup>[73]</sup>. MDB are ubiquitinated keratins and cytoskeletal structures within the proteasome (Figure 1E). p62 and ubiquitin are immunomarkers that label MDB<sup>[74]</sup>. By trichrome stain, MDB can be blue or green (Figure 1F). In NASH, MDBs are often less well-formed than in alcoholic hepatitis or alcoholic steatohepatitis<sup>[75]</sup>. Finally, ballooning has been associated with several relevant clinical markers such as measures of insulin resistance<sup>[76]</sup>, increased serum cholesterol<sup>[76,77]</sup> and serum markers of necroinflammation<sup>[78]</sup>. Similar correlations have also been noted in the pediatric population<sup>[79]</sup>.

Lobular inflammation, typically more prominent than portal inflammation in uncomplicated adult NAFLD, is mostly mononuclear, but also includes Kupffer cell clusters, microgranulomas with or without lipid droplets, and larger lipogranulomas (Figure 1G). Lefkowitz noted prominent enlargement and aggregation of Kupffer cells around terminal hepatic venules in NASH, in contrast to the even distribution in normal livers and uncomplicated steatosis<sup>[80]</sup>. Kupffer cells, the largest resident macrophage



**Figure 1** Histologic features, grading, and staging of nonalcoholic fatty liver disease. A: Mixed large and small droplet steatosis, single droplet, with nucleus pushed to one side, HE stain, 600 ×; B: Microvesicular steatosis, nuclei in the center with foamy cytoplasm, and megamitochondria HE stain, 600 ×; C: Ballooned hepatocytes with flocculent cytoplasm, HE stain, 600 ×; D: Loss of cytoplasmic expression of keratin 8/18 in ballooned hepatocytes, 600 ×; E: Mallory-Denk body, HE stain, 600 ×; F: Mallory-Denk body in blue-green color and dense perisinusoidal fibrosis, Trichrome stain, 600 ×; G: Portal lipogranuloma, HE stain, 400 ×; H: Mallory-Denk bodies and satellitosis HE stain, 600 ×; I: Delicate perisinusoidal fibrosis, Trichrome stain, 600 ×; J: Bridging fibrosis, Trichrome stain, 200 ×.

population in liver, are an important component of the innate immune system<sup>[81]</sup> and are implicated in the development and the progression of steatohepatitis<sup>[82,83]</sup> as well as in fibrosis<sup>[83]</sup>.

Occasional polymorphonuclear leukocytes may also occur as a part of lobular inflammation. When intense and encircling hepatocytes (*i.e.*, satellitosis), one should consider alcoholic hepatitis (Figure 1H). The intensity



and distribution of the inflammation varies within the lobule. In some cases, the intense inflammation in zone 3 may be confused with a portal area with the duct obscured by inflammation. This may be particularly true in the cases when an artery branch is readily appreciated in zone 3<sup>[84]</sup>.

Portal inflammation in NAFLD/NASH can be seen in 4 situations in increased amounts. In most cases, however, it is usually milder than lobular inflammation, and mononuclear cells are typically predominant<sup>[85]</sup>. Increased portal inflammation in active NASH has been associated with increased steatosis, ballooning and fibrosis in a series of 728 adult and 205 pediatric biopsies<sup>[86]</sup>. Additionally, portal inflammation was noted to predominate along with portal fibrosis in a study of 100 pediatric NAFLD biopsies<sup>[87]</sup>. When portal inflammation is unusually prevalent, or when lymphoid aggregates occur in adult NAFLD or NASH, one should consider the possibility of a concurrent liver disease such as viral hepatitis, autoimmune liver disease, as examples<sup>[36,88]</sup>. Liver biopsy data has shown that pathologists are capable of diagnosing NASH concurrently with another serologically positive liver disease, in particular, HCV, but the criteria may differ. This concept was shown by 3 separate studies; 2 retrospective reviews of large biopsy series<sup>[36,38]</sup> and a prospective study<sup>[89]</sup>. One group emphasized the necessity of stricter histologic criteria when evaluating NASH with other diseases by focusing on the characteristic zone 3 perisinusoidal fibrosis of the former that does not occur in the latter<sup>[36]</sup>. The fourth consideration is increased portal inflammation compared to lobular in adult patients following otherwise effective intervention<sup>[29]</sup>. Finally, a different type of portal inflammation, *i.e.*, polymorphonuclear leukocytes accompanying periportal ductular reaction may be indicative of ALD with pancreatitis or other forms of biliary obstruction.

Studies have demonstrated expansion of the periportal progenitor cell compartment in NASH. Hepatic progenitor cells (HPC) reside within the canal of Hering, along the limiting plate. They are rarely visualized by light microscopy unless activated. HPC are characterized by high N:C, round to spindle cytoplasm, ovoid nuclei and positivity for keratin 7 and 19; these characteristics are altered with progressive stages of development towards hepatocellular or biliary epithelium. In certain circumstances of liver injury and repair, stem cell markers, Hedgehog pathway markers and others are also reported in activated HPC<sup>[90]</sup>. Roskams *et al.*<sup>[91]</sup> demonstrated increased number of progenitor cells in patients with NASH and ALD; the study further showed correlation between numbers of hepatic progenitor cells and fibrosis stage. In a multi-center study of subjects before and after various forms of treatment, the investigators demonstrated expansion of progenitor cell component, correlation between ductular reaction, steatosis, amount of portal inflammation, and NASH activity grade<sup>[19]</sup>. The group went on to demonstrate that the ductular reaction correlated with p21 positive replicative arrest in hepatocytes which

was also associated with NASH activity and with insulin resistance. This area of epithelial-mesenchymal communication is one of ongoing interest in NASH.

Apoptotic hepatocytes are common both in NASH and ALD. Investigators have found that increased apoptosis is associated with disease severity<sup>[92,93]</sup>, as well as fibrosis in NASH<sup>[92]</sup>.

Isolated arteries observed in zone 3 correlated with advanced fibrosis in NASH<sup>[84]</sup>. Care must be taken to not confuse this region for a portal tract when there is marked inflammation.

Iron deposition, typically mild, can be noted as punctate granules within reticuloendothelial lining cells and as granules or blush within hepatocytes. It has been reported in 15%-55% of cases<sup>[94]</sup>. Reticuloendothelial iron deposition was associated with steatosis, ballooning, portal inflammation, and fibrosis in a study of 849 patient biopsies from the NASH CRN<sup>[95,96]</sup>. The relationships of iron deposition, hepcidin, iron regulatory genetics, advanced fibrosis and insulin resistance in fatty liver disease are complex and under intense investigation<sup>[96-99]</sup>.

The typical pattern of fibrosis in adult NASH is initially located in zone 3 in the perisinusoidal spaces in a pattern that is described as pericellular. When delicate, it is best appreciated with Masson trichrome or other collagen stains (Figure 1I). As the disease progresses, the fibrosis becomes denser in zone 3 perisinusoidal spaces and, with further progression, portal and periportal fibrosis can be appreciated. At that point, ductular reaction is often present. In time, central-central, central-portal, or portal-portal bridging, architectural remodeling and finally cirrhosis may occur (Figure 1J). In the advanced stages of fibrosis and remodeled architecture, perisinusoidal fibrosis may no longer be present. Cartoon depiction of progression may be seen in texts<sup>[100]</sup>.

## GRADING AND STAGING THE LESIONS OF NAFLD/NASH

In 1999, a semi-quantitative grading and staging system to describe and unify the approach of pathologists to the histopathologic lesions of NASH and fibrosis along with architectural alterations, was proposed by Brunt *et al.*<sup>[101]</sup>. The system was developed from evaluation of 51 liver biopsies of NASH, and followed the broad method recently developed for chronic hepatitis of separating activity (grade) from fibrosis (stage), with the recognition that NASH was not a portal-based process<sup>[102]</sup>. A semi-quantitative activity grade was assigned by a combination of parameters including steatosis, lobular and portal inflammation, and hepatocyte ballooning (Table 1). Fibrosis staging was based on fibrosis patterns of adult NASH, and reflects the progression of fibrosis as well as subsequent architectural remodeling. Figures 1I and J illustrate Stages 1 and 3. The details of staging system can be seen in Table 2.

In 2002 the Brunt grading and staging was revised by

**Table 1 Brunt grading system**

Grade	Steatosis	Ballooning	Inflammation
Mild (1)	1-2 (< 66%)	Minimal	L: 1-2 P: 0-1
Moderate (2)	2-3	Present-zone 3	L: 2 P: 1-2
Severe (3)	2-3	Marked-zone 3	L: 3 P: 1-2

Reproduced with permission<sup>[101]</sup>. Steatosis: grade 1: < 33%; grade 2: > 33%-66%; grade 3: > 66%. Lobular inflammation: grade 1: < 2 foci per 200 × field; grade 2: 2-4 foci per 200 × field; grade 3: > 4 foci per 200 × field. Portal inflammation: grade 0: None; grade 1: Mild; grade 2: Moderate; grade 3: Severe. Ballooning: grade 1: Rare; grade 2: Prominent ballooning. L: Lobular/acinar inflammation; P: Portal inflammation.

**Table 2 Brunt staging system**

Stage	Zone 3 PSF, focal or extensive	Portal, periportal	Bridging	Cirrhosis
1	+	0	0	0
2	+	+	0	0
3	+/-	+/-	+	0
4	+/-	+/-	+/-	+

Reproduced with permission<sup>[101]</sup>. PSF: Perisinusoidal fibrosis.

NASH CRN for use as a feature-based system in clinical trials. The system was published in 2005, and has come to be known as “NAFLD Activity Score (NAS)”<sup>[65]</sup>. The NASH CRN Scoring system includes the entire spectrum of lesions that can be seen in the full range of NAFLD and NASH, including pediatric liver disease (Table 3). The disease activity score represents the unweighted sum of scores for steatosis, hepatocyte ballooning, and lobular inflammation. The fibrosis stage is an expansion of the Brunt scoring, with additional subdivisions to stage 1 (1a-mild perisinusoidal fibrosis, 1b-moderate perisinusoidal fibrosis, and 1c-portal fibrosis only, as occurs in pediatric NAFLD).

Alkhouiri *et al*<sup>[103]</sup> recently published a calculated pediatric NAFLD histological score (PNHS), using the histologic parameters in NAS. The PNHS consists of weighted sum of steatosis, hepatocyte ballooning, lobular inflammation and portal inflammation. The “borderline NASH” category has been eliminated from the pediatric scoring system and high scores are associated with a diagnosis of NASH in this patient group<sup>[103]</sup>. The reader is referred to the reference for the calculation.

Most recently, an algorithmic approach to scoring has been proposed by Bedossa *et al*<sup>[104]</sup> based on over 600 bariatric patient liver biopsies. The SAF (steatosis, activity, fibrosis) system is a sum of scores of steatosis, activity (hepatocyte ballooning + lobular inflammation) and fibrosis. Many details are modeled on the criteria by NASH CRN<sup>[65]</sup>. The SAF system differs from NAS in three major areas: it includes fibrosis into the final score, it excludes steatosis from the activity score and one is able to derive a diagnosis of NASH from a numeric value of SAF. It is noteworthy that the proponents of this system do not take patterns of any of the lesions or any as-

**Table 3 Nonalcoholic steatohepatitis clinical research network nonalcoholic fatty liver disease scoring system**

Steatosis grade	Lobular inflammation	Liver cell ballooning
0: < 5%	0: No foci	0: None
1: 5%-33%	1: < 2 foci per 200 × field	1: Few ballooned hepatocytes
2: 34%-66%	2: 2-4 foci per 200 × field	2: Many ballooned hepatocytes
3: > 66%	3: > 4 foci per 200 × field	

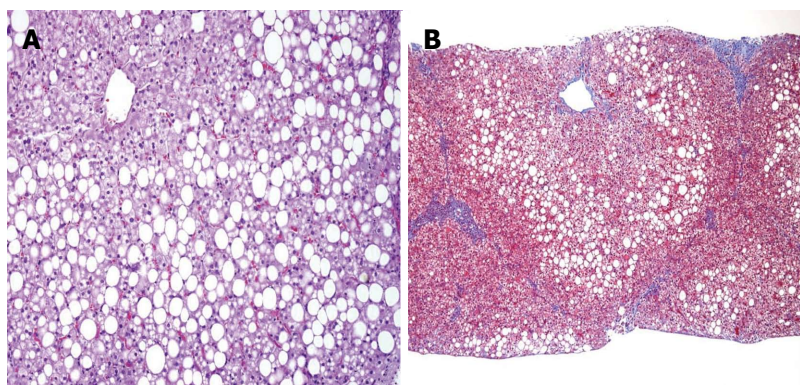
Reproduced with permission<sup>[65]</sup>. Nonalcoholic fatty liver disease activity score (NAS): Steatosis + inflammation + ballooning. Fibrosis, 0: None; 1: Perisinusoidal or periportal; 1A: Mild, zone 3 perisinusoidal; 1B: Moderate, zone 3 perisinusoidal; 1C: Portal, periportal; 2: Perisinusoidal and portal/periportal; 3: Bridging fibrosis; 4: Cirrhosis.

sessments of portal inflammation into account. Further validation of this method in non-morbidly obese subjects is awaited.

In summary, grading and staging systems are useful tools in terms of providing a standard in pathology reporting, monitoring response to treatment and/or progression of disease both in patient care and clinical trials. However, the diagnosis of NAFLD depends on interpreting a variety of histologic findings and patterns and, cannot be replaced or reflected with a single number or score.

## PEDIATRIC NAFLD

One of the most consistent observations of pediatric NAFLD that differed from adults was the difference in distribution of the fat, and the preferential accentuation of portal inflammation and fibrosis (Figure 2A, B). Steatosis is commonly either panacinar or distinctly periportal (zone 1)<sup>[87,105-107]</sup>. From a study that resulted in a hierarchical analysis of 100 pediatric biopsies, Schwimmer proposed two dominant subtypes of pediatric NAFLD: Type 1, the least common, but seen in Caucasian girls and with the similar zone 3 accentuation as with adult NAFLD, and Type 2, the most common, seen more often in boys and characterized by either panacinar or periportal steatosis, portal-predominant inflammation and portal-based fibrosis, and most commonly encountered in Asian, Hispanic or Native American ethnic groups. The remainder of the biopsies were “overlap” or steatosis<sup>[87]</sup>. Carter-Kent *et al*<sup>[108]</sup> studied a large multi-ethnic overweight biopsy population from several North American centers and found less ability to clearly separate the biopsies into discrete patterns with an overlap of the two main patterns in 82% of cases. Nobili *et al*<sup>[109]</sup> also noted more overlap (52.4%) than either Type 1 or 2 in a series of 84 Italian overweight subjects. The NASH CRN refers to zone 1 (periportal) pattern as “borderline, zone 1”, and zone 3 pattern as “borderline, zone 3”, and has found similar ethnic correlations to Patton *et al*<sup>[110]</sup>. Both patterns have been seen in blinded biopsy reviews by the Central Pathology Committee in adult biopsies, albeit in small numbers. Interestingly, to date, there is yet to be a definitive agreement amongst expert



**Figure 2** Pediatric nonalcoholic fatty liver disease. A: Periportal accentuation of steatosis with sparing of zone 3, pediatric nonalcoholic fatty liver disease (NAFLD), HE stain, 200  $\times$ ; B: Portal fibrosis without zone 3 perisinusoidal fibrosis, pediatric NAFLD, Trichrome stain, 100  $\times$ .

pathologists in the field for pediatric NASH histology, unless there are the very same characteristics found in adult NASH, as described above. There is also no knowledge about when or how a transition may occur from pediatric patterns to adult patterns of disease, but an initial retrospective cross-sectional review of 186 NASH CRN biopsies has shown that comparing biopsies from children during prepuberty, puberty and post puberty, there is less steatosis and portal inflammation, but increased steatohepatitis and Mallory-Denk bodies with the change in age<sup>[111]</sup>. These findings are strongly suggestive that with the changes of aging and associated hormonal alterations and shifts of insulin sensitivity, the liver is more susceptible to the injury of increased free fatty acids and lipotoxicity. Natural history studies with prospective biopsies are needed in this growing population.

## DIFFERENTIATION FROM ALCOHOLIC LIVER DISEASE

Some histologic features of NASH and ALD such as steatosis, hepatocyte injury (including ballooning, necrosis and apoptosis, MDBs), and lobular inflammation are shared<sup>[112]</sup>. However, in ALD lobular inflammation may show clusters of PMNs; when present, the lesion is known as “satellitosis”. The lesion is a clue to the presence of MDB, often in apoptotic hepatocytes (Figure 1H). Steatosis is not a diagnostic requirement for ALD. An unusual form of ALD is nearly all microvesicular steatosis and is referred as “alcoholic foamy degeneration”; there is no equivalent described in NASH. Canalicular cholestasis and features of pancreatitis or biliary obstruction (ductular reaction accompanied by marked acute inflammation and edema) can occur in ALD; these lesions have not been described in NASH. Thickening and perivenular fibrosis of terminal hepatic venules and, veno-occlusive lesions are described in ALD<sup>[113]</sup>. Sclerosing hyaline necrosis (obliteration of terminal hepatic venules, hepatocyte necrosis and MDBs) is exclusive to severe alcoholic hepatitis<sup>[112,113]</sup>.

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