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TOPIC HIGHLIGHT

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Histopathology of nonalcoholic fatty liver disease

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Abstract

Histological analysis of liver biopsies remains a standard against which other methods of assessment for the presence and amount of hepatic injury due to nonalcoholic fatty liver disease (NAFLD) are measured. Histological evaluation remains the sole method of distinguishing steatosis from advanced forms of NAFLD, i.e. nonalcoholic steatohepatitis (NASH) and fibrosis. Included in the lesions of NAFLD are steatosis, lobular and portal inflammation, hepatocyte injury in the forms of ballooning and apoptosis, and fibrosis. However, patterns of these lesions are as distinguishing as the lesions themselves. Liver injury in adults and children due to NAFLD may have different histological patterns. In this review, the rationale for liver biopsy, as well as the histopathological lesions, the microscopically observable patterns of injury, and the differential diagnoses of NAFLD and NASH are discussed.

Key words: Fatty liver; Steatosis; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Fibrosis

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INTRODUCTION

Liver biopsy evaluation was the foundation for the proposals that certain individuals who do not consume excess alcohol could, in fact, suffer from a form of chronic progressive liver disease characterized by lesions that had been considered those of alcohol, namely, steatosis, ballooned hepatocytes with Mallory-Denk hyaline bodies, and the particular form of fibrosis of alcohol. Several authors had been evaluating this concept[1-5], when the seminal manuscript of Ludwig *et al*[6] was published in 1980 that described 20 subjects. Since that time, much of the progress that has been made in studies of natural history[7-12], clinical associations of histological assessments of activity and fibrosis, correlations with metabolic syndrome[13,14] and serum markers of cardio-vascular disease[15], the variability of ethnic susceptibility[16-18], and the presence of hepatocellular carcinoma in cirrhotics and non-cirrhotics with nonalcoholic fatty liver disease (NAFLD)[19-21] have occurred by evaluating liver biopsies.

The clinical and scientific needs for imaging studies and serum-based assays to "predict" the presence of steatohepatitis, fibrosis, and/or inflammation are based on a well-recognized concept that liver biopsy is invasive, potentially harmful, and may suffer from "sampling error"[22,23]. Furthermore, liver biopsy cannot be considered

a screening tool for population studies. However, it is also recognized that, imperfect as it is, liver biopsy evaluation remains the standard against which other assays and clinical algorithms must be matched, and prospectively validated.

Liver biopsy does, indeed, suffer from challenges. Many can be overcome by the realization that even an adequate biopsy represents only 1/50 000-1/65 000 of this large organ. Therefore, high quality biopsy techniques, such as utilization of appropriately sized needles[24], careful choice of sampled area, and appropriate tissue preparation and interpretation are all important considerations for liver biopsy. Pathologists recognize that wedge biopsies are inferior to appropriately-sized and placed needle core biopsies for evaluation of a diffuse parenchymal disease. In addition, there might be differences between right and left lobes of the liver, as the left lobe is relatively smaller, is covered by more capsule per unit area, and the subcapsular portal tracts can be deceptively more fibrous than those of deeper parenchyma. Differences in histopathological findings have been demonstrated in studies of bariatric patients biopsied concurrently from the right and left lobes[25]; these differences were abrogated somewhat by the use of a large bore needle in another study[24]. Adequate sample length has also been noted as a correlative factor with histological evaluation in NAFLD[26], with biopsies $\geqslant$ 1.5 cm long having a higher yield of definitive nonalcoholic steatohepatitis (NASH) diagnosis compared to those measuring < 1 cm in length (29% *vs* 56%)[26]. Small biopsies (< 1.6 cm) show higher variability for NAFLD fibrosis stage than longer biopsies[27]. It has been proposed that sampling error might be reduced by using larger gauge needles[24], obtaining longer ($\geqslant$ 1.5 cm) biopsies[28], or by taking more than one tissue core when possible[26]. Once in the laboratory, liver biopsies should not be prepared on tissue "sponges", as these create space-distorting artifacts[29]. Of at least equal, if not greater, significance, however, is pathologists' training and focus in the field. Just as one doesn't ask a non-specialized surgeon to perform cardiac bypass surgery, one should also recognize the need for trained liver pathologists. Studies in chronic hepatitis C (CHC) liver biopsy evaluation that compared the expertise of liver pathologists in academic practice with community pathologists showed this to be a potential source of discordance in histological evaluation[30]. The number of independent readings has also been shown to correlate with greater yield of findings[26].

VALUE OF LIVER BIOPSY EVALUATION IN NONALCOHOLIC FATTY LIVER DISEASE

Pro's and Con's of liver biopsy

The large series of over 350 liver biopsies done for clinically unexplained liver test elevation in adults by Skelly *et al*[31] highlights the value of liver biopsy evaluation in this setting. While two-thirds of the biopsies showed some form of nonalcoholic fatty liver disease, the remainder were either "normal" (6%) or had histopathological features of subsequently clinically confirmed liver disease, such as hereditary hemochromatosis, drug-induced liver injury, and autoimmune liver disease. Several series have now shown the lack of correlation of alanine aminotransferase (ALT) levels and both necroinflammatory activity and fibrosis, including cirrhosis, in subjects with known NAFLD[32-34], including obese children[35]. Liver biopsy evaluation is also important for a patient with a clinical suspicion of autoimmune liver disease. Up to 20% of NAFLD have positive serologies for antinuclear antibody, antismooth muscle antibody, and/or antimitochondrial antibody[36-38]; therefore, liver biopsy evaluation might be the only means of determining the underlying cause of the disease. Furthermore, surgeons have shown that intraoperative visualization is commonly not correct for the prediction of either steatosis or advanced fibrosis, and thus liver biopsy is currently being recommended in the morbidly obese bariatric population at the time of surgery[39].

The final "pro" of liver biopsy is both confirmation of the diagnosis, as well as evaluation and semiquantitation of necroinflammatory lesions and fibrosis, and evaluation of architectural remodeling. To date, noninvasive tests have shown to be effective for the extremes: either lack of, or abundance of, inflammation or fibrosis, but overall sensitivity and specificity for the lesions in between are less impressive[22]. A recently studied modification of the European Liver Fibrosis score, named the Enhanced Liver Fibrosis score, has shown the most promise in terms of predicting over 75% of adult patients who would not need a liver biopsy, regardless of stage[40]. Measurement of serum keratin 18 (K18) fragments, a marker of hepatocyte apoptosis, has emerged as a promising biomarker for NASH, with sensitivity and specificity of up to 77% and 92%, respectively[41]. Transient elastography measuring liver stiffness has been recently shown to be a useful noninvasive test to exclude advanced fibrosis in Chinese NAFLD patients, with a negative predictive value of 97%[42]. Limitations and need for further testing of this modality are reviewed[43].

The arguments against liver biopsy in NAFLD include the invasive nature of the procedure, the lack of effective treatments beyond recommendations for dietary changes, weight loss and exercise, the fact that only a minority of NAFLD patients have the progressive lesions of NASH[44], and the known improvements of diagnosis with clinically-derived tests[45,46] and imaging techniques[47]. However, at present, the final correlation for any of the new tests is liver biopsy evaluation.

HISTOLOGY OF NAFLD IN ADULTS

The histological spectrum of NAFLD includes various forms of small and large droplet macrovesicular steatosis, with or without lobular and portal inflammation, and steatohepatitis, which is characterized by steatosis, inflammation, and cell injury, i.e. NASH. Progression of fibrosis and architectural remodeling is thought to develop in 10%-15% of NASH patients, and cirrhosis

in 15%-25%. Thus, of all individuals with some form(s) of fatty liver, 3%-5% may develop cirrhosis[48]. Cirrhosis due to NASH and cryptogenic cirrhosis might both result in hepatocellular carcinoma (HCC)[19,49]. Rarely, HCC may occur in non-cirrhotic NASH[21,50].

NAFLD

The main histological characteristic of NAFLD, as its name implies, is the accumulation of fat in the form of triglycerides within hepatocytes, a lesion originally termed "steatosis" after the ancient Greek word for fat, "stear". The presence of > 5% steatotic hepatocytes in a liver tissue section is now accepted as the minimum criterion for the histological diagnosis of NAFLD[51,52].

Steatosis in NAFLD is usually macrovesicular, referring to hepatocytes with a single large intracytoplasmic fat droplet or smaller well defined droplets displacing the nucleus to the cell periphery. Mixed steatosis might also occur, when, in addition to macrovesicular steatosis, groups of hepatocytes with centrally placed nuclei and numerous minute lipid droplets in the cytoplasm are observed. Pure microvesicular steatosis, needing special stains such as Oil-Red-O to identify the intracytoplasmic material as fat, is not a common feature of NAFLD; however, small, azonal collections of hepatocytes might have this type of fat. In simple NAFLD, in addition to steatosis, foci of lobular inflammation, mild portal inflammation, and lipogranulomas may be seen. However, features of hepatocellular injury and fibrosis, indicating progression to steatohepatitis, are not observed, by definition[51].

The extent of steatosis is commonly evaluated and reported semi-quantitatively. The most reproducible method follows the acinar architecture dividing the liver parenchyma in thirds and assessing percentage involvement by steatotic hepatocytes: 0%-33% (or 0%-5%, 5%-33%)-mild, 33%-66%-moderate or > 66% - severe steatosis[53,54].

One of the distinctive features of steatosis in adult NAFLD, in contrast to most pediatric NAFLD cases, is its predilection to start in acinar zone 3 (perivenular). When steatosis is severe it can occupy the whole acinus. Steatosis might not persist during the progression of fibrosis to remodeling and cirrhosis; thus, steatosis might not be reliably identified in a cirrhotic liver[55].

Adult NASH

Most hepatopathologists agree that the minimal criteria for the histological diagnosis of adult NASH include steatosis, hepatocyte injury, usually in the form of ballooning, and lobular inflammation, typically localized in acinar zone 3. Fibrosis, as in other forms of chronic hepatitis, is not a required diagnostic feature of NASH[56-58].

Hepatocellular injury in NASH can take the form of ballooning, apoptosis, or lytic necrosis[51]. Ballooning is a feature of major importance in NASH as its presence has been associated in prognostic studies with more aggressive disease and high incidence of cirrhosis[59]. Furthermore, the presence of ballooned hepatocytes on liver biopsy has been linked with features of the metabolic syndrome[60]. Ballooned hepatocytes are enlarged, with swollen, rarefied, pale cytoplasm and, usually, show a large, hyperchromatic nucleus, often with a prominent nucleolus. They might be the result of alterations in intermediate filament cytoskeleton[61] and/or presence of microvesicular steatosis[62]. The histological recognition of hepatocellular ballooning can show significant inter-observer variation[63]. Loss of the normal hepatocyte keratins, 8 and 18, as detected by immunostaining, might help in the objective identification of ballooned hepatocytes[64].

Apoptotic (acidophil) bodies, another form of hepatocyte injury and a feature of programmed cell death, are common in NASH[65]. They can easily be identified on routine stains, but are further highlighted by immunohistochemistry for keratin 18 fragments, the same antigen that has been recently proposed as a biomarker of NASH[41,66]. The number of acidophil bodies per mm^2 of liver tissue (acidophil body index) has been proposed to serve as a complementary histological feature when diagnosis of NASH is uncertain[67].

Lobular inflammation is usually mild, and consists of a mixed inflammatory cell infiltrate, composed of lymphocytes, some eosinophils, and, occasionally, a few neutrophils. Polymorphs are occasionally observed surrounding ballooned hepatocytes in a lesion known as "satellitosis". Foci of chronic lobular inflammation, consisting mainly of lymphocytes, are occasionally seen. Scattered lobular microgranulomas (Kupffer cell aggregates) and lipogranulomas are common[57,68].

Portal chronic mononuclear cell inflammation in adult NASH is not uncommon and is usually mild. When severe or disproportionate to the acinar lesions, the possibility of concurrent CHC should be excluded[56,68,69]. In untreated NAFLD patients, increased portal inflammation has been proposed as a marker of severe disease[70], and has been correlated with the diagnosis of definite steatohepatitis[71].

Fibrosis in adult NASH usually starts in acinar zone 3 and has a characteristic "chicken wire" pattern due to deposition of collagen and other extracellular matrix fibers along the sinusoids of zone 3 and around the hepatocytes. Portal fibrosis without perisinusoidal/pericellular fibrosis has been reported in some cases of morbid obesity-related NASH[72,73] and in pediatric NASH (discussed below). In advanced disease, bridging fibrosis and cirrhosis might develop[51]. NASH-related cirrhosis is most commonly macronodular or mixed. At the cirrhotic stage, perisinusoidal fibrosis and other features of "active" disease may or may not be evident. Therefore, in the absence of a previous biopsy with NASH or other disease-specific histology, the cirrhosis may be labeled "cryptogenic". In a recent meta-analysis of ten longitudinal histological studies of NASH, presence of inflammation in the initial biopsy and age emerged as independent predictors of progression to advanced fibrosis in patients with NASH[74].

Sinusoidal collagen formation in NASH, as in other chronic liver diseases, is likely to be the result of hepatic stellate cell (HSC) activation[75,76]. The HSC activation score, as measured by alpha-smooth muscle actin immunohistochemistry, was shown to predict progression of fibrosis in NAFLD[77]. Portal fibrosis, on the other

hand, has been linked to the ductular reaction (i.e. ductular proliferation at the portal tract interface arising from progenitor cells in the periportal area and accompanied by neutrophils and stromal changes). These findings have been correlated with insulin resistance, impaired hepatocellular replication, and advanced stages of fibrosis, indicating that it might provide a pathway for progressive fibrosis[78]. Recent work has found evidence of epithelial-to-mesenchymal transition of ductular cells *via* activation of the hedgehog pathway, a process by which mature epithelial cells differentiate into cells with a mesenchymal phenotype and function, in NAFLD[79].

Other histological lesions that may be seen in NASH include Mallory-Denk bodies (MDB), megamitochondria, glycogenated nuclei, and iron deposition. MDB (previously called Mallory bodies or Mallory's hyaline)[61] are eosinophilic intracytoplasmic inclusions commonly seen close to the nucleus of ballooned hepatocytes in zone 3, usually in areas of perisinusoidal fibrosis. They are composed of misfolded intermediate filaments (keratins 8 and 18), ubiquitin, heat shock proteins, and p62[80]. MDB have been correlated with increased necroinflammatory activity[53] and with a higher incidence of cirrhosis[59]. Although not a requirement for the histological identification of NASH, the presence of MDB strengthens the diagnosis. MDB are not, however, restricted to NASH and can be seen in steatohepatitis due to other causes (alcoholic, drug-induced, etc), as well as in chronic cholestasis, metabolic diseases, and hepatocellular neoplasms[81].

Megamitochondria (giant mitochondria) are round or needle-shaped, eosinophilic, intracytoplasmic inclusions more commonly observed in hepatocytes with microvesicular steatosis. The are not zonally restricted[82]. Ultrastructural studies have shown that these abnormal mitochondria show loss of cristae, multilamellar membranes, and paracrystalline inclusions[82,83]. Megamitochondria in NASH may be the result of injury from lipid peroxidation or represent an adaptive change[84]. Glycogenated nuclei are vacuolated nuclei usually observed in periportal hepatocytes. Their presence in biopsies with steatohepatitis is supportive of nonalcoholic etiology (obesity and/or diabetes) because they are very rarely seen in biopsies of alcoholic steatohepatitis (ASH)[85].

Finally, hepatic siderosis might be seen in NAFLD biopsies. It is usually mild (1+, 2+) and occurs in periportal hepatocytes and/or pan-acinar reticulo-endothelial cells. The significance of liver tissue iron deposition and abnormal iron metabolism genetics in the pathogenesis and progression of NAFLD are under investigation[86,87].

HISTOLOGY OF NAFLD IN ADULTS: PATHOLOGIST REPRODUCIBILITY

Reproducibility studies have shown excellent to good agreement for the extent of steatosis, presence of perisinusoidal fibrosis, and stage of fibrosis in adult NAFLD; the feature with the least agreement between histopathologists is lobular inflammation[54,88,89]. Intra-observer agreement is generally better than inter-observer agreement for all histological features and diagnostic categories.

HISTOLOGICAL FINDINGS OF NAFLD/NASH IN CONCURRENCE WITH OTHER LIVER DISEASE

The increased frequency of NAFLD in the general population makes it important for pathologists to recognize the lesions, especially those of NASH, when concurrent with other clinically suspected liver diseases. The frequency of co-existent NASH in a series of > 3000 non-allograft liver biopsies undertaken for clinical suspicion of CHC, autoimmune hepatitis, hemochromatosis, alpha-1-antitrypsin deficiency, or chronic hepatitis B was 2.6%[90]. This number most likely underestimates the actual prevalence of the concurrence given the very stringent criteria for the diagnosis of SH used in the study[56]. Other groups, however, have found similar results[91].

In CHC, which is the most common chronic liver disease diagnosed with concomitant NASH (5%-10%)[90-93], the presence of NAFLD can accelerate disease progression and decrease response to antiviral treatment with negative consequences on patients' prognoses[94,95]. Other studies have shown that co-existent fatty liver or NASH in CHC, more common in patients with genotype 3, was not related to metabolic disturbances[93]. In a subsequent study, however, this same group showed that insulin resistance was increased in CHC, particularly genotypes 1 and 4, and was independently related to advanced fibrosis[94]. Thus, the data for CHC and NAFLD is still under investigation.

The diagnosis of concurrent NASH can be a challenge. One study required the identification of the characteristic zone 3 perisinusoidal fibrosis[90], as this is not a typical finding in other forms of chronic liver disease; another study only required the prominence of steatosis and inflammation in zone 3, along with hepatocellular ballooning[93]. It is important to understand that hepatocellular ballooning, steatosis, and even MDB, have been documented in CHC[96].

Recently, the co-existence of NAFLD and alcoholic liver disease (ALD) has been increasingly recognised in clinical settings. The presence of NAFLD in alcoholic patients has been linked with progression of ALD[97], while some have shown that moderate alcohol consumption is associated with fibrosis progression in NAFLD[98]. Interestingly, in contrast, several studies have shown a beneficial effect of moderate alcohol intake. Moderate alcohol use was shown to be protective against insulin resistance in morbidly obese individuals[73], was associated with decreased prevalence of fatty liver in asymptomatic individuals[99], and with decreased prevalence of steatohepatitis in NAFLD patients[100]. The pathologist is not able to recognize the relative contributions of NAFLD or ALD to the liver injury in a biopsy with steatohepatitis.

HISTOLOGICAL FINDINGS OF RESOLUTION OF NASH

Resolution of the histological lesions of NASH is one of the primary end-points of many treatment trials. It is now apparent that "spontaneous" resolution of liver injury can occur without treatment (i.e. studies of placebo groups)[101-103]. This observation, therefore, indicates the need for adequate sample size as well as randomized controlled trial for treatment trials of NASH.

Improvement of the major histological features of disease activity (i.e. steatosis, lobular inflammation, and ballooning), grade of steatohepatitis and, occasionally, of fibrosis following therapy using different agents, including thiazolidinediones (pioglitazone and rosiglitazone), metformin, vitamins E and C, dietary interventions, or bariatric surgery have been reported[104]. However, after discontinuation of medical treatment, the initial histological improvement might not be maintained, indicating the need for longer (permanent?) duration of therapy[105]. Nevertheless, the results of one long-term treatment trial with the insulin sensitizer roziglitazone showed that there is no additional anti-steatogenic effect with longer treatment, despite an increase in insulin sensitivity[106]. In contrast, reportedly, surgical intervention for obesity appears to have long-lasting beneficial effects on liver histology[107,108].

Histological evaluation of post-treatment liver biopsies in a small trial[102] showed that increased portal inflammation is a feature related to resolution of NASH; a subsequent review of published treatment trials has confirmed this finding, which is independent of the type of therapeutic intervention (medical, dietary, or surgical)[56]. In some cases, successful treatment with resolution is associated with the change in the quality of zone 3 perisinusoidal fibrosis from dense to delicate[102].

HISTOLOGY OF NAFLD IN PEDIATRIC SUBJECTS

Diagnosis of nonalcoholic fatty liver disease in children continues to be a challenge for pathologists. Clinicopathological studies have confirmed that the lesions may or may not resemble those of adults, including cirrhosis with and without steatosis and inflammation[109-119].

Utilizing a mathematic algorithm of the lesions following evaluation of 100 obese children and their liver biopsies, Schwimmer *et al*[120] proposed the currently utilized schema of types 1 and 2 steatohepatitis in pediatric fatty liver disease. Type 1, the least common overall, but the most common in girls, resembles the adult pattern, with zone 3 accentuation of steatosis. Type 2 is more common in boys, and consists of either zone 1 accentuation of steatosis, or panacinar steatosis. Ballooning was uncommon in both types. Zone 3 perisinusoidal fibrosis was uncommon in type 2, whereas, when fibrosis was present, only portal-based fibrosis was seen. A small percent of cases did not fit completely into either category in the Schwimmer study, and were referred to as "overlap". A subsequent clinicopathological series from children from a single site in Rome[112], as well as multiple centers across North America[115], have found types 1 and 2 less common than overlap patterns, and individual features such as zone 3 steatosis, ballooning, and zone 3 perisinusoidal fibrosis less common in children than in adults[121]. However, no author has yet specified the features and/or patterns of injury that would result in an unequivocal diagnosis of "steatohepatitis" in pediatric cases if there were changes other than the adult-pattern. Thus, this is an area of ongoing investigation in pathology. In addition, there are no natural history studies to date showing evolution of the pediatric pattern in children; whether it evolves to the adult pattern with increasing age or not is unknown. The rate and processes involved in progression to cirrhosis, an unfortunate but growing problem[114] in obese children, are also unknown.

HISTOLOGY OF NAFLD IN SPECIAL POPULATIONS

Asians

It is recognized that the body habitus of individuals of Asian descent differs from that of Caucasoids; there is greater body fat for a given body mass index (BMI) in Asians[122], thus, the prevalence and incidence of NAFLD and advanced fibrosis are related to factors other than BMI. However, histological features, as described, do not appear to differ from those described for Caucasian adults.

Bariatric patients

To date, there is no dedicated literature related to the bariatric population, other than straightforward observations of changes of steatosis, inflammation, and fibrosis following surgery. Many studies are done without a specific protocol requiring follow-up biopsies, and most workers do not require pre and post biopsies to be obtained from the same lobe of the liver, or for surgical biopsies to be obtained as early as possible in the operation to avoid the accrual of polymorphonuclear leukocytes within the lobules (i.e. surgical hepatitis). The former is important because of potential differences between the lobes (capsule: parenchyma ratio) and the latter for obtaining correct lobular inflammation scores. The extant literature is reviewed[108] and highlights the fact that the majority of bariatric patients have varying amounts of steatosis. In addition, a recent study of surgeon ability to detect steatosis and/or cirrhosis has shown that this is not reproducible; thus reinforcing the need for liver biopsy in all bariatric patients[123]. To this, the authors would add unpublished personal observations of the non-cirrhotic biopsies from bariatric surgery, that (1) it is common to see enlarged, prominent portal tracts from biopsies obtained from the left lobe; (2) steatosis is commonly mild and often not in zone 3; and (3) zone 3 perisinusoidal fibrosis is uncommon. It is possible that the very low calorie diet that commonly precedes the actual surgical procedure influences

NAFLD and even NASH that may have been present. Finally, a large European study has shown that after five years, if insulin resistance has not improved, fibrosis might actually show progression[124].

NASH IN THE TRANSPLANTED LIVER

NAFLD can occur in the allograft liver as recurrence or as a *de novo* process[125-136].

The incidence of recurrent steatosis in patients transplanted for cryptogenic cirrhosis or NASH-cirrhosis ranges from 25%-100%, while NASH development is observed in 10%-37.5% of these cases without leading to early allograft failure (reviewed in[135]). Recurrent NASH can progress to advanced fibrosis and cirrhosis, but only rare cases have been re-transplanted as a result of decompensation due to severe NASH in the allograft[137].

Development of *de novo* steatosis and/or NASH in the liver allograft might be the result of metabolic disturbances, as transplanted patients are at increased risk for obesity, diabetes, hypertension, and hypercholesterolemia and have decreased physical activity levels. Moreover, anti-rejection medications, in particular steroid treatment and cyclosporine, are known risks for fatty liver development in the allograft, probably by affecting diabetogenic pathways[51,138]. Therefore, the occurrence of NASH in an allograft liver of a patient transplanted for "cryptogenic cirrhosis" does not justify attribution of the original disease to "burned-out" NASH without appropriate clinico-pathological correlation[139-141].

DIFFERENTIAL DIAGNOSIS

Clinico-pathological correlation is essential for highlighting the underlying cause of steatohepatitis in a liver biopsy, as the histology is similar irrespective of etiology. The differential diagnoses of NAFLD and NASH include ASH, drug toxicity (tamoxifen, glucocorticoids, and highly active antiretroviral therapy in human immunodeficiency virus patients), metabolic diseases (such as Wilson disease, tyrosinemia, and citrin deficiency), lipodystrophy, surgical procedures (such as jejunoileal bypass and biliopancreatic diversion), total parenteral nutrition, and malnutrition[51].

ASH vs NASH

The ongoing discussions of the limits of alcohol intake in "nonalcoholic" fatty liver[142] revolve around the complex interactions of host (genetic and metabolic) factors[143,144], the type of alcohol utilized, patterns of drinking[144], and the potentially beneficial interactions of modest alcohol with insulin resistance[73].

Hepatopathologists agree that the histology of uncomplicated NAFLD and alcoholic steatosis are indistinguishable. Further, NASH might be indistinguishable from ASH; however, there are some histological features observed in ALD that have not yet been described in NAFLD. These include sclerosing hyaline necrosis (a combination of dense perivenular fibrosis, MDB accumulation, and hepatocyte necrosis in zone 3), veno-occlusive lesions, alcoholic foamy degeneration (zone 3 microvesicular steatosis), and cholestasis[51]. Generally, there is consensus that the overall histopathological appearance of NASH is milder than that observed in ASH[145], with the understanding that alcoholic steatosis can be as "mild" as NAFLD.

Certain histological lesions, including severe steatosis, glycogenated nuclei, and lipogranulomas, are more frequent in NAFLD compared to ALD; however, their utility in differential diagnosis on an individual basis is limited[145]. On the other hand, numerous, well formed MDB and dense fibrosis, composed mostly of collagen type Ⅲ might be indicative of an alcoholic rather than metabolic origin[146-148]. One group utilized immunohistochemical detection of the insulin receptor (IR) and increased expression of protein tyrosine phosphatase 1B (PTP1B), a protein that acts as a negative regulator of IR expression, to aid in the histological distinction of ASH and NASH. IR receptor was more frequent in NASH in contrast to normal expression for IR in conjunction with low level expression of PTP1B noted in ASH[149]. The clinical utility of this method, however, has not been tested. The same group also proposed a noninvasive model for the differential diagnosis between ALD and NAFLD using the ALD/NAFLD Index (ANI). This is based on the mean corpuscular volume, aspartate aminotransferase (AST)/ALT ratio, BMI and gender. An ANI greater than 0 indicates ALD, while ANI less than 0 is diagnostic of NAFLD[150]. There has been no published validation of this test.

GRADING AND STAGING OF THE HISTOLOGICAL LESIONS IN NAFLD

The first system for grading necroinflammatory activity and staging fibrosis in NASH appeared in 1999[53] in response to the growing need for standardization of the histological criteria used for the diagnosis of NASH and its differentiation from steatosis, and to meet the necessity for the development of a histological scoring system similar to those already used for other forms of chronic hepatitis. In this scoring system, the features that were shown to contribute the most to the severity of the disease, such as steatosis, lobular and portal inflammation, and hepatocellular ballooning were semi-quantitatively assessed to produce a three-tier global grade of activity (Table 1). Staging was based on the characteristic pattern and evolution of fibrosis in NASH with initial involvement of perisinusoidal spaces in zone 3 (stage 1) and subsequent development of portal/periportal fibrosis (stage 2), bridging fibrosis (stage 3), and cirrhosis (stage 4) (Table 1)[53].

Among subsequently published scoring systems for grading histological activity in NAFLD[54,151] and NASH[152], the current most widely used histological scoring system is the one published in 2005 by the NASH Clinical Research Network (CRN), sponsored by the National Institute of Diabetes, Digestive and Kidney Diseases[54]. The NASH CRN system has several advantages: it is a validated system created from blinded reviews by nine pathologists, it is applicable to the entire histological

Table 1 Grading activity and staging fibrosis in nonalcoholic steatohepatitis according to Brunt *et al* (adapted from[53,54])

	Steatosis (1: ⩽ 33%, 2: 33%-66%, 3: ⩾ 66%)	Ballooning (zonal location and severity recorded)	Inflammation[a]	
			L-Lobular [(0-3): 0: absent, 1: < 2, 2: 2-4, 3: > 4 foci]	P-Portal [(0-3): 0: absent, 1: mild, 2: moderate, 3: severe]
Grade 1 (mild)	1-2	Minimal, zone 3	L = 1-2	P = 0-1
Grade 2 (moderate)	2-3	Present, zone 3	L = 1-2	P = 1-2
Grade 3 (severe)	2-3	Marked, predominantly zone 3	L = 3	P = 1-2

[a]Counted in 20 × fields. Stage of fibrosis: 1: Zone 3 perisinusoidal fibrosis, focal or extensive; 2: As above with focal or extensive periportal fibrosis; 3: Bridging fibrosis; 4: Cirrhosis, probable or definite.

Table 2 Nonalcoholic steatohepatitis Clinical Research Network system for scoring activity and fibrosis in nonalcoholic fatty liver disease (modified from[54])

Steatosis grade (S)	Lobular inflammation[a] (L)	Hepatocyte ballooning (B)
0: < 5%	0: None	0: None
1: 5%-33%	1: < 2	1: Few ballooned cells
2: 34%-66%	2: 2-4	2: Many ballooned cells
3: > 66%	3: > 4	

[a]Counted in 20 × fields. Nonalcoholic fatty liver disease Activity Score: S + L + B (range 0-8). Stage of fibrosis (Fibrosis score): 0: None; 1: 1a, mild, zone 3 perisinusoidal fibrosis, 1b, moderate, zone 3 perisinusoidal fibrosis, 1c, portal/periportal fibrosis only; 2: Zone 3 perisinusoidal fibrosis and portal/periportal fibrosis; 3: Bridging fibrosis; 4: Cirrhosis, probable or definite.

spectrum of NAFLD, it scores both adult and pediatric NAFLD biopsies, and it generates a numeric score for grading activity and NAFLD activity score (NAS) for comparing pre- and post-treatment biopsies in therapeutic trials. The NAS derives from the summation of individual scores for steatosis, lobular inflammation, and hepatocellular ballooning and ranges from 0 to 8 (Table 2). In the validation study, NAS of 1 or 2 corresponded to definitely not NASH, while a NAS score 5-8 correlated with definite NASH. Activity scores 3 and 4 were noted in borderline cases that did not fulfill the pathologists' criteria for definite NASH. It is important to note, however, that the numbers generated by NAS were not created to be used as a substitute for histological diagnosis; properly utilized, the NAS is applied by the pathologist after reaching final diagnosis.

Fibrosis scoring with the NASH CRN system is based on the prototype staging method proposed by Brunt *et al*[53], with the difference that stage 1 is further subdivided into three sub-stages to differentiate between delicate perisinusoidal zone 3 fibrosis (stage 1a), dense perisinusoidal zone 3 fibrosis (stage 1b), and portal fibrosis only (stage 1c)[54]. NASH CRN stage 1c refers to the pattern of fibrosis sometimes seen in severely obese patients and in pediatric NASH (Table 2). The value of histological scoring systems for grading and staging in NAFLD in routine practice has not yet been adequately assessed and, to date, they are mainly used in treatment trials and in natural history studies. Furthermore, the recognition of the significance of other histological features associated with definite NASH and advanced fibrosis, such as portal chronic inflammation[70], and the proposed use of serum biomarkers of hepatocyte apoptosis for risk-stratifying patients with NAFLD[41], indicate that the currently used systems could be improved by including these histological features in the semi-quantitative histological scoring of both adult and pediatric NAFLD[58].

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