Nonalcoholic Fatty Liver Disease and the Ongoing Role of Liver Biopsy Evaluation

Elizabeth M. Brunt

Nonalcoholic fatty liver disease (NAFLD) is one of the most common underlying causes of chronically elevated liver tests and liver disease in adults and children worldwide and may be strongly suspected if not diagnosed by ever evolving and available serologic and imaging-based noninvasive tests. However, the definitive diagnosis of the most progressive form of NAFLD, nonalcoholic steatohepatitis, and the identification of fibrosis stage still require liver biopsy evaluation as noninvasive testing has not replaced some of the specifics or the totality of information obtainable from liver biopsy. In this review, both the role and value of a liver biopsy evaluation in NAFLD/ nonalcoholic steatohepatitis are examined from publications related to a selected variety of settings. Details of the most commonly used semiquantitative methods of analysis are discussed, and some useful potential pitfalls for differential diagnostic consideration in liver biopsy interpretation are given. (Hepatology Communications 2017;1:370–378)

Introduction

The role of microscopic liver tissue evaluation in nonalcoholic fatty liver disease (NAFLD), primarily obtained as a needle core biopsy, appropriately continues to be examined as noninvasive modalities become increasingly more competent, available, and cost effective in documenting hepatic steatosis and markers of liver fibrosis. The open-ended discussion concerning lessons learned as well as the ongoing value of liver biopsy evaluation in the foreseeable future are presented from a pathologist’s viewpoint in this review.

Liver Biopsy Evaluation in Patient Care Considerations

Expert reviews[1,2] and International Liver Society clinical practice guidelines for patient care[3-6] recognize that liver biopsy (evaluation) remains the sole, although invasive, procedure to distinguish nonalcoholic fatty liver (NAFL) from nonalcoholic steatohepatitis (NASH) and to establish stage of fibrosis in adults and children.[7] Liver biopsy evaluation is thus considered essential for the diagnosis of NASH, even with the recognized concerns of tissue sampling variability[8] and interobserver pathologist differences.[9,10] Often not discussed but necessarily included in establishing the diagnosis is the less likely but documented possibility of finding alternative nonserologic causes of liver test abnormalities by liver biopsy evaluation. For example, of 354 biopsies for nonserologically diagnosable liver test elevations, 66% were NAFLD, but 12.7% were other significant clinical diseases (primary biliary cholangitis, sarcoid, autoimmune hepatitis [AIH], hereditary hemochromatosis, drug-induced liver injury), 9% were cryptogenic, and 5.9% were considered normal.[11,12] A recent study of 347 children...
10 years or older who were referred to a gastroenterology practice for the evaluation of suspected NAFLD due to overweight/obesity had elevated alanine aminotransferase (ALT) levels, which confirmed the findings. Liver biopsy of 273 children showed NAFLD in 55% of overall referrals, 75% of those biopsied (NASH in 54% of NAFLD), and importantly, other diagnoses (AIH, primary sclerosing cholangitis, alpha one anti-trypsin deficiency, drug-induced liver injury, and other) in 24% of biopsied subjects (18% of overall referrals); three biopsies (1% of biopsies, 0.8% of referrals) were normal. The authors additionally highlighted the value of liver biopsy evaluation for the accurate detection of advanced fibrosis found in 11% of the overall study population and greater in NAFLD than other disease diagnoses. One conclusion of the study was the importance of obtaining liver biopsy in suspected NAFLD in obese children for positive identification and staging of fibrosis or for identification of an alternative potentially reversible diagnosis.

Additionally, liver biopsy evaluation has proven useful in the evaluation of subjects with clinical features of NAFLD in whom nonorgan-specific autoantibodies are present. Anti-nuclear antibodies (ANAs), anti-smooth antibodies, or both or anti-mitochondrial autoantibodies have been reported in adult and pediatric cohorts from around the world. The minority of reports have indicated greater histologic activity and fibrosis and thus a possible relationship to progression of disease; however, others have not shown this, regardless of the demographics of the patient population. Loria et al. suggested a relationship with insulin resistance (IR) in high-titer ANAs and found a small number of cases with overlapping features of AIH and NASH. The largest study, from the NASH Clinical Research Network (CRN) included 864 well-characterized subjects; of the 21% with autoantibodies (ANA ≥ 1:160 or anti-smooth muscle actin ≥ 1:40 or both), the only difference found was an independently associated lower prevalence of moderate to severe steatosis. No other histologic features of NASH, including the diagnosis of definite steatohepatitis, hepatocellular ballooning, lobular inflammation, or advanced fibrosis, differed from those without autoantibodies. As indicated by other investigators, the International Autoimmune Hepatitis Group scoring system may not be effective in distinguishing autoimmune hepatitis from NAFLD/NASH in an individual with autoimmune antibodies and clinical features of NAFLD; it is these individuals who specifically benefit from biopsy analysis prior to initiation of treatment.

In the clinical situations in which serologic evidence for another liver disease coexists with clinical evidence concerning NAFLD/NASH in diseases, liver biopsy evaluation can be informative, if not challenging. De Luca-Johnson et al. reviewed 73 clinical records and baseline biopsies from a single center over a 38-year period from pretreatment type 1 AIH subjects (after exclusions of alcoholic liver disease, hepatitis B virus [HBV], and hepatitis C virus [HCV]); in this cohort, a second review of all the material, including the biopsies, confirmed AIH alone in 70% and found 14% AIH plus coexistent NAFL and 16% AIH plus coexistent NASH. Eighteen percent of the subjects with AIH had cirrhosis compared to 50% of the AIH plus NASH biopsies. Liver-related and all-cause mortality were greater in subjects with AIH plus NASH than with AIH (or AIH plus NAFL).

Prior to the more effective treatments for chronic HCV infection with direct-acting antiviral agents, liver biopsy was often a component in patient care and clinical trials for management of HCV. Contemporaneously, studies investigated the possibility of concurrent disease with fatty liver disease when the field of NASH as a bona fide liver disease was becoming more

ARTICLE INFORMATION:
From the Department of Pathology and Immunology, Washington University School of Medicine, St Louis, MO.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:
Elizabeth M. Brunt, M.D.
Emeritus Professor
Department of Pathology and Immunology
Washington University School of Medicine
Campus Box 8118
St Louis, MO 63110
E-mail: ebrunt@wustl.edu
Tel.: +1-314-362-0143
widely accepted. Careful criteria were warranted, however, as the primary challenge was distinguishing the known 40%-80% steatosis that could be seen in liver biopsies due to HCV from steatosis due to underlying metabolically driven factors related to NAFLD/NASH. Retrospective reviews of two large biopsy databases from the United States showed that up to 5% of subjects with HCV also had NASH. One group suggested the means of separating the shared histologic features of chronic HCV (and any other form of serologically diagnosable liver disease) from NASH by pathology could be based on stricter criteria than those for NAFLD/NASH alone; this was the presence of perisinusoidal fibrosis in zone 3 as this is a location not seen for early fibrosis in most other nonvascular forms of chronic liver disease. Studies confirmed a correlation of HCV plus NASH and more advanced fibrosis compared to HCV alone, with increased weight and diabetes as independent predictors of advanced fibrosis. These are two factors also shown in biopsy-based studies of coexistent NAFLD/NASH and AIH, alcohol, hemochromatosis, and even drug-induced liver injury. Bedossa et al. prospectively studied the specific role of histologically diagnosed steatohepatitis in HCV genotype (gt)1 and gt3. The histologic criteria for diagnosis of steatohepatitis included cytologic evidence of ballooning and zone 3 perisinusoidal fibrosis. One study conclusion was that histologic features of NASH, including ballooning, could occur in HCV gt3 without the commonly related underlying metabolic complications of IR, whereas features of NASH in gt1 were more related to clinical features associated with IR. An extensive body of literature, reviewed by Adinolfi et al., has resulted from clinical observations supporting biopsy results of the complex role of steatosis in HCV in a gt-specific manner in progression of disease, risk of hepatocellular carcinoma (HCC), and response to both conventional and direct-acting antiviral treatments.

The relationship of HBV and hepatic steatosis (NAFLD and NASH) continues to be evaluated but is not yet as clear as that of HCV, even though the presence of NAFLD throughout the world is well known and thus may likely be a concurrent process with HBV. A large volume of literature on the topic exists as investigators continue to probe this problem; a sampling of this is presented. Nonbiopsy-based studies from Asia and Europe have shown discordant results. Wong et al. showed that HBV patients have lower serum triglycerides as well as clinical features of metabolic syndrome, while Pais et al. noted that while steatosis is less commonly detected in chronic HBV than chronic HCV, its presence is associated with greater body mass index, waist circumference, and IR as measured by the homeostasis model assessment score and thus is host derived rather than a viral feature. The 2011 meta-analysis by Machado et al. of 17 histology-based studies of HBV, of which eight also included HCV, showed that the 29.6% prevalence of steatosis in the 4,100 HBV patient population was similar to the prevalence of the overall study populations but lower than in the eight studies that also included 945 subjects infected with HCV. Steatosis in HBV was related to metabolic factors as well as alcohol and male sex. Association with fibrosis, common in HCV, was not seen. The authors suggested that the negative association of steatosis with HBV DNA raised the consideration of a protective effect of HBV. In a recent tissue-based study of 270 well-characterized HBV subjects without alcohol use, Chan et al. documented fatty liver (the presence of steatosis ≥5%) in 39.6% of the subjects. This is in contrast to 13.9% fatty liver in the 91 subjects positive for hepatitis B surface antigen (HBsAg) negative. Fourteen percent of both eAg-positive and eAg-negative subjects had fatty liver by intrahepatic triglyceride measurement, defined as >5%. In the 9 years of follow-up of the biopsied subjects, 11 subjects developed HCC, 9 (82%) of whom had had concomitant fatty liver on biopsy; the actual amount of steatosis was not an associated risk factor. This latter study evaluated several gene polymorphisms associated with fatty liver and/or HCC. The adjusted hazard ratio for developing HCC with fatty liver was 7.27 (95% confidence interval [CI], 1.52-34.76; \( P = 0.013 \)) and with the \( APOC3 \) rs2854116 CT/TT gene polymorphism was 3.93 (95% CI, 1.30-11.84; \( P = 0.013 \)). The \( PNPLA3 \) rs738409 polymorphism, on the other hand, rendered similar results as the prior study in the general population and showed no association with metabolic factors, cirrhosis, or HCC. PNPLA3 rs738409 was, however, associated with the coexistence of fatty liver and positive HBsAg in the biopsy cohort. Possible reasons for the discordance between these two studies include the small number of HBV subjects (91) in the 2012 study and demographic and clinical differences between a...
population accrual study of the former and a retrospective but biopsied population in the 2017 study. A recent study with humanized mice and human liver tissue confirms pathway alterations due to gene expression changes for bile acids, lipids, and cholesterol following HBV binding to its receptor site Na/taurocholate cotransporter, NTCP (SLC10A1). Interestingly, the 2016 American Association for the Study of Liver Disease guidelines for HBV recognize obesity and diabetes as factors for HCC in HBV but do not discuss a role for evaluation of hepatic steatosis. A 2016 European Association for the Study of Liver special conference on HBV likewise did not mention evaluation for hepatic steatosis. The 2016 Asian Pacific Guidelines for HBV, on the other hand, do suggest evaluation for metabolic factors, including hepatic steatosis, and specifically indicate that the latter is not virally induced. It appears the role of liver pathology evaluation in this viral infection may continue as a valuable source of information.

Value of Liver Biopsy in Fibrosis Assessment in NAFLD

Liver biopsy evaluation assessment of fibrosis has become the foundation for prognosis in NAFLD as studies have shown that fibrosis is the feature with which all-cause and liver-related mortality are related. A recent meta-analysis of 1,495 subjects with 17,452 patient years of follow-up has shown exponential increases in the liver-related mortality rate with each histologic stage of fibrosis. This meta-analysis confirmed the significance of the presence of all stages of fibrosis, not just advanced fibrosis, in long-term and liver-related outcomes as had been shown from a longitudinal study of 619 subjects over 12.6 years. Other highly quoted studies have shown the significance of stages 3 and 4 (advanced) fibrosis with adverse overall and liver-related outcomes. The meta-analysis of Dulai et al. also importantly included a recent study of a group of biopsied adult subjects with NAFLD reported from Hong Kong; to date, this is the largest tissue-based outcome study of Asian subjects with NAFLD. Nearly one quarter of the 307 subjects were nonobese (body mass index < 25 kg/m²). While the follow-up time in this last study was less than three of the other four studies of the Dulai et al. meta-analysis (median 4.1 years compared to a median of 5 years, 12.1 years, 12.6 years, and 26.4 years), the result was similar: fibrosis stage was the most significant histologic feature in overall and liver-related outcomes. The nonobese group had more subjects with lower stages of fibrosis but did show the full spectrum of stages of fibrosis. Death and HCC occurred only in the obese subjects, but nonobese and obese alike suffered cardiovascular events, liver-related morbidity, and other types of malignancies, although in fewer numbers. Having established the significance of the presence of fibrosis in fatty liver disease, it is perhaps important that the specifics of determination of fibrosis stage histologically are revisited. Once these are described, it may be more apparent why the challenge(s) of noninvasive markers to actually replace the information obtained from liver biopsy exists. To the pathologist, fibrosis stage not only is the presence of abnormal collagen and other matrix components but also describes the microscopic parenchymal location of the deposition as well as alterations of the hepatic microarchitecture that have occurred due to ongoing tissue inflammation, necrosis, hepatocyte loss, and matrix deposition. Thus, within the four widely accepted stages in NASH, information is relayed regarding amount and location of matrix deposition as well as hepatic vascular relationships. The first site of matrix deposition in adult fatty liver disease is in the perisinusoidal spaces in zone 3; this has been referred to as pericellular or chicken-wire fibrosis because of the exquisite manner in which the matrix appears to outline hepatocytes and form a pattern similar to the eponymous fence. The fibrosis emanates particularly from the perivenular region in zone 3 perisinusoidal spaces rather than in isolated patches and can be seen in several of these zones along a biopsy. The fibrosis may be dense enough to be appreciated solely on the slide stained with hematoxylin and eosin; in the NASH CRN staging system, this is referred to as stage 1b, whereas fibrosis that requires the Masson trichrome, a stain to highlight collagen, is referred to as stage 1a. On rare occasions in adults, but not uncommonly in pediatric NAFLD, fibrosis may first be appreciated as small spikes emanating from enlarged portal tracts; occasional hepatocytes appear to be trapped between connective tissue spikes. This is referred to as stage 1c. Stage 2 includes any form of stage 1 plus the portal/perportal fibrosis described. Stages 3 and 4 are the stages most often referred to as advanced fibrosis, and both involve some amount of bridging fibrosis; in stage 3, this may be focal or extensive. Bridging is a term that applies to...
Liver Biopsy Evaluation in NAFLD: Methods for Assessment

There is ongoing discussion related to the value of histologic features as outcomes in treatment trials for NASH as noted in a recent overview by the lead pathologists of the American NASH CRN and the European Fatty Liver Inhibition of Progression (FLIP) centers. Both have proposed, validated, and published methods for grading and staging the lesions of NAFLD/NASH, the first primarily for use in clinical trials, the second additionally for clinical use. The former method is referred to as the nonalcoholic fatty liver disease activity score (NAS) and does not include the separately reported fibrosis stage. The latter is referred to as steatosis, activity, fibrosis (SAF) and does include the stage of fibrosis.

While the basic histologic lesions focused on and evaluated by both are the same (steatosis, lobular inflammation, ballooning), there are differences between these methods, the most apparent being the use of the system. The NAS (plus fibrosis stage) was created as a means for comparison of biopsies after therapeutic intervention but was not intended to replace the pathologist’s separately reported diagnostic classification of the overall disease process, which relies on patterns of the lesions and injury. The SAF numeric values were created as a means to actually differentiate NAFL and NASH. This will be discussed below. The SAF is reported with subscripts for each component, i.e., \(S_0, 3A_{(0-4)}F_{(0-4)}\), whereas the NAS is reported as a single numeric value, the unweighted sum of steatosis + lobular inflammation + ballooning, \((0-8)\). As can be seen, the SAF/FLIP algorithm does not include the steatosis amount in assessing activity as it is in the NAS; rather, A is a combined score of lobular inflammation \((0-2)\) and ballooning \((0-2)\) for an activity score in the SAF/FLIP algorithm. Thus, A weights the diagnostic category, and specifically a score of \(\geq 1\) is required for ballooning for NASH. The ranges and/or definitions of scores also differ. Lobular inflammation can be \((0-3)\) in the NAS but is \((0-2)\) in the SAF/FLIP
algorithm. Steatosis (0-3) and ballooning (0-2) numeric scores are the same, but definitions of ballooning scores differ. In the NAS, ballooning is scored according to none (0), few (1), or many (2). For the SAF/FLIP algorithm, ballooning is scored none (0), clusters of rounded hepatocytes with pale and possibly reticulated cytoplasm (1), and similar to (1) with some enlarged hepatocytes (2). Both grade steatosis based on percentage of the parenchyma involved (<5% = 0; 5%-33% = 1; 34%-66% = 2; >66% = 3).

As noted, a final diagnostic category can be determined in the SAF/FLIP algorithm by the numerics of the SAF. If \( S > 0 \) and \( A \geq 2 \), with at least 1 point from each of the components (lobular inflammation and ballooning), the diagnosis is NASH.\(^{56,57}\) This method was shown to correlate with serum liver tests when first proposed in a study of morbidly obese subjects\(^{57}\) and resulted in high kappa coefficients for interobserver studies in the validation study of non-morbidly obese biopsies\(^{57}\) and a subsequent study by both academic and community pathologists.\(^{55}\) The first study\(^{57}\) confirmed findings reported by the NASH CRN of lack of concordance in a numeric NAS and diagnosis. The NASH CRN had shown in a study of 976 adult biopsies that the numeric NAS and separately rendered diagnostic category from choices of Definite steatohepatitis, Borderline steatohepatitis (zone 3), or NAFL not steatohepatitis were clearly separate processes and were giving overlapping but also separate information.\(^{59}\) Definite steatohepatitis as a diagnostic category, regardless of the NAS, strongly correlated with features commonly associated with this disease: older age, female sex, elevated ALT, IR calculations, and diabetes. Higher values of NAS (i.e., NAS \( \geq 5 \)) and diagnosis of steatohepatitis were both individually associated with higher liver tests (serum ALT, aspartate aminotransferase) as well as metabolic syndrome defined by the National Cholesterol Education Program.\(^{60}\) diabetes, homeostasis model assessment of insulin resistance, and quantitative insulin-sensitivity check index, but only the diagnostic category of definite steatohepatitis remained associated with all the features tested in a model that included both NAS \( \geq 5 \) and diagnostic category. The primary purpose of the study was to examine the use of only the NAS \( \geq 5 \) as a surrogate for entry criteria into a clinical trial. One of the points made in the study was that using NAS \( \geq 5 \) could miss a diagnosis of definite steatohepatitis in up to 25% of cases; alternatively, definite steatohepatitis was diagnosed in 29% of cases with NAS \( \leq 4 \). An interpretation of these findings is that the process of scoring specific lesions for a study differs from that of evaluating the overall features of injury and their relationships (i.e., pattern) to derive a clinically useful diagnosis. Thus, not only is the presence of the lesions of steatohepatitis important for categorization but also their locations and lesions other than those determined to be important for a score are considered in a diagnostic evaluation. An obvious example is steatosis. Macrophagel vascular steatosis is what is meant by the term steatosis in discussions of NASH. When present in adults, there is often zone 3 accentuation. In many cases of pediatric NAFLD, particularly in boys, steatosis may be accentuated in the periportal regions.

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diagnosis. As the SAF/FLIP algorithm by definition results in diagnostic categories, the final results are numeric. Kleiner and Bedossa\(^{(54)}\) pointed out that while the NAS may show a decrease in numeric value (i.e., improvement), the diagnostic category may actually not be altered from definite steatohepatitis. This was noted in the NASH CRN FLINT\(^{(53)}\) trial. All biopsies were reviewed by the pathology committee blinded to all clinical information, site origination of the material, and/or inclusion in a treatment trial. Forty-five percent of treated subjects met the primary endpoint of histologic improvement (decrease of NAS by \(\geq 2\), no worsening of fibrosis stage) compared with 21% of the placebo group \((P = 0.0002)\). Only 22% of treated subjects (and 13% of placebo) had criteria for resolution of the diagnosis of definite steatohepatitis \((P = 0.08)\), one of the secondary endpoints. For that study, resolution had been defined as a change from baseline diagnosis of definite steatohepatitis to end-of-study diagnosis of Not NAFLD or NAFLD not NASH. Not NAFLD is a relatively straightforward diagnosis as by definition there is <5% steatosis. NAFLD not NASH, may be straightforward steatosis with or without inflammation but can also be more of a challenge. This category can include cases in which either some features (commonly ballooning) or some pattern is not diagnostic; if there is a zonal accentuation of lesions, this can be expressed as borderline zone 3 or borderline zone 1. Thus, there is steatosis by definition of at least 5%; lobular and portal chronic inflammation may be present. There may or may not be hepatocytes that are at most indeterminate for ballooning, but no classically ballooned hepatocytes or ballooned hepatocytes with large ropey Mallory-Denk bodies are present. Fibrosis may or may not be present; delicate strands may represent regression of matrix from prior injury; on the other hand, cirrhotic septa and remodeling may be seen without any ongoing activity. Thus, as mentioned, the lesions are interpreted together for a final diagnostic categorization, and in some situations, while improvement may occur and is documented by a score, the underlying disease category remains unchanged. The significance of this can only be realized with larger groups of subjects exposed over longer periods of time to understand if true efficacy (i.e., lack of progression of disease) has been met by the pharmaceutical intervention.

Pediatric NAFLD is gaining clarification with the aid of liver biopsy evaluation as noted above, but challenges remain for pathologists as there are noted differences in the histologic features between adults and young children. Several of these challenges were discussed in the recent report of the 52-week cysteamine bitartrate delayed release (CyNCh) trial of the NASH CRN.\(^{(65)}\) Even though serum liver enzymes (ALT, aspartate aminotransferase, and gamma-glutamyltransferase) showed rapid, sustained, and significant response to cysteamine bitartrate delayed release capsules compared to placebo, the only equivalent histologic signal was found in subgroup analyses of children \(\leq\) 13 years of age \((43\% \text{ versus } 21\% \text{ in placebo}; \text{relative risk}, 2.3; 95\% \text{ CI}, 1.0-5.2; \text{ } P = 0.04)\) and/or \(\leq 65\) kg \((50\% \text{ versus } 13\% \text{ in placebo}; \text{relative risk}, 4.0; 95\% \text{ CI}, 1.3-12.3; \text{ } P = 0.005)\). Neither children >13 years of age nor >65 kg showed any differences in response compared with appropriate placebo subjects. The authors pointed out the three histologic phenotypes of NAFLD in children recognized to date: a panacinar (diffuse) macrosteatosis pattern, a periportal steatosis pattern with portal chronic inflammation, and a zone 3 pattern more akin to that seen in adults. Relationships of these patterns to age and sex have been noted in cross-sectional studies.\(^{(61,62)}\) Long-term natural history studies based on paired biopsies in pediatrics beyond abstracts have not been published.

In conclusion, liver biopsy evaluation has provided and will continue to provide pertinent information for clinical decision making and care as well as for investigation into pathogenesis, progression, and disease correlates and effectiveness of therapeutic interventions in clinical trials in NAFLD. Focused examples in each of these areas have been discussed in this review.

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