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.[11] 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.[13] 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.[13] 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.[14] 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[15,16] and thus a possible relationship to progression of disease; however, others have not shown this,[17-19] regardless of the demographics of the patient population. Loria et al.[14] 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)[20] 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.[19,21]

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.[21] 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.[22,23] 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.[22] 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.[23-25] These are two factors also shown in biopsy-based studies of coexistent NAFLD/NASH and AIH,[21] alcohol, hemochromatosis, and even drug-induced liver injury.[26,27] Bedossa et al.[28] 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.,[29] has resulted from clinical observations supported by 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[30,31] 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.[32] showed that HBV patients have lower serum triglycerides as well as

clinical features of metabolic syndrome, while Pais et al.[33] 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.[34] 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.[34] In a recent tissue-based study of 270 well-characterized HBV subjects without alcohol use, Chan et al.[35] 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 in their prior proton magnetic resonance spectroscopy study of 1,013 subjects from the general population (after exclusion of alcohol). None of the subjects with HBV had advanced fibrosis by elastography, and 85% were hepatitis B e antigen (HBeAg) negative. Fourteen percent of both eAg-positive and eAg-negative subjects had fatty liver by intrahepatic triglyceride measurement, defined as >5%.[32] In the 9 years of follow-up of the biopsied subjects,[35] 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[32] and showed no association with metabolic factors, cirrhosis, or HCC. *PNPLA3* rs738409 was, however, associated with the coexistence of fatty liver and positive HBeAg 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.[35] 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).[36,37]

Interestingly, the 2016 American Association for the Study of Liver Disease guidelines for HBV[38] 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.[39] 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.[40] 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.[41,42] 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.[43] 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.[41] Other highly quoted studies have shown the significance of stages 3 and 4 (advanced) fibrosis with adverse overall and liver-related outcomes.[44-46] The meta-analysis of Dulai et al.[47] 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,[46] 12.1 years,[44] 12.6 years,[41] and 26.4 years[45]), 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.[47]

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 chickenwire 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,[48] 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/periportal 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

septum formation between vascular structures within the hepatic parenchyma; thus, septal formations may occur between terminal hepatic venules or between terminal hepatic venules and portal tracts or between portal tracts. All three of these result in differing architectural alterations and potentially differing amounts of parenchymal nodularity. The septa themselves may be delicate strands of connective tissue but more often are actually larger regions comprised of confluent parenchymal extinction[49] that may contain intact portal tracts embedded in scar tissue, numerous and dilated lymphatics, and mixed chronic inflammation. When septa surround and intersect the residual hepatic parenchyma, typically giving the overall appearance of nodules, stage 4 (cirrhosis) is diagnosed. If the terminal hepatic venule has been incorporated into a septum, it will no longer be microscopically observable; occasionally, a residual terminal hepatic venule can be identified within a nodule. Stage 3 is a continuum in the process of bridging fibrosis from incomplete to nearly complete remodeling of the hepatic architecture, but the numeric alone gives little to no information related to the extent of bridging, size of septa, or architectural alterations.

The significant contributions that liver biopsy evaluation has offered in the maturing years of NAFLD research have been long-term observations of fibrosis. There is now hard evidence not only that NAFL/NASH is a potentially progressive process but also, through clinical reports, clinical trials, and meta-analysis, that fibrosis can progress but may also regress. Rates of progression and concepts of fast and slow progressors have been introduced[50,51]; however, not only did the studies chosen for the meta-analysis show progression of fibrosis, 11 of 12 studies showed a small proportion of subjects who remained stable over the observation period and 9 of 12 studies had cases that showed improvement. There were insufficient data available for histologic correlations with improvement (or progression). An abstract from the NASH CRN recently showed improvement in fibrosis regardless of the treatment arm in 38.5% of the 221 subjects in the Pioglitazone Versus Vitamin E Versus Placebo for the Treatment of Nondiabetic Patients With Nonalcoholic Steatohepatitis (PIVENS) trial; resolution of the diagnosis of steatohepatitis (see below) had the strongest odds ratio (OR) of all histologic features associated with the improvement in fibrosis (OR, 3; 95% CI, 2.0-7.6; $P < 0.001$), followed by a nonalcoholic fatty liver disease activity score (NAS) decrease ≥ 2 (OR, 2.4; 95% CI, 1.3-4.3; $P = 0.003$).[52] As in PIVENS, improvement in fibrosis was noted in the Farnesoid X

nuclear receptor ligand obeticholic acid for noncirrhotic, nonalcoholic steatohepatitis trial (FLINT)[53] in both the treated (35%) and placebo (19%) arms ($P = 0.004$). Evaluation of histologic associations as noted in PIVENS is ongoing.

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.[54] Both have proposed, validated, and published methods for grading and staging the lesions of NAFLD/NASH,[55,56] the first primarily for use in clinical trials, the second additionally for clinical use. The former[55] 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.[56,57] While the basic histologic lesions focused on and evaluated by both are the same (steatosis, lobular inflammation, ballooning), there are differences between these methods,[58] 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., $S_{(0-3)}A_{(0-4)}F_{(0-4)}$, 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 ≥ 1 is required for ballooning for NASH.[57] 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 ≥ 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 ≥ 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 ≥5 and diagnostic category. The primary purpose of the study was to examine the use of only the NAS ≥5 as a surrogate for entry criteria into a clinical trial. One of the points made in the study was that using NAS ≥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 ≤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. Macrovesicular steatosis is what is meant by the term steatosis in discussions of NAFLD. 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. These differences in pattern have been informative in developing an understanding of pediatric NAFLD, discussed below.[61-63] Another example commonly found in liver biopsies for numerous clinical indications is portal chronic inflammation. This lesion is not present in either the NAS or the SAF/FLIP algorithm for scoring (or diagnosing) NASH but is usually present to some extent in NAFLD. If portal chronic inflammation is marked or if it is duct centered, pathologists commonly raise queries concerning concomitant HCV[64]; if there is prominent interface activity, an evaluation of many forms of chronic viral or nonviral hepatitis may be pursued. If portal inflammation is comprised predominantly of acute inflammatory cells, further evaluation of the biopsy for possible biliary disease may be warranted, particularly if ductular reaction with polymorphs is also present. The presence of Mallory-Denk bodies within zone 3 perivenular hepatocytes greatly eases the considerations of ballooned hepatocytes if in fact steatohepatitis is the diagnosis under consideration. If, however, the Mallory-Denk bodies are noted in periportal hepatocytes, differential diagnostic considerations of chronic cholestasis or Wilson disease must occur. Further, if the Mallory-Denk bodies are seemingly in apoptotic hepatocytes surrounded by polymorphonuclear leukocytes, alcoholic hepatitis enters the differential diagnoses. Thus, without detailing further examples, it is location, surrounding features, and of course clinical input that are essential for appropriate assignation of any given lesion from a liver biopsy.

The approaches to endpoint analyses for histologic evaluation in clinical trials aim at evaluating the effects of the intervention: improvement, no change, or worsening. These can apply to the individual components of the disease, such as can be done by use of the NAS (plus fibrosis stage), SAF/FLIP algorithm, or diagnostic categories, or any combination of score and

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 trial.[53] 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 ≥ 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 ≤ 13 years of age (43% versus 21% in placebo; relative risk, 2.3; 95% CI, 1.0-5.2; $P = 0.04$) and/or ≤ 65 kg (50% versus 13% in placebo; relative risk, 4.0; 95% CI, 1.3-12.3; $P = 0.005$). 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.

REFERENCES

1) Rinella ME, Sanyal AJ. Management of NAFLD: a stage-based approach. Nat Rev Gastroenterol Hepatol 2016;13:196-205.

2) Rinella ME. Nonalcoholic fatty liver disease: a systematic review. JAMA 2015;313:2263-2273.

3) Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. Hepatology 2012;55:2005-2023.

4) European Association for the Study of the Liver (EASL); European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. J Hepatol 2016;64:1388-1402.

5) Duseja A, Singh SP, Saraswat VA, Acharya SK, Chawla YK, Chowdhury S, et al. Non-alcoholic fatty liver disease and metabolic syndrome. Position paper of the Indian National Association for the Study of the Liver, Endocrine Society of India,

Indian College of Cardiology and Indian Society of Gastroenterology. J Clin Exp Hepatol 2015;5:51-68.

6) Italian Association for the Study of the Liver (AISF). AISF position paper on nonalcoholic fatty liver disease (NAFLD): Updates and future directions. Dig Liver Dis 2017;49:471-483.

7) Vos MB, Abrams SH, Barlow SE, Caprio S, Daniels SR, Kohli R, et al. NASPGHAN Clinical practice guideline for the diagnosis and treatment of nonalcoholic fatty liver disease in children: recommendations from the Expert Committee on NAFLD (ECON) and the North American Society of Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN). J Pediatr Gastroenterol Nutr 2017;64:319-334.

8) Ratziu V, Charlotte F, Heurtier A, Gombert S, Giral P, Bruckert E, et al. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. Gastroenterology 2005;128:1898-1906.

9) Younossi ZM, Gramlich T, Liu YC, Matteoni C, Petrelli M, Goldblum J, et al. Nonalcoholic fatty liver disease: assessment of variability in pathologic interpretations. Mod Pathol 1998;11: 560-565.

10) Juluri R, Vuppalanchi R, Olson J, Unalp A, Van Natta ML, Cummings OW, et al. Generalizability of the nonalcoholic steatohepatitis Clinical Research Network histologic scoring system for non-alcoholic fatty liver disease. J Clin Gastroenterol 2011;45:55-58.

11) Skelly MM, James PD, Ryder SD. Findings on liver biopsy to investigate abnormal liver function tests in the absence of diagnostic serology. J Hepatol 2001;35:195-199.

12) Bianchi L. Liver biopsy in elevated liver function tests? An old question revisited. J Hepatol 2001;35:290-294.

13) Schwimmer JB, Newton KP, Awai HI, Choi LJ, Garcia MA, Ellis LL, et al. Paediatric gastroenterology evaluation of overweight and obese children referred from primary care for suspected non-alcoholic fatty liver disease. Aliment Pharmacol Ther 2013;38:1267-1277.

14) Loria P, Lonardo A, Leonardi F, Fontana C, Carulli L, Verrone AM, et al. Non-organ-specific autoantibodies in nonalcoholic fatty liver disease: prevalence and correlates. Dig Dis Sci 2003; 48:2173-2181.

15) Adams LA, Lindor KD, Angulo P. The prevalence of autoantibodies and autoimmune hepatitis in patients with nonalcoholic fatty liver disease. Am J Gastroenterol 2004;99:1316-1320.

16) Niwa H, Sasaki M, Haratake J, Kasai T, Katayanagi K, Kurumaya H, et al. Clinicopathological significance of antinuclear antibodies in non-alcoholic steatohepatitis. Hepatol Res 2007;37: 923-931.

17) Cotler SJ, Kanji K, Keshavarzian A, Jensen DM, Jakate S. Prevalence and significance of autoantibodies in patients with non-alcoholic steatohepatitis. J Clin Gastroenterol 2004;38: 801-804.

18) Cho DH, Choi MS, Kim DH, Kim DY, Shim SG, Lee JH, et al. A prospective study on the prevalence and clinical significance of autoantibodies in patients with suspected nonalcholic fatty liver disease. [in Korean] Korean J Hepatol 2005;11:261-267.

19) Yatsuji S, Hashimoto E, Kaneda H, Taniai M, Tokushige K, Shiratori K. Diagnosing autoimmune hepatitis in nonalcoholic fatty liver disease: is the International Autoimmune Hepatitis Group scoring system useful? J Gastroenterol 2005;40:1130-1138.

20) Vuppalanchi R, Gould RJ, Wilson LA, Unalp-Arida A, Cummings OW, Chalasani N, et al. Clinical significance of serum autoantibodies in patients with NAFLD: results from the nonalcoholic steatohepatitis clinical research network. Hepatol Int 2012;6:379-385.

21) De Luca-Johnson J, Wangensteen KJ, Hanson J, Krawitt E, Wilcox R. Natural history of patients presenting with

autoimmune hepatitis and coincident nonalcoholic fatty liver disease. Dig Dis Sci 2016;61:2710-2720.

22) Brunt EM, Ramrakhiani S, Cordes BG, Neuschwander-Tetri BA, Janney CG, Bacon BR, et al. Concurrence of histologic features of steatohepatitis with other forms of chronic liver disease. Mod Pathol 2003;16:49-56.

23) Sanyal AJ, Contos MJ, Sterling RK, Luketic VA, Shiffman ML, Stravitz RT, et al. Nonalcoholic fatty liver disease in patients with hepatitis C is associated with features of the metabolic syndrome. Am J Gastroenterol 2003;98:2064-2071.

24) Hourigan LF, Macdonald GA, Purdie D, Whitehall VH, Shorthouse C, Clouston A, et al. Fibrosis in chronic hepatitis C correlates significantly with body mass index and steatosis. Hepatology 1999;29:1215-1219.

25) Ong JP, Younossi ZM, Speer C, Olano A, Gramlich T, Boparai N. Chronic hepatitis C and superimposed nonalcoholic fatty liver disease. Liver 2001;21:266-271.

26) Powell EE, Jonsson JR, Clouston AD. Steatosis: co-factor in other liver diseases. Hepatology 2005;42:5-13.

27) Clouston AD, Jonsson JR, Powell EE. Steatosis as a cofactor in other liver diseases: hepatitis C virus, alcohol, hemochromatosis, and others. Clin Liver Dis 2007;11:173-189.

28) Bedossa P, Moucari R, Chelbi E, Asselah T, Paradis V, Vidaud M, et al. Evidence for a role of nonalcoholic steatohepatitis in hepatitis C: a prospective study. Hepatology 2007;46: 380-387.

29) Adinolfi LE, Rinaldi L, Guerrera B, Restivo L, Marrone A, Giordano M, et al. NAFLD and NASH in HCV infection: prevalence and significance in hepatic and extrahepatic manifestations. Int J Mol Sci 2016;17:pii:E803

30) Loomba R, Sanyal AJ. The global NAFLD epidemic. Nat Rev Gastroenterol Hepatol 2013;10:686-690.

31) Farrell GC, Wong VW, Chitturi S. NAFLD in Asia--as common and important as in the West. Nat Rev Gastroenterol Hepatol 2013;10:307-318.

32) Wong VW, Wong GL, Chu WC, Chim AM, Ong A, Yeung DK, et al. Hepatitis B virus infection and fatty liver in the general population. J Hepatol 2012;56:533-540.

33) Pais R, Rusu E, Zilisteanu D, Circiumaru A, Micu L, Voiculescu M, et al. Prevalence of steatosis and insulin resistance in patients with chronic hepatitis B compared with chronic hepatitis C and non-alcoholic fatty liver disease. Eur J Intern Med 2015;26:30-36.

34) Machado MV, Oliveira AG, Cortez-Pinto H. Hepatic steatosis in hepatitis B virus infected patients: meta-analysis of risk factors and comparison with hepatitis C infected patients. J Gastroenterol Hepatol 2011;26:1361-1367.

35) Chan AW, Wong GL, Chan HY, Tong JH, Yu YH, Choi PC, et al. Concurrent fatty liver increases risk of hepatocellular carcinoma among patients with chronic hepatitis B. J Gastroenterol Hepatol 2017;32:667-676.

36) Oehler N, Volz T, Bhadra OD, Kah J, Allweiss L, Giersch K, et al. Binding of hepatitis B virus to its cellular receptor alters the expression profile of genes of bile acid metabolism. Hepatology 2014;60:1483-1493.

37) Geier A. Hepatitis B virus: the "metabolovirus" highjacks cholesterol and bile acid metabolism. Hepatology 2014;60:1458-1460.

38) Terrault NA, Bzowej NH, Chang KM, Hwang JP, Jonas MM, Murad MH; American Association for the Study of Liver Diseases. AASLD guidelines for treatment of chronic hepatitis B. Hepatology 2016;63:261-283.

39) Lampertico P, Maini M, Papatheodoridis G. Optimal management of hepatitis B virus infection-EASL Special Conference. J Hepatol 2015;63:1238-1253.

40) Sarin SK, Kumar M, Lau GK, Abbas Z, Chan HL, Chen CJ, et al. Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. Hepatol Int 2016;10:1-98.

41) Angulo P, Kleiner DE, Dam-Larsen S, Adams LA, Bjornsson ES, Charatcharoenwitthaya P, et al. Liver fibrosis, but no other histologic features, is associated with long-term outcomes of patients with nonalcoholic fatty liver disease. Gastroenterology 2015;149:389-397.e310.

42) Ekstedt M, Hagström H, Nasr P, Fredrikson M, Stål P, Kechagias S, et al. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. Hepatology 2015;61:1547-1554.

43) Dulai PS, Singh S, Patel J, Soni M, Prokop LJ, Younossi Z, et al. Increased risk of mortality by fibrosis stage in nonalcoholic fatty liver disease: systematic review and meta-analysis. Hepatology 2017;65:1557-1565.

44) Younossi Z, Stepanova M, Rafiq N, Makhlouf H, Younoszai Z, Agrawal R, et al. Pathologic criteria for nonalcoholic steatohepatitis: Interprotocol agreement and ability to predict liver-related mortality. Hepatology 2011;53:1874-1882.

45) Ekstedt M, Hagstrom H, Nasr P, Fredrikson M, Stal P, Kechagias S, et al. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. Hepatology 2015;61:1547-1554.

46) Sebastiani G, Alshaalan R, Wong P, Rubino M, Salman A, Metrakos P, et al. Prognostic value of non-invasive fibrosis and steatosis tools, hepatic venous pressure gradient (HVPG) and histology in nonalcoholic steatohepatitis. PLoS One 2015;10: e0128774.

47) Leung JC, Loong TC, Wei JL, Wong GL, Chan AW, Choi PC, et al. Histological severity and clinical outcomes of nonalcoholic fatty liver disease in nonobese patients. Hepatology 2017; 65:54-64.

48) Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313-1321.

49) Wanless IR, Wong F, Blendis LM, Greig P, Heathcote EJ, Levy G. Hepatic and portal vein thrombosis in cirrhosis: possible role in development of parenchymal extinction and portal hypertension. Hepatology 1995;21:1238-1247.

50) Singh S, Allen AM, Wang Z, Prokop LJ, Murad MH, Loomba R. Fibrosis progression in nonalcoholic fatty liver vs nonalcoholic steatohepatitis: a systematic review and meta-analysis of paired-biopsy studies. Clin Gastroenterol Hepatol 2015;13:643-654.e1-9.

51) Harrison SA. Nonalcoholic fatty liver disease and fibrosis progression: the good, the bad, and the unknown. Clin Gastroenterol Hepatol 2015;13:655-657.

52) Brunt EM, Kleiner DE, Wilson L, Unalp-Arida A, Neuschwander-Tetri BA. Histologic predictors of improvement of fibrosis in NASH: results from the NASH Clinical Research Network PIVENS trial (abstract). Hepatology 2012;56:1480A.

53) Neuschwander-Tetri BA, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, et al. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. Lancet 2015;385:956-965.

54) Kleiner DE, Bedossa P. Liver histology and clinical trials for nonalcoholic steatohepatitis-perspectives from 2 pathologists. Gastroenterology 2015;149:1305-1308.

55) Kleiner D, Brunt E, Van Natta M, Behling C, Contos M, Cummings O, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005; 41:1313-1321.

56) Bedossa P; FLIP Pathology Consortium. Utility and appropriateness of the fatty liver inhibition of progression (FLIP) algorithm and steatosis, activity, and fibrosis (SAF) score in the evaluation of biopsies of nonalcoholic fatty liver disease. Hepatology 2014; 60:565-575.

57) Bedossa P, Poitou C, Veyrie N, Bouillot JL, Basdevant A, Paradis V, et al. Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. Hepatology 2012;56:1571-1579.

58) Brunt EM. Nonalcoholic fatty liver disease: pros and cons of histologic systems of evaluation. Int J Mol Sci 2016;17:pii:E97.

59) Brunt EM, Kleiner DE, Wilson LA, Belt P, Neuschwander-Tetri BA; NASH Clinical Research Network (CRN). Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. Hepatology 2011;53:810-820.

60) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive summary of the third report of the National Cholesterol Education Program (NCEP) Expert Panel On Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). JAMA 2001;285:2486-2497.

61) Schwimmer JB, Behling C, Newbury R, Deutsch R, Nievergelt C, Schork NJ, et al. Histopathology of pediatric nonalcoholic fatty liver disease. Hepatology 2005;42:641-649.

62) Suzuki A, Abdelmalek MF, Schwimmer JB, Lavine JE, Scheimann AO, Unalp-Arida A, et al. Association between puberty and features of nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol 2012;10:786-794.

63) Africa JA, Behling CA, Brunt EM, Zhang N, Luo Y, Wells A, et al. In children with nonalcoholic fatty liver disease, zone 1 steatosis is associated with advanced fibrosis. Clin Gastroenterol Hepatol 2017; doi: 10.1016/j.cgh.2017.02.030. [Epub ahead of print]

64) Brunt EM, Clouston AD. Histologic features of fatty liver disease. In: Bataller R, Caballeria J, eds. Nonalcoholic Steatohepatitis (NASH). Barcelona: Permanyer, 2007;95-110.

65) Schwimmer JB, Lavine JE, Wilson LA, Neuschwander-Tetri BA, Xanthakos SA, Kohli R, et al. In children with nonalcoholic fatty liver disease, cysteamine bitartrate delayed release improves liver enzymes but does not reduce disease activity scores. Gastroenterology 2016;151:1141-1154.e1149.