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Pathological Assessment for Clinical Trials of Non-Alcoholic Fatty Liver Disease

Tong X[1], Wang Q[1], Sun Y[1], Chen S[1], Ou X[1], Jia J[1] and You H[1*]

[1]Liver Research Center, Beijing Friendship Hospital, Capital Medical University; Beijing Key Laboratory of Translational Medicine on Liver Cirrhosis; National Clinical Research Center of Digestive Diseases; 95 Yong-An Road, Xi-Cheng District, China

***Corresponding author:**

Hong You,
Liver Research Center, Beijing Friendship Hospital, Capital Medical University, Beijing Key Laboratory of Translational Medicine in Liver Cirrhosis, National Clinical Research Center of Digestive Diseases, 95 Yong'an Road, Xicheng District, Beijing 100050, China, E-mail: youhongliver@ccmu.edu.cn; sheinchung.chow@duke.edu

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1. Abstract

Non-Alcoholic Fatty Liver Disease (NAFLD) has increasingly become a common cause of liver cirrhosis, hepatocellular carcinoma, and liver-related death. Currently, there are no therapeutic drugs that have proven efficacy and safety through randomized controlled clinical trials. Therefore, there is an urgent need for effective drug therapy to improve clinically relevant endpoints. NASH is a severe form of NAFLD, but the disease progression is slow. Therefore, it would not be practical to observe the hard clinical endpoints. These characteristics pose challenges to the development of new NASH drugs. The selection of an appropriate surrogate endpoint is not only closely related to the clinical outcome but also obtainable in the short term. At present, multiple important international guidance recommends histopathology as the primary therapeutic endpoint of NAFLD's new drug development. However, histology as a surrogate endpoint still has some issues that need to concern, such as consistency, placebo effect, and the association with long-term clinical outcomes. This review will focus on the major concerns of pathological assessment in the development of new NASH drugs.

Non-alcoholic fatty liver disease (NAFLD) is a liver disease related to metabolic syndromes such as obesity, insulin resistance, type 2 diabetes, hypertension, and hyperlipidemia [1]. Recently, a panel of experts proposed that the name of the disease be changed from NAFLD to MAFLD (Metabolic associated fatty liver disease) to better reflect the metabolic pathogenesis [2-3]. The full spectrum of NAFLD is classified as simple fatty liver (non-alcoholic fatty liver, NAFL), non-alcoholic steatohepatitis (NASH), NASH-related liver fibrosis, and cirrhosis [4]. Over time, the incidence and prevalence of NAFLD have increased dramatically. About 25% of the global population is affected by NAFLD [1]. In 2019, the incidence of NAFLD in Asia has reached 29.62% [5]. NASH is a severe form of NAFLD characterized by hepatocellular injury, hepatocyte ballooning, lobular inflammation, and varying degrees of fibrosis [6]. Obviously, NASH is an essential link of liver fibrosis, cirrhosis, hepatocellular carcinoma, and liver-related death, which cause significant health and economic burden on patients and society [1, 7].

To date, there is no approved pharmacotherapy for the treatment of NASH, therefore, the development of new drugs is an urgent clinical need. The trend of disease naming from NAFLD to MAFLD has clarified the pathogenesis and the direction of clinical trials of new drugs. Clinical outcomes are the hard endpoint for evaluating the efficacy of new drugs. However, for patients with NASH, especially those without cirrhosis, the observation of clinical outcomes usually takes many years. Therefore, the rationality of using "surrogate endpoint" in NASH drug research has been affirmed in several international guidance. The "surrogate endpoint" should be able to reflect the clinical outcome and benefit. The pathological manifestations of NASH are related to long-term prognosis. The advanced fibrosis of NASH is closely related to mortality. Based on the close relationship between NASH pathology and clinical outcome, both the U.S. Food

and Drug Administration (FDA) and European Medicines Agency (EMA) have recommended histological evaluation as a surrogate endpoint for the new NASH drug development.

2. Pathological Manifestations and Evaluation Systems of NASH

The main histological features of NASH include steatosis, ballooning of hepatocytes, scattered lobular inflammation [8]. NAFLD has steatosis which is more than 5% by definition. Steatosis of NASH is often of the macrovesicular type with most intense around the central veins (predominantly in zones 2 and 3). The inflammation of NASH is usually mild and nonspecific. Clusters of mononuclear cells including T cells and macrophages infiltrate into hepatocyte plates [9]. Although portal vein inflammation is not necessary for diagnosis, its severity is related to the degree of fibrosis [10, 11]. Ballooned hepatocytes are one of the cardinal features of NASH and essential evidence for NASH diagnosis [12]. Typical ballooned hepatocytes are generally more swelling and larger than the surrounding hepatocytes. The cytoplasm of these hepatocytes appears rarefied or finely reticulate, with or without the presence of Mallory-Deck Bodies. NASH fibrosis usually begins in the peri-central region, showing a typical "chicken wire" pattern. Periportal fibrosis develops after perisinusoidal fibrosis. As the disease progress, eventually, it develops bridging fibrosis and liver cirrhosis.

The pathological evaluation systems of NAFLD and NASH are necessary for disease diagnosis and important efficacy indicators in clinical trials. The currently generally accepted NAFLD/NASH central pathological evaluation systems include the Brunt system [13], the NASH-CRN system [14], the SAF/FLIP system [15], and the Children's NAFLD scoring system [16].

The Brunt system is mainly used as a method for grading the severity of NASH in adults. The NAFLD Activity Score (NAS) scoring system was proposed by the NASH Clinical Research Network (CRN) in 2005 and is now widely used in clinical trials. This system evaluates the disease activity of NASH from three typical pathological manifestations: steatosis, lobular inflammation, and ballooning.

The injury severity of NASH is the sum of the scores of the three components varying from 0 to 8. SAF/FLIP scoring system was developed by the European NASH research team. This system includes steatosis (S, 1-3), activity (A, inflammation and ballooning varies 0-4), and fibrosis (F, 0-4). The SAF/FLIP scoring system evaluates both NASH and Fibrosis, and there is no area of the borderline, so it is widely used in clinical diagnosis.

The pathological characteristics of NASH in children are somewhat different from those in adults. Therefore, the NAFLD children's scoring system was designed to evaluate the severity of NASH and fibrosis in children [16].

3. Histology as an Essential Efficacy Evaluation and Surrogate Endpoint for NASH New Drug Development

The progression of NASH disease is slow, and it takes decades to observe the hard clinical endpoints. Therefore, current new drug research uses surrogate markers to evaluate the efficacy of new drugs. Surrogate markers need to be demonstrated to predict clinically meaningful endpoint results, which can reflect the changes in the disease process and are closely related to the pathogenesis of the disease. Surrogate endpoints usually need to be predictive of clinical benefit, morbidity, or mortality.

Surrogate endpoint based on histology meets the needs of NASH's new drug development. Histology was flexible and sensitive to evaluate the dynamic changing of disease in the short term. The typical manifestations of NASH histopathology provide information for the progression or regression of the disease. Many recent studies have shown that histology manifestation has a close relationship with the long-term prognosis of NAFLD. Large cohort studies based on histological with long-term follow-up have demonstrated that the degree of fibrosis is the essential predictable factor of liver-related mortality [17-20]. The study from Kleiner's team showed that improvement or worsening of disease activity of NAFLD might be associated with fibrosis regression or progression [21]. The activity of NASH is a factor that promotes fibrosis [22, 23].

As a surrogate endpoint for clinical trials of new drugs, histology requires scientific evaluation criteria to reflect the dynamic changes of the disease and predict clinical benefits. In 2018, the U.S. FDA issued a draft guideline for developing therapeutic drugs for NASH associated with liver fibrosis [24]. In this guideline, the FDA recommended that the late phase II program should explore the treatment effect on histological endpoints. A successful phase II program should provide evidence of efficacy on a histological endpoint to support the initiation of phase III trials.

In the phase III development, histology could be used as therapeutic endpoints in patients with moderate or bridging fibrosis (F2 and F3) to accelerate the approval pathway under regulations:

- Resolution of steatohepatitis (on overall histopathological reading) and no worsening of liver fibrosis based on the NASH CRN fibrosis score. Resolution of steatohepatitis is defined as absent fatty liver disease or isolated or simple steatosis without steatohepatitis and a NAS score of 0–1 for inflammation, 0 for ballooning, and any value for steatosis.
- Improvement in liver fibrosis greater than or equal to one stage (NASH CRN fibrosis score) and no worsening of steatohepatitis (defined as no increase in NAS for ballooning, inflammation, or steatosis)
- Both Resolution of steatohepatitis and improvement in fibrosis (as defined above).

In 2019, the joint Report of AASLD and EASL on NAFLD clinical trial endpoints confirmed that histopathology could be accepted as a surrogate endpoint for NASH new drug trials [25]. This report supplements the histological surrogate endpoints of the NASH Phase IIb clinical trial: NASH resolution, fibrosis does not worsen (NAS

score reduction ≥ 2 points, of which at least 1 point is lobular inflammation or ballooning); Or at least one stage reduction in fibrosis and NASH does not worsen (Figure 1).

For clinical trials of patients with compensated cirrhosis, it is still recommended to use endpoint evaluation based on the improvement of clinical outcomes combined with surrogate model end-stage liver disease (MELD) score and hepatic venous pressure gradient (HVPG). However, histology endpoint is still required for the phase IIb/III clinical trials in patients with compensated cirrhosis, which is at least a 1-point improvement in fibrosis with no worsening of NASH [25].

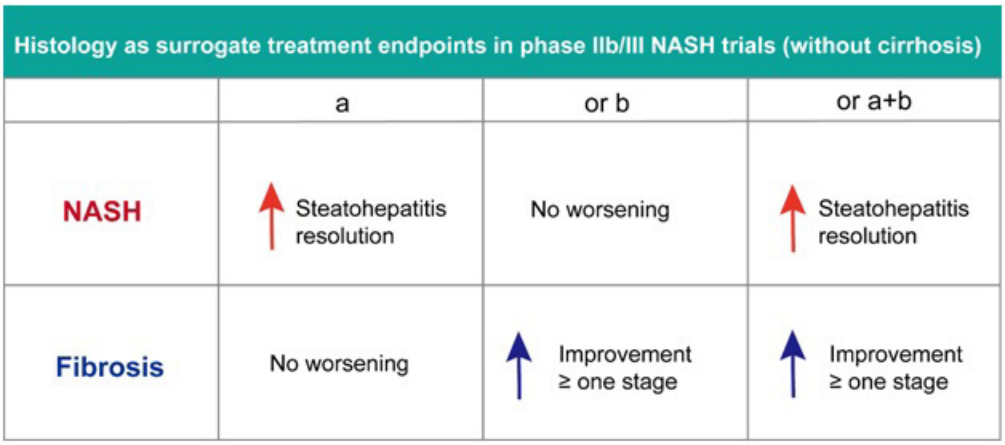

Figure 1: Histology endpoints used in phase IIb/III clinical trials of NASH.

4. Further Considerations with Histology as A Surrogate Endpoint

Histology can be used as a surrogate endpoint for new drug research of NASH mainly based on the relationship between histology and prognosis in previous studies, combined with clinical treatment experience and pathophysiological evidence. Histology is considered to be reasonably possible to predict clinical benefits [26]. However, there is still insufficient evidence to prove that those who have reached the histological surrogate endpoint, the NASH-related liver cirrhosis, decompensation, hepatocellular carcinoma, liver-related death, and all-cause mortality are also synchronously reduced. In the future development of NASH drugs, studies are needed to prove histological improvement and clinical benefits.

Recently, the Department of Hepatology and Nutrition of the FDA issued the current thinking on the development of new NASH drugs [27]. This guidance emphasized that if new drug development is accelerated approved by the histological efficacy endpoint, it is still necessary to conduct a phase IV clinical trial to verify the drug efficacy and clinical benefits. Through the efficacy verification of phase IV clinical trials, the drug can finally be marketed. In addition to evaluating the outcome mentioned above events, patients with NASH without cirrhosis also include progression to cirrhosis [28].

One stage of histological fibrosis improvement is a commonly accepted surrogate treatment endpoint. However, studies have shown that many patients in the placebo group also have histological improvement. That is, patients taking a placebo also have a good performance on the surrogate endpoint. A meta-analysis about placebo effects of clinical trials in NASH showed that about 25% of patients in the placebo group has a more than 2- points increase in NAS score (95% CI, 21%-29%) [29]. The process of participating in clinical trials allows subjects to receive and actively follow dietary recommendations and start exercising, so they can achieve weight loss, which leads to a higher placebo effect than expected.

However, the difference between the experimental drugs and placebo is lower than expected. Therefore, some researchers have proposed whether more stringent standards should be established, such as two stages of fibrosis improvement, to increase the specificity and reduce the placebo effect. However, the more stringent standards may increase the sample size and prolong the time of trial. Also, setting the bar too high for anti-fibrotic treatments may lead to false-negative trials and experts were concerned about using a two stages improvement as a secondary endpoint [30].

Histology is the gold standard for the diagnosis and grading of NASH. Accurate and reproducible interpretation of Nonalcoholic Fatty Liver Disease (NAFLD) histology has significant clinical and research-related implications. However, it still has limitations, such as serious (about 40%) sampling error [31], the insufficiency of sample length and portal areas numbers [32], which may lead to poor pathological evaluation and inaccurate grading. Histological scoring systems do not quantify the absolute amount of fibrosis across the entire liver, diagnose accuracy, therefore, relies not only on a pathologist's judgment but also on the quality of liver biopsy specimens. Another critical challenge is the consistency of diagnosis between different pathologists (inter-group differences), sometimes even inconsistent with their previous diagnosis (intra-group differences).

In a recent study, digitized slides from 678 biopsies for 339 patients with paired biopsies in the MSDC-0602K study were re-evaluated by three hepatopathologists [33]. The inter-reader unweighted kappas were 0.400 for the diagnosis of NASH, 0.396 for NASH resolution without worsening fibrosis, and 0.366 for fibrosis improvement with-

out worsening NASH. The kappa coefficients in this research values of 0.21-0.40, which have been suggested as "fair", meaning better than "slight" (0.01-0.20) but did not reach "moderate" (0.41-0.60) level. About 46.3% of the patients included in the study did not meet the study's histologic inclusion criteria by at least one of the three hepatopathologists. In this study, the inter-reader linearly weighted kappas were 0.609, 0.484, 0.328, and 0.517 for steatosis, fibrosis, lobular inflammation, and ballooning, which was lower than the reports of NASH CRN (steatosis 0.79, fibrosis 0.84, lobular inflammation 0.45, and ballooning 0.56) [14]. Lobular inflammation and ballooning are the two components with the worst consistency. In another research, biopsies from 100 Iranian NAFLD patients were read twice by four pathologists, the inter-observer ICCs for lobular inflammation and ballooning were only 0.288 and 0.012 [34].

Pathological evaluation is crucial for the inclusion criteria and the primary treatment endpoints of NASH trials. However, the inconsistency in the evaluation between pathologists makes it difficult to identify who is the true responder accurately. The low consistency of pathological evaluation may be one of the reasons why many trials of NASH hard to succeed. Detailed information regarding studies in the inter-observer and intra-observer agreement on pathological evaluation of NAFLD is provided in (Table 1).

Table 1: Studies of the inter-observer and intra-observer agreement on pathological evaluation of NAFLD

Studies	Patients, *n*	Pathologists, *n*	Statistic method	Inter-observer agreement				Intra-observer agreement				
				Steatosis	Lobular inflammation	Ballooning	Fibrosis		Steatosis	Lobular inflammation	Ballooning	Fibrosis
Davison[33], *et al*, 2020	339	3	kappa	0.609	0.328	0.517	0.484	Pathologist A (Qualifying vs. Repeated)	0.666	0.227	0.487	0.679
								Pathologist B (Individual vs. Paired)	0.863	0.662	0.840	0.854
Kleiner[21] *et al*, 2019	446	NA	kappa	0.77	0.46	0.54	0.75					
Pavlides[44] *et al*, 2017	65	3	kappa				0.54					
Pournik[34], *et al*, 2014	100	4	ICC	0.654	0.288	0.012	0.504		0.75		0.563	0.744
Gawrieh[45], *et al*, 2011	65	2	kappa	Pre 0.65	0.23	0.28	0.54	Pre	0.72	0.37	0.32	0.64
				Post 0.74	0.2	0.18	0.56	Post	0.75	0.48	0.56	0.75
Kleiner[14] *et al*, 2005	32	9	kappa	0.79	0.45	0.56	0.84		0.83	0.60	0.66	0.85

5. The Accuracy and Reliability of the Pathological Evaluation of NASH Could Be Improved

The U.S. FDA recommends that sponsors should pay more attention to the pathology-related processing procedures in the plan, especially before the Phase III clinical trial. To improve the inter-reader concordance rate for key components of both the NAS score (inflammation, ballooning, and steatosis) as well as the NASH fibrosis score, the FDA emphasizes pathologists' training and recommends an adjudication committee of central pathologists read baseline and treatment slides together to decide how each of the components of the NAS system will be interpreted [27]. The Chinese Food and Drug Administration requires that NASH clinical trials strictly follow the Standard Operation Procedure (SOP) of pathological samples. To reduce the difference in histopathological evaluation, the pathological image should be read in the center. It is recommended that two or more liver pathologists performed double-blind reading [28].

Recently, Artificial Intelligence (A.I.) has been applied in the [29-34] pathological evaluation of liver disease. Sampling size variability of liver biopsy remains a significant limitation in the assessment of liver fibrosis. A Chinese study used a fully quantitative method (second harmonic generation/two-photon excitation fluorescence technology, SHG/TPEF based) to evaluate the "short" liver biopsy samples of chronic hepatitis B patients [35]. They found that the use of SHG/TPEF-based image technology may give useful suggestive information in the evaluation of liver fibrosis for the short sample (biopsy length>0.5 cm). In this way, the A.I.-assisted systems could compensate for the lack of information caused by insufficient liver biopsy length. Another study put SHG/TPEF-based image technology into the fibrosis evaluation of NASH [36]. Automated quantification of fibrosis-related parameters (q-FPs) was used in their study,

and it was found that q-FP is highly accurate in assessing fibrosis in NAFLD patients. The inter-and intra-observer concordance of q-FP could be equal to or more than 0.8. The receiver-operating-characteristic (ROC) curves of any stage of fibrosis versus no fibrosis were 0.88, versus cirrhosis was 0.93. This technology can assist NASH clinical trials to more accurately screen F2-F3 subjects, and it can also help the evaluation of dynamic change of fibrosis in the efficacy judgment.

In the pathological evaluation of NASH clinical trials, the grade of lobular inflammation and ballooning is the most controversial. The consistency of the two features is lower than other parts. To solve this problem, a study used SHG/TPE-based technology to develop and validate a computational algorithm that quantifies key histological features of NASH: fibrosis (qFibrosis), lobular inflammation (qInflammation), ballooning (qBallooning), and steatosis (qSteatosis) [37]. Automated qFIBS analysis outputs showed strong correlation with each respective component of the NASH CRN scoring (qFibrosis [r=0.776], qInflammation [r=0.557], qBallooning [r=0.533], and qSteatosis [r=0.802])) and was able to distinguish differing stages of histological disease.

A European team developed a high-throughput, machine learning-based quantification of the four cardinal pathological components of NAFLD. The interobserver and intraobserver agreement of this software ranged from 0.95 to 0.99, which was higher than that of semiquantitative scoring systems [38]. The application of artificial intelligence may provide a good tool that potentially improves the reproducibility and standardization of pathological assessments required for NASH clinical trials.

6. Correlation of Non-Invasive Tests with Histological Assessment

More and more non-invasive markers are used in clinical and scientific research of NASH. In phase Ib and IIa trials, MRI-PDFF is commonly used as the primary efficacy endpoint to evaluate steatosis. A recent study showed that a higher proportion of liver steatosis (15.7%) measured by MRI-PDFF was associated with a higher risk of fibrosis progression [39]. Another study demonstrated that a relative reduction of 29% in liver fat on MRI-PDFF was associated with a histologic response in NASH [40]. Some non-invasive diagnostic methods based on serum biomarkers, liver stiffness determined by elastography-based methods, including Vibration-Controlled Transient Elastography (VCTE) and Magnetic Resonance Elastography (MRE), have shown a good correlation with pathology [41-43]. However, there is insufficient evidence that non-invasive testing is associated with long-term clinical outcomes of NASH. Those underlying data are still too limited to make these tests sufficiently reliable for pivotal trials. The non-invasive tests cannot replace pathology in IIb/III NASH new drug development; however, non-invasive tests should be captured alongside biopsy to support validation and to mitigate biopsy sampling error if a consistent direction of change was observed in histology and biomarkers (Figure 2).

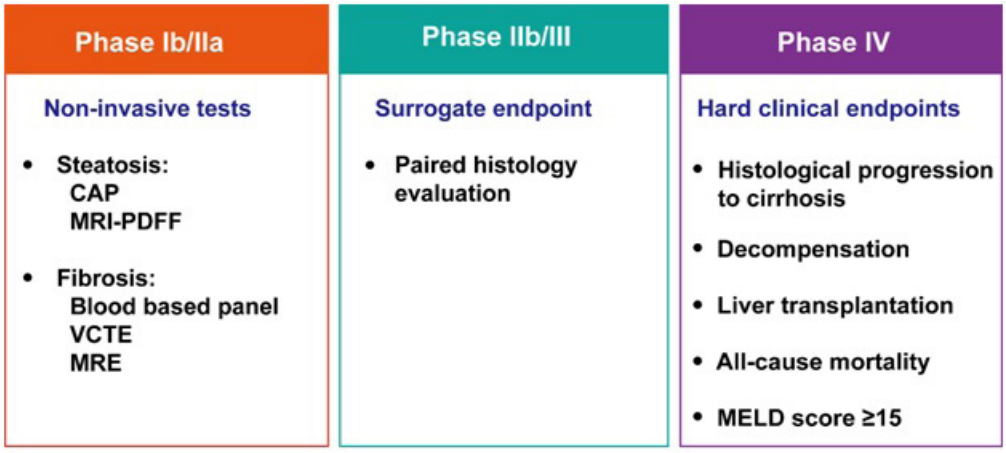

Figure 2: Different phases of NASH clinical trials use different markers as the primary therapeutic endpoint.

7. Conclusions

Although pathological evaluation faces challenges and unresolved problems, it is still essential and irreplaceable in new NASH drugs development. The consistency of NASH pathology evaluation needs to be improved. In addition to central image reading and training, A.I. technology can be used to assist pathologists in reading images. Whether the existing histological improvement by new drugs for NASH can represent real clinical benefits and are related to long-term clinical outcomes still needs to be verified by phase IV clinical trials.

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