

# Pathology of Non-Alcoholic Fatty Liver Disease

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## Introduction

Due to pandemic of over-nutrition and its related metabolic risks including central obesity, glucose intolerance, dyslipidaemia and hypertension, non-alcoholic fatty liver disease (NAFLD) becomes an alarming global public health issue. NAFLD is the most common metabolic liver disease in the world with prevalence of 10-45% in different countries [1, 2]. The prevalence of NAFLD is not significantly different between Western countries, [3] and most Asian countries under the influence of “westernized” sedentary lifestyle. NAFLD leads to substantial morbidity and mortality associated with cirrhosis, hepatocellular carcinoma and cardiovascular disease, and becomes the most rapidly growing indication for liver transplantation [2]. Accurate diagnosis of NAFLD is important to facilitate timely and proper management of patients to minimize morbidity and mortality. NAFLD is composed of a full spectrum of conditions from steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis. Different non-invasive tests, based on clinical, laboratory and radiological tests, have been developed to assess the degree of steatosis, steatohepatitis and fibrosis in NAFLD [2, 4, 5]. Although liver biopsy is an invasive procedure associated with uncommon but severe complications and limited by sampling error, it remains the gold standard for evaluating hepatic pathology in patients with NAFLD, and is recommended in patients with NAFLD at high-risk of steatohepatitis and advanced fibrosis [bridging fibrosis and cirrhosis], and concurrent chronic liver disease of other aetiology [6]. Understanding pathological terminologies of NAFLD is not only important for pathologists in daily diagnostic practice but also essential for hepatologists in communication with pathologists and patients. One should keep in mind that steatosis and even steatohepatitis are not only exceptional to NAFLD and alcoholic liver disease (ALD) but also found in viral hepatitis C, drug-induced liver injury (e.g. methotrexate, tamoxifen, steroid), Wilson disease and various metabolic liver diseases. This review article provides a brief overview of fundamental pathological changes of NAFLD and practical tips for general pathologists.

## Pathological patterns of NAFLD

Many essential data on the natural history, clinical characteristics,

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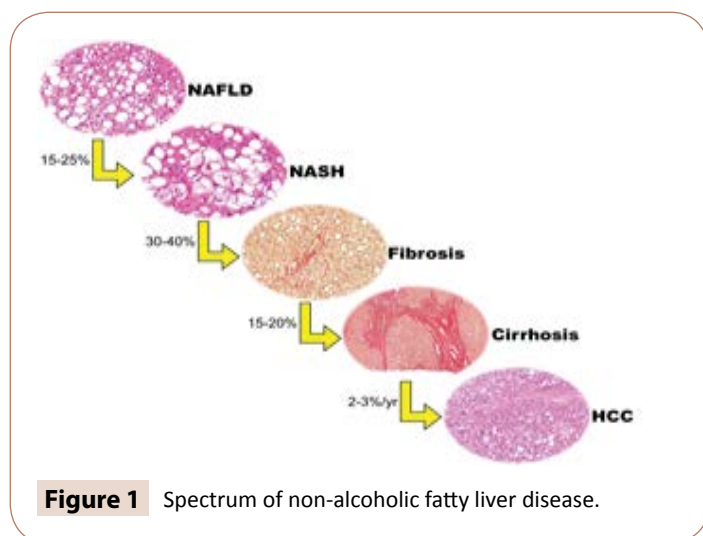
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management and pathological features of NAFLD have been provided by Non-Alcoholic Steatohepatitis Clinical Research Network [NASH-CRN]. NASH-CRN has proposed several histological patterns of NAFLD, which have been already extensively applied in clinical and research settings [7] (**Figure 1**).

## Steatosis with or without inflammation

Hepatic steatosis (fatty change) is the core pathological change of NAFLD and represents the cytoplasmic accumulation of fat droplets, mainly triglyceride, in hepatocytes. The amount of 5% is used as the cut-off differentiating between physiological and pathological steatosis according to studies by lipid content measurement and imaging [8]. Macrovesicular and microvesicular steatosis are two morphological forms of hepatic steatosis. Macrovesicular steatosis is traditionally described as a hepatocyte containing a single large fat droplet pushing the nucleus to the periphery (**Figure 2A**). However, it is not unusual to observe hepatocytes with multiple small to medium-sized fat droplets (**Figure 2B**) in vicinity to those hepatocytes with a single



large fat droplet. A single large fat droplet is believed to be formed by the fusion of multiple small to medium-sized fat droplets [9]. Hence, the term “macrovesicular steatosis” should be broadened to include those hepatocytes with small to medium-sized fat droplets. On the other hand, microvesicular steatosis is featured by the accumulation of much smaller uniform minute fat droplets dispersed throughout the hepatocytes. The hepatocyte with microvesicular steatosis has centrally located nucleus and foamy cytoplasm. Diffuse microvesicular steatosis is typically observed in potentially life-threatening clinical conditions associated with hepatic encephalopathy and acute liver failure secondary to the underlying mitochondrial dysfunction and fatty acid oxidation defect. Reye syndrome, acute fatty liver of pregnancy and acute alcoholic foamy degeneration are classical examples of diffuse microvesicular steatosis. Diffuse microvesicular steatosis is not a pathological feature of NAFLD, whereas focal microvesicular steatosis could be found in 10% of liver biopsies in patients with NAFLD. The presence of focal microvesicular steatosis in background of conventional macrovesicular steatosis in NAFLD is associated with higher disease severity: higher grades of steatosis, hepatocellular ballooning, inflammation and advanced fibrosis [10].

The degree and distribution of steatosis in a liver biopsy should be recorded in the pathological report. Low power histological examination [i.e., at most 10x and usually 4x objective] is sufficient for evaluation. Assessment of steatosis at higher power should be avoided because the severity of steatosis can be overvalued. The degree of steatosis is semi-quantitatively classified in to 3 grades: mild [5 to 33%], moderate (>33 to 66%) and marked (>66%) [11]. The degree of steatosis is correlated with lobular inflammation and centrilobular fibrosis and not associated with hepatocellular ballooning, Mallory-Denk bodies or portal/advanced fibrosis [12]. Predominant zonal distribution of steatosis should be also commented unless steatosis is too mild or the biopsy is too fragmented. There are 4 different patterns of zonal distribution: zone 3 (centrilobular), zone 1 (periportal), panacinar periportal and azonal. Zone 3 and panacinar distribution patterns are usual patterns in adult NAFLD. Predominant zone 1 distribution is rare in adult patients [1%] but more typically in paediatric patients [12%] [11]. Azonal distribution is more commonly found in more severe disease with hepatocellular ballooning, Mallory-Denk bodies and advanced fibrosis [12].

A diagnostic label of “steatosis with inflammation” can be given in the presence of steatosis with lobular and/or portal inflammation. By definition, steatosis with inflammation is not equivalent to steatohepatitis [7]. The inflammatory cells are usually composed of lymphocytes, mononuclear cells and occasionally eosinophils. Compared with ALD, neutrophilic infiltrate is uncommon in NAFLD. Lobular inflammation is featured by small aggregates of macrophages [microgranuloma] or lymphocytes, resembling spotty necrosis in chronic viral hepatitis (**Figure 2C**). In 80% of NAFLD patients, the degree of lobular inflammation is usually mild [i.e., <2 foci/20x] [10]. Portal inflammation, which is a typical pathological feature in chronic viral hepatitis, is usually absent or mild in NAFLD (76% and 77% in adults and children, respectively) [13]. “More than mild” portal inflammation is defined when at least one portal area shows a moderate to marked density of inflammation and/or the presence of lymphoid aggregates. Although its presence is associated with steatohepatitis and advanced fibrosis [13], one should consider other causes of chronic hepatitis, particularly viral hepatitis C which is characterized by moderate/marked portal lymphocytic inflammation with lymphoid follicles and mild steatosis. Predominant portal inflammation exceeding lobular inflammation is more common in paediatric patients [14].

Simple steatosis has been also applied to steatosis with or without inflammation. Simple steatosis is believed to be a benign and non-progressive condition with long-term survival similar to the general population, while steatohepatitis is associated with increased liver-related mortality [15,16]. However, Wong et al. showed that 58% and 28% of patients with simple steatosis had increased disease activity and fibrosis progression in follow-up biopsies in a 3-year interval, respectively [17]. Moreover, hepatic steatosis could induce oxidative fat injury, endoplasmic reticulum dysfunction and abnormalities of the cytoskeleton resulting in hepatocellular ballooning, which is the characteristic feature of steatohepatitis [18]. Although larger studies are still required to clarify that simple steatosis is potentially progressive lesion [19], one should be reminded that simple steatosis is not always quiescent.

## Steatohepatitis

Steatohepatitis is a characteristic pathological pattern featured by steatosis more than 5%, inflammation and hepatocellular ballooning. Hepatocellular ballooning is the hallmark to distinguish steatohepatitis from steatosis with inflammation, and is characterized by cellular swelling, rarefaction of the hepatocytic cytoplasm and clumped strands of intermediate filaments (**Figure 2D**). Substantial accumulation of fat droplets, dilatation of the endoplasmic reticulum and cytoskeletal injury contribute to the formation of ballooned hepatocytes [18].

In the early stage of steatohepatitis, ballooned hepatocytes predominantly found in the centrilobular region. Centrilobular distribution of hepatocellular ballooning disappears in later stage or very active steatohepatitis. Mallory-Denk bodies, as known as Mallory bodies and Mallory hyalines are often found in ballooned hepatocytes. They are deeply eosinophilic, ropey intracytoplasmic inclusions (**Figure 2D**), and represent misfolded protein aggregates composed of primary ubiquitinated cytokeratin 8/18 [CK8/18] and sequestosome 1/p62 [20]. High fat diet contributes to Mallory-Denk bodies through CK8/18 accumulation, CK8

hyperphosphorylation with subsequent transglutaminase 2-mediated CK18 crosslinking [21]. Although identification of ballooned hepatocytes is crucial in establishing the diagnosis of steatohepatitis, there are significant interobserver ( $\kappa$  0.52-0.56 and 0.22 for adult and paediatric cases, respectively) and intraobserver ( $\kappa$  0.62-0.66) variabilities in recognizing hepatocellular ballooning [11, 22, 23]. Ballooned hepatocytes should have a clear, not steatotic, cytoplasm with loss of sharp angles regardless of cell size. By using immunohistochemical stain of CK8/18, ballooned hepatocytes show characteristic loss of cytoplasmic expression of CK8/18, whereas residual immunoreactivity is confined to their Mallory-Denk bodies if present (**Figure 2E**) [24]. However, the identification of hepatocellular ballooning is still relied on conventional H&E section and immunohistochemistry may serve as an ancillary tool in equivocal cases. Although this characteristic CK8/18 is useful to differentiate ballooned hepatocytes in steatohepatitis from hydropic hepatocytes in acute hepatitis, autoimmune hepatitis and chronic viral hepatitis, it is not entirely unique to NASH and ALD and can be shown among hepatocytes with feathery degeneration in chronic cholestatic diseases [24].

## Fibrosis

Fibrosis is a histological parameter signifying chronicity and disease progression. Although it is not one of the diagnostic criteria of steatohepatitis, it is commonly observed in >80% patients with NASH irrespective of age [11]. Centrizonal fibrosis and pericellular/perisinusoidal fibrosis (**Figure 2F**) are the characteristic patterns of fibrosis in fatty liver disease, and represents deposition of fibrous tissue in the space of Disse associated with activation of stellate cells. They are typical in early stage of fibrosis in adult NAFLD/NASH, similar to that in ALD. The fibres tend to be more slender and less marked in NAFLD/NASH than ALD. Periportal fibrosis and bridging fibrosis will be developed as the disease progresses. Eventually, cirrhosis will be established after repetitive hepatic injury, fibrosis, parenchymal extinction and hepatocellular regeneration. In order to properly assess the fibrosis, a good quality connective tissue stain is essential. Common connective tissue stains used in hepatopathology include Masson trichrome, Gordon-Sweets reticulin and Sirius red stains. A good trichrome stain requires an adequate step of differentiation, usually by phosphomolybdic acid. Inadequate or excessive differentiation leads to over- or understaining, which may lead to over- or underestimation of the degree of fibrosis. Sirius red stain is recommended for morphometric quantitation of fibrosis because it provides highly detailed and contrasted staining and is more sensitive in identifying mild perisinusoidal fibrosis [25]. Collagen proportional area [CPA] determined by computer-assisted image analysis could better quantify fibrosis than histological stage, [26] and Sirius red staining for CPA determination was more accurate and reliable for quantifying fibrosis compared with trichrome staining [25]. One pitfall in pathological assessment of fibrosis should be highlighted. The centrizonal fibrotic scar contains aberrant arterioles in about 40% of patients with NASH, and 55% of these abnormally arterIALIZED centrizonal scar contains ductular reaction [27]. Pathologists may potentially misinterpret these arterIALIZED centrizonal regions with ductular reaction as portal tracts, resulting in either missed diagnosis or inaccurate staging of NAFLD/NASH. To prevent this misinterpretation, accurate appreciation of normal liver

histology is essential. In a normal portal tract, a hepatic artery is usually (>90%) accompanied by a nearby (within a distance two to three times that of its diameter) interlobular bile duct of similar diameter [28]. Portal tracts have more organized and uniform collagen and elastin deposition than centrizonal scar regions, and well-demarcated limiting plates separating from periportal hepatocytes. However, arterIALIZED centrizonal fibrous scars do not contain any portal vein, or those arterioles/ductule structures are embedded adjacent to or among hepatocytes without separation from the limiting plate [27].

## Borderline steatohepatitis

Definite steatohepatitis is applied for cases fulfilling all diagnostic features of steatohepatitis typically with a predominantly centrizonal distribution. Borderline steatohepatitis is reserved for those cases immediate between steatosis with/without inflammation and definite steatohepatitis. NAFLD-CRN has mentioned 2 forms of borderline steatohepatitis. The first one is zone 3 borderline steatohepatitis and used for those do not have full-blown unequivocal histological features of definite steatohepatitis, including those cases with characteristic centrizonal/ perisinusoidal fibrosis in absence of hepatocellular ballooning, and those cases with equivocal hepatocellular ballooning. The second one is zone 1 borderline steatohepatitis featured by portal-based injury (periportal steatosis, predominantly portal inflammation and portal fibrosis) [11]. Hepatocellular ballooning is usually absent or minimal. This distinctive form of borderline steatohepatitis is a characteristic histological pattern preferentially found in paediatric patients with NASH [75%]. It is also sometimes referred as type 2 NASH [in contrast to "type 1" NASH in adult] or paediatric NASH in the literature. Boys, younger age, and Asian and Hispanic ethnicity are factors more commonly associated with zone 1 borderline steatohepatitis [14].

## Cryptogenic cirrhosis

Cryptogenic cirrhosis is established after exclusion of viral hepatitis, metabolic, autoimmune and cholestatic liver diseases after an extensive evaluation. It is a common indication for liver transplantation and accounts for 7-14% of patients requiring liver transplantation. NAFLD is one of leading causes of cryptogenic cirrhosis [29]. The prevalence of diabetes mellitus and obesity in patients with cryptogenic cirrhosis is similar to that of patients with NAFLD and far exceeds that of patients with cirrhosis associated with chronic viral hepatitis and autoimmune hepatitis [30]. Steatosis and/or necroinflammatory activity may resolve or "burn out" as disease progresses to advanced fibrosis in patients with NAFLD/NASH. Careful searching for residual hepatocellular ballooning, Mallory-Denk bodies and perisinusoidal fibrosis, as well as clinical correlation with underlying metabolic risks, are helpful to establish a diagnosis of "burnt-out" NAFLD in cryptogenic cirrhosis [31].

## Other pathological lesions in NAFLD

Some pathological changes that are used to classify the pattern of the disease are briefly mentioned here. Lipogranuloma is composed of a loose aggregate of lymphocytes and histiocytes surrounding a fat globule (**Figure 2G**), and can be found in NAFLD, ALD and ingestion of mineral oil in food and medication. Glycogenated nuclei represent the nuclear accumulation of

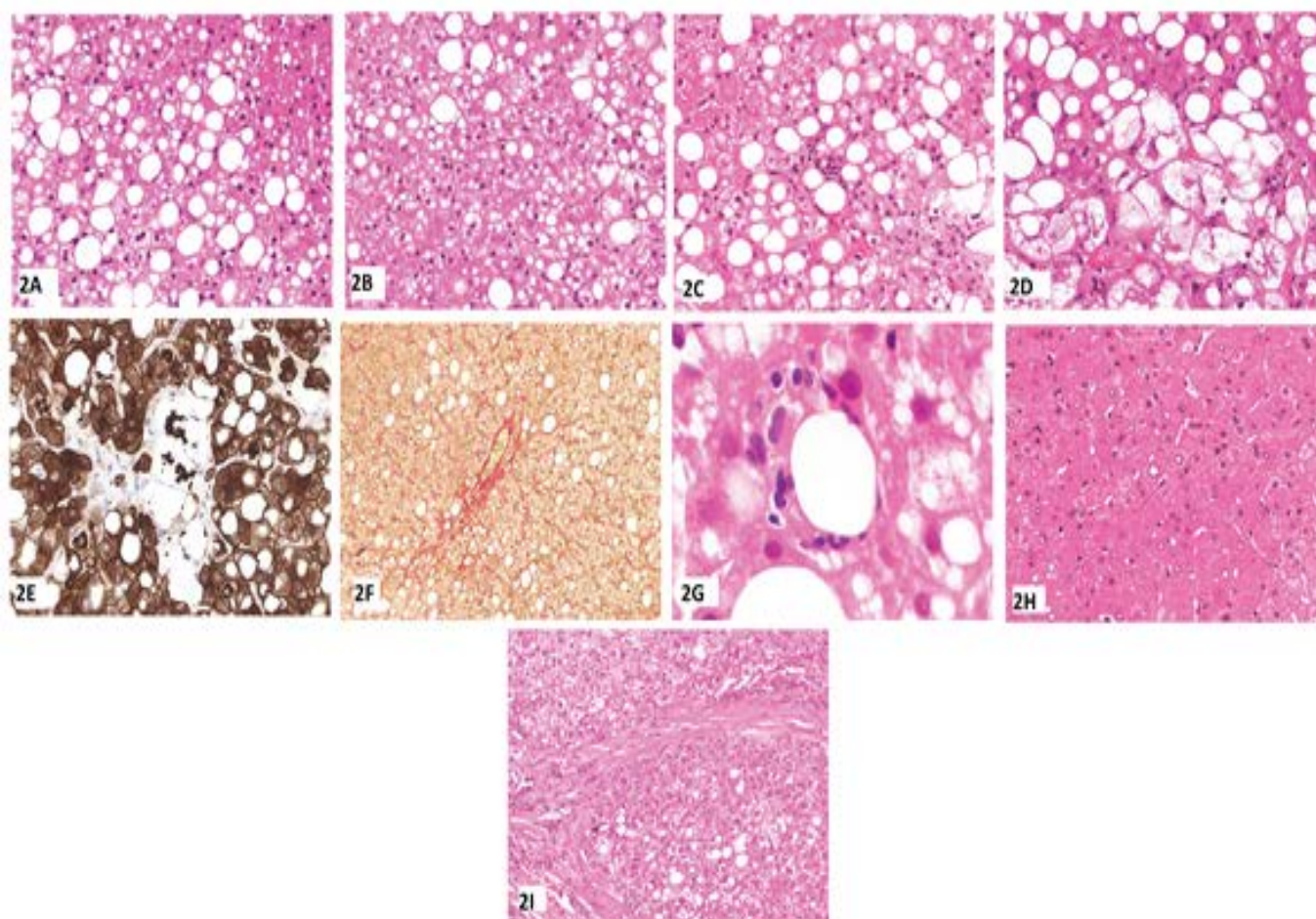
glycogen (**Figure 2H**), and are observed more commonly in NAFLD than ALD. Although they are probably resulted from impaired glucose tolerance or insulin resistance, they are not pathognomic for NAFLD. They may occur normally in children and young adults (11% and 4% in the 20s and early 30s, respectively) [32], and in other liver diseases including glycogen storage disease, Wilson disease, and other copper overload disorders. Giant mitochondria, as known as megamitochondria, are eosinophilic globular or needle-shaped intracytoplasmic inclusions larger than the nucleus of hepatocytes. Although they are usually found in alcoholic and non-alcoholic fatty liver diseases [33], they may be occasionally observed in different physiologic and pathologic conditions including aging, acute fatty liver of pregnancy, glycogen storage disease and urea cycle defects.

Metabolic syndrome is a significant risk factor for hepatocellular carcinoma (HCC; odds ratio 2.13; 95% CI: 1.96-2.31) and

intrahepatic cholangiocarcinoma (odds ratio 1.56; 95% CI: 1.32-1.83) [34]. Patients with NAFLD-related cirrhosis have an increased risk of developing HCC with incidence of 2-3% per year [35]. A recently described histological variant of HCC, steatohepatic HCC, is characterized by HCC exhibiting features of steatohepatitis [steatosis in more than 5% of tumour cells, hepatocellular ballooning, Mallory-Denk bodies, intratumoral inflammatory infiltrate and perisinusoidal fibrosis], and associated with underlying NAFLD and metabolic risks [36, 37] (**Figure 2I**).

### Pathological grading, staging and scoring systems

Grading is measure of disease activity and staging is an indicator of disease chronicity. Grading and staging systems have been used in chronic viral hepatitis for decades to provide semi-quantification assessment of severity and progression of chronic



**Figure 2** [A] Macrovesicular steatosis: Hepatocytes with predominantly a single large-sized fat droplet. [B] Macrovesicular steatosis: Hepatocytes with multiple small to medium-sized fat droplets. [C] Spotty necrosis: A small clusters of small lymphocytes and histiocytes replace hepatocytes. [D] Hepatocellular ballooning: Swollen hepatocytes exhibit rarefaction of the hepatocytic cytoplasm and clumped strands of intermediate filaments. Some clumped strands of intermediate filaments are qualified as Mallory-Denk bodies. [E] Characteristic loss of cytoplasmic expression in ballooned hepatocytes, whereas residual immunoreactivity is confined to their Mallory-Denk bodies [Immunohistochemistry of cytokeratin 8/18]. [F] Centrilobular and perisinusoidal fibrosis [Sirius red stain]. [G] Lipogranuloma. [H] Glycogenated nuclei. [I] Steatohepatic hepatocellular carcinoma.

viral hepatitis [38], and helping in preparing clinical guideline, standardizing pathology reporting and facilitating research studies. The first grading and staging system for NAFLD was proposed by Brunt et al. in 1999. It was derived on liver biopsies from 51 patients with NAFLD. The disease activity grade (0-3) was based by a constellation of histological features including steatosis, lobular and portal inflammation, and hepatocellular ballooning. The fibrosis stage (0-4) was assessed according to fibrosis patterns of adult NAFLD from centrilobular/perisinusoidal to periportal, bridging and cirrhosis [39]. Six years later, NASH-CRN released a revised Brunt's system or NASH-CRN system in 2005 [11]. The disease activity grade, known as NAFLD Activity Score (NAS), was the unweighted sum of scores (0-8) for steatosis (0-3), hepatocellular ballooning (0-2), and lobular inflammation (0-3). In the fibrosis staging, early disease (stage 1) was further divided into 1a (mild perisinusoidal fibrosis visualized by connective tissue stain only), 1b (moderate perisinusoidal fibrosis visualized by H&E section) and 1c (portal/periportal fibrosis only). In a validation study of the NASH-CRN system in 976 patients, cases with NAS of 0 to 2 were largely considered not diagnostic of definite steatohepatitis (99%: simple steatosis 75% and borderline steatohepatitis 24%), whereas most cases with scores of 5 or more were diagnosed as definite steatohepatitis (86%) [40]. Cases with NAS of 3 and 4 were distributed almost evenly between all three patterns: steatosis (27%), borderline steatohepatitis (32%) and definite steatohepatitis (41%). One should be reminded that NAS should not be used as the diagnostic criteria for steatohepatitis (i.e., diagnosis of steatohepatitis only if NAS is 5 or more), although an NAS value of 5 or more has been employed as an inclusion criteria of clinical trials treating patients with NASH [11, 40]. In 2012, Bedossa et al. introduced an algorithm and a scoring system based on a cohort of 679 obese patients receiving bariatric surgery [41]. The FLIP [fatty liver inhibition of progression] algorithm is proposed for segregating lesions into normal liver, NAFLD or NASH by semiquantitative evaluation of steatosis, hepatocellular ballooning, and lobular inflammation. After using this algorithm, the agreement of the diagnosis [NAFLD vs. NASH] increased among expert hepatopathologists from moderate (kappa 0.54) to substantial [kappa 0.66], and much more significantly from fair (kappa 0.35) to substantial (kappa 0.61) among general pathologists received training in liver pathology [42]. The SAF [steatosis, activity, fibrosis] score is the combination of scores of steatosis, activity [hepatocellular ballooning and lobular inflammation] and fibrosis. Compared to the NASH-CRN system, steatosis is no longer a part of the activity score because the prognostic significance of steatosis in disease

progression remains controversial. The clinical application of FLIP/SAF system requires further investigations.

### Limitations of liver biopsy

Although liver biopsy is the gold standard diagnostic tool for NAFLD/NASH, it has a few limitations. First, it is an invasive procedure with the incidence of serious complications and mortality of 0.57% and <0.01%, respectively [43, 44]. Transient discomfort at the biopsy site, pain requiring analgesic, vasovagal attack, and transient mild hypotension are common minor complications. Severe complications include haemoperitoneum, pneumothorax, haemothorax, punctures of other organs and biliary peritonitis. The frequency of complications increases with the number of passes performed, and reduces in experienced hands and under imaging guidance [43]. Second, liver biopsy only samples a small proportion (0.001-0.002%) of the entire liver, and hence sampling error is another limitation. In studies using simultaneous paired biopsies, there were substantial to high agreement (kappa 0.64-0.88) for steatosis grade, fair to high agreement (kappa 0.45-0.87) for hepatocellular ballooning, fair to good agreement (kappa 0.43-0.65) for centrilobular/perisinusoidal fibrosis, and fair agreement (kappa 0.47) for the overall staging [22, 23]. Last but not least, intraobserver and interobserver variability is a substantial problem. The consistency in evaluation of steatosis and fibrosis is good to high among different pathologists (kappa 0.64-0.79 and 0.60-0.84, respectively) and for the same pathologist (kappa 0.74-0.83 and 0.69-0.85, respectively), while the consistency in assessment of hepatocellular ballooning and lobular inflammation is only fair to good among different pathologists [kappa 0.52-0.56 and 0.33-0.45, respectively] and for the same pathologist (kappa 0.62-0.66 and 0.37-0.60, respectively) [11, 22, 23].

### Conclusion

Understanding different pathological patterns of NAFLD is important to establish an accurate diagnosis. Grading and staging systems are valuable tools to providing a standard reference in pathology reporting, monitoring disease progression and therapeutic response in daily practice and clinical trials. We should be reminded that the pathological diagnosis of NAFLD/NASH should be relied on interpreting a constellation of histological findings and patterns, and could not be simply replaced by numeric scores. Systematic histological evaluation, full consideration of clinical and laboratory parameters, and good communications with hepatologists are crucial for making an accurate diagnosis of NAFLD and all other medical liver diseases.

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