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Diagnosis and assessment of NAFLD: definitions and histopathological classification

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Abstract

Non-alcoholic fatty liver disease comprises a spectrum of clinical and histopathological changes including 'simple' steatosis, steatosis with inflammation, steatohepatitis, cirrhosis and hepatocellular carcinoma. It was initially described in the context of drug-induced liver injury and acute liver disease following jejuno-ileal bypass surgery but since the early 1980s it has been widely acknowledged as the hepatic manifestations of the metabolic syndrome. It now represents a burgeoning public health crisis and is fast becoming the main indication for liver transplantation in some parts of the world. Its true incidence and prevalence is unknown although estimates have been made from large imaging studies. Liver biopsy interpretation is still regarded as the gold standard for making accurate diagnoses in NAFLD although sampling limitations are recognized. Furthermore, clear definitions for key histopathological components have been lacking and partly as a result of significant inter-observer variations in making a diagnosis of steatohepatitis. This review considers some aspects of classification and variant forms of NAFLD such as that occurring in children. It provides an update on grading and staging systems and histopathological prognostic factors and, finally, addresses the role of liver biopsy in contemporary clinical care of patients with NAFLD.

Key words: non-alcoholic fatty liver disease, steatosis, steatohepatitis, histopathology, metabolic syndrome

Introduction and historical perspective

Alcoholic liver disease (ALD) is the archetypal fatty liver disease comprising a spectrum of pathological changes from the simple accumulation of lipid in hepatocytes (steatosis) through fat and inflammation (steatohepatitis) to cirrhosis and hepatocellular carcinoma¹. That such a spectrum of disease could also be associated with other conditions was not widely acknowledged until the late 1970s when reports appeared of steatosis and steatohepatitis, histologically indistinguishable from that of alcoholic liver disease, occurring in patients following jejuno-ileal bypass surgery², taking therapeutic agents such as perhexilene³ and in patients that were diabetic and obese⁴. Since then it is recognized that fatty liver disease (FLD) is a common phenomenon in patients with the metabolic syndrome. As with ALD there appear to be host factors that determine the degree (if any) of liver disease with some patients with BMIs greater than 40 showing no steatosis or steatohepatitis. Initially, it was considered that fatty liver disease in the metabolic syndrome was a more indolent process than in alcoholic liver disease with relatively slow progression but metabolically associated FLD is now one of the most common indications for orthotopic transplantation in many centres around the world. Increasingly it is also being seen as the underlying aetiology in the development of hepatocellular carcinoma where it may arise before cirrhosis is established.

The incidence and prevalence of FLD has been assessed by a number of investigators but the true current and projected public health burden from this condition remains uncertain. The situation is confounded by inconsistent histopathological and clinical definitions for diagnosis and the existence of a number of semi-quantitative scoring systems to assess the extent of morphological damage. Furthermore there is often a lack of appreciation of the impact of co-morbid liver disease. This review focuses on the histopathology of non-alcoholic fatty liver disease (NAFLD) and addresses issues of classification and definitions particularly in the context of the metabolic syndrome. It also considers the place of liver biopsies in the contemporary clinical management of patients with NAFLD.

The first use of the term “fatty liver” in the English medical literature is attributed to Thomas Addison from Newcastle upon Tyne, England⁵, better known today for the description of the eponymous disease. Several years later, Karl Rokitansky, one of the forefathers of pathology from Vienna, Austria, observed in autopsy material that fat accumulation in the liver may be aetiologically related to cirrhosis⁶. Addison and Rokitansky most likely referred to alcoholic liver disease but an association of fatty liver with obesity and starvation was made by Bartholow in 1885⁷ and by Statkewitsch in 1894⁸, respectively. Pepper (1884)⁹ was first to describe fatty liver in a diabetic patient and in 1938 Charles Connor highlighted an aetiological link and described in detail FLD and its association with cirrhosis development in diabetics¹⁰. Thereafter, pathologists have recognized similarities between ALD and histological changes in the liver of diabetic and morbidly obese patients in a number of publications in the 1950s, 1960s and 1970s (summarized in Brunt et al 2012¹¹). Ludwig et al first used the term nonalcoholic steatohepatitis (NASH) in 1980 describing 20 patients who did not misuse alcohol but had chronic progressive liver disease with histopathological features of ALD including steatosis, hepatocellular ballooning, Mallory-Denk bodies (MDB) and zone 3 perisinusoidal/pericellular fibrosis. Since then, clinical and research interest in NAFLD has increased considerably with numerous studies documenting its heritability, highlighting the pathophysiological link with features of the metabolic syndrome (central obesity, insulin resistance or diabetes, dyslipidaemia, hypertension), focusing on non-invasive diagnosis of NASH, evaluating the natural history, and developing new pharmacological treatments¹².

Histopathological diagnosis of NAFLD/NASH in the setting of the metabolic syndrome

Steatosis

Hepatic steatosis refers to the accumulation of lipid droplets within hepatocytes and is considered pathological when it affects >5% of hepatocytes¹¹. In adults, it usually first affects acinar zone 3 (centrilobular) hepatocytes, while in children zone 1 (periportal) predilection or a panacinar pattern is more common¹³. Morphologically, steatosis is classified as

macrovesicular, when a large lipid droplet (*large droplet subtype*) or several lipid droplets of variable size (*small droplet subtype*) occupy the cytoplasm and displace the nucleus and organelles to the cell periphery, and *microvesicular*, when the hepatocyte nucleus remains central and numerous minute lipid droplets, difficult to discern by light microscopy, fill the cytoplasm giving it a “foamy” appearance. *Mixed steatosis*, when both macrovesicular and microvesicular types co-exist, is common in NAFLD. In these cases, microvesicular steatosis is seen in small parenchymal patches with a non-zonal distribution and its presence correlates with increased steatosis severity and progressive disease¹⁴. Pure microvesicular steatosis, however, has not been reported in NAFLD to date although it may be diagnosed in alcoholics (so-called alcoholic foamy degeneration) where it may resemble the changes seen in Reye syndrome, acute fatty liver of pregnancy and drug toxicity¹⁵.

Lipid droplets are metabolically active and dynamic organelles composed of a central core of triacylglycerols and/or cholesterol esters and a peripheral single layer of phospholipids with associated proteins that belong to the perilipin/PAT family, including TIP47, MLDP, adipophilin and perilipin¹⁶. Recent studies have shown that during the formation of intracellular lipid droplets these proteins are expressed sequentially with TIP47- and MLDP-positive microvesicular steatosis evolving with time to adipophilin- and perilipin-positive macrovesicular steatosis. The differences in PAT-proteins during lipid droplet evolution may underlie the different clinical significance of the two main types of steatosis and may aid the differentiation between acute and chronic steatosis¹⁷. The rs738409, I148M sequence polymorphism in patatin-like phospholipid domain containing protein (PNPLA3) has recently been seen to correlate with NAFLD development. The inactive PNPLA3 accumulates on the surface of lipid droplets and is associated with an increase in macrovesicular steatosis¹⁸. In NAFLD, the accumulation of triacylglycerols within intracytoplasmic droplets may actually protect hepatocytes from the detrimental effect of non-droplet bound lipotoxic saturated free fatty acids¹⁹.

An important recent observation in NAFLD is that of reticulin loss, a finding more prominent in extensive steatosis and not related to the presence of inflammation or fibrosis²⁰.

The pathophysiological effects of disruption of the connective tissue framework of the sinusoids remain to be determined. Steatosis may not persist as NAFLD progresses and may often be absent in cirrhotic specimens.

Histological assessment of the extent of steatosis is usually semi-quantitative and is based on the percentage of hepatocyte involvement. Most commonly the affected parenchyma is divided in thirds i.e. 5-33%, 33-66%, >66% and consequently the severity of steatosis may be converted into mild, moderate or severe, respectively^{21,22}. Histopathologists have a tendency to overestimate the extent of steatosis, especially when it is severe, therefore more accurate and objective methods for its quantitation have been devised mainly based on digital image analysis (DIA)²³. It has been recently shown that the accuracy of microscopical fat estimation may be increased with the use of guideline images²⁴. Conventional non-invasive imaging methods for assessing hepatic steatosis, such as ultrasound, computed tomography or magnetic resonance imaging (MRI), cannot detect relatively low amounts of hepatic fat (involving <30% hepatocytes) and therefore maybe inaccurate for NAFLD diagnosis. In contrast, novel imaging techniques, such as the ultrasound-based controlled attenuation parameter (CAP), MRI-estimated proton density fat fraction (PDFF), and ¹H-magnetic resonance spectroscopy correlate well with histologically-detected steatosis in both adult²⁵ and paediatric NAFLD²⁶. MRI, in particular, was shown to be more sensitive than liver histology in quantifying changes in hepatic fat²⁷ and may prove useful for the non-invasive diagnosis of adult NAFLD in longitudinal natural history studies and therapeutic trials²⁸.

Steatosis and inflammation

Steatosis in NAFLD only rarely is an “isolated” finding and is frequently accompanied by a chronic mononuclear cell inflammatory infiltrate of variable intensity located in the acini and composed of lymphocytes (mainly T cells), rare plasma cells, and monocytes. Mixed inflammation with neutrophils is less frequent while eosinophils are usually seen in relation to lipogranulomas (see below). Mild chronic or mixed portal inflammation may also be present.

Single ceroid-laden PAS-diastase-positive Kupffer cells, solitary or in groups (microgranulomas), signpost previous inflammatory activity and may be seen diffusely in the acini. Lipogranulomas, composed of a central steatotic hepatocyte or fat droplet, an occasional eosinophil and peripheral collections of mononuclear cells and macrophages, are a frequent finding. Lipogranulomas are not indicative of active inflammation in NAFLD and are not included in the evaluation of necroinflammatory activity. Kupffer cells are thought to play a significant role in NAFLD pathogenesis and progression by regulating hepatic triglyceride storage, mediating inflammation, contributing to hepatocyte injury and initiating fibrosis^{29,30}. Their density in NAFLD correlates with necroinflammatory activity, progressive injury and the extent of fibrosis³⁰. Abnormal innate immune signaling may trigger inflammation in NAFLD³¹ while oxidative stress can contribute to disease progression by stimulating both humoral and cellular immune responses³².

Studies on NAFLD pathogenesis have shown that inflammation is a driver for the development and progression of the disease³⁰. Although, traditionally, steatosis or steatosis with inflammation were considered “innocent” and non-progressive, recent data from studies with paired liver biopsies have shown that both can progress to steatohepatitis with clinically significant fibrosis (albeit infrequently)^{33,34}.

Steatosis and fibrosis

According to traditional definitions of NAFLD, hepatocellular injury and fibrosis, considered features of progression to steatohepatitis, are not observed in steatosis injury^{11,35}. In adult NAFLD, mild fibrosis, either portal or zone 3 sinusoidal without hepatocellular injury may occasionally be encountered in cases of steatosis or steatosis with chronic inflammation. These cases possibly represent an intermediate stage in the dynamic process of NAFLD development and the presence of fibrosis could be indicative of prior episodes of active steatohepatitis. Sampling variability, discussed in more detail below, may also be responsible for the absence of hallmarks of hepatocellular injury in some cases of fibrotic NAFLD.

Steatohepatitis

It is important to draw a distinction between steatosis with mild inflammation and steatohepatitis. Most experts agree that the minimal requirements for the morphological diagnosis of NASH includes hepatocyte ballooning in addition to steatosis and inflammation^{36,37}. These key lesions are typically accentuated in zone 3 of the hepatic acinus. Ballooned hepatocytes are characterized by a rounded shape and usually enlarged, lightly stained cytoplasm on routine histology (cellular diameter >30µm). The mechanisms underlying hepatocellular ballooning are not fully defined. However, oxidative stress-driven alterations of microtubules, loss of the intermediate filament cytoskeleton (which consists of keratins 8 and 18 (K8/18)), fluid retention, modification of small droplet fat and dilation of the endoplasmic reticulum are among the factors thought responsible³⁸⁻⁴². Ballooning is an important component of all currently used grading systems of NAFLD activity (see below).

Morphological features frequently encountered but not necessary for the diagnosis of NASH are Mallory-Denk bodies (MDB), glycogenated nuclei in periportal hepatocytes, acinar lipogranulomas, megamitochondria, apoptotic hepatocytes and pericellular fibrosis^{36,37}. MDB are frequently found in the cytoplasm of ballooned hepatocytes. They are irregularly formed hyaline proteinaceous inclusions consisting mainly of the intermediate filaments K8/18, the oxidative stress-induced 1/p62 sequestosome (p62) and ubiquitin⁴³. Ballooned hepatocytes are often found in the vicinity of steatotic hepatocytes in zone 3 and are frequently surrounded by pericellular collagenous fibres (pericellular fibrosis). Hedgehog signalling (Shh) activated in ballooned hepatocytes and adjacent stromal cells may be one of the mechanisms responsible for increased pericellular matrix deposition⁴⁴.

None of the single morphological features described above is specific for NASH (see below). Importantly, there is a very marked overlap of the morphological spectrum of NAFLD and ALD. Therefore, significant alcohol consumption has to be excluded in order to warrant a diagnosis of NASH. However, there is no universally accepted threshold that allows for the definition of excess alcohol consumption and indication that fatty liver disease in a given

patient is related to alcohol rather than insulin resistance. Data range between 10-20 g/day for women and 20-40 g/day for men⁴⁵⁻⁴⁹. Furthermore ALD and NAFLD share obesity and overweight as risk factors^{50,51}. Both conditions can co-exist and aggravate liver injury⁵². The distinction between ASH and NASH may be very difficult or impossible on morphological grounds in an individual patient.

The histological diagnosis of NASH has important clinical and prognostic implications. However, intra- and inter-observer variability in the interpretation of the morphological key features of NASH have been noted as important limitations of the utility of liver biopsy in assessing severity of disease^{35,53,54}. In general, intra-observer variation is lower than inter-observer variation. While substantial agreement for steatosis was reported in two studies, the strength of inter-observer agreement for inflammation and ballooning were only fair to moderate^{22,55}. However, it should be noted that substantial inter-observer agreement has been found in a more recent study⁵⁶. There is some evidence that the use of immunohistochemistry may facilitate the identification of morphological features, reducing the level of inter-observer variation. This particularly applies to the detection and quantification of ballooning, a morphologically ill-defined feature of hepatocellular injury that may be discrete in mild NASH³⁵ and that is also a feature of several liver diseases including acute and chronic viral and autoimmune hepatitis, chronic cholestasis, copper toxicity, and ischemia-reperfusion injury in the liver allograft. Significant diminution or loss of immunohistochemically demonstrable cytoplasmic expression of K8/18⁴² is only a feature in steatohepatitis, cholate stasis, copper toxicity and ischaemia-reperfusion injury. Therefore, loss of cytoplasmic K8/18 immunohistochemical staining can be regarded a marker for a certain type of hepatocellular injury and is useful in objectively detecting ballooned cells with higher sensitivity than conventional H&E histology in NAFLD⁵⁷. In addition, presence of small MDB is easily appreciated by p62 and ubiquitin immunohistochemistry^{58,59}. The presence of MDB and expression of Shh in ballooned hepatocytes have been shown to correlate with severity of NASH and disease progression^{60,61}.

Although initially considered a disease confined to affluent industrialised Western countries, obesity and insulin resistance are not restricted to the West and over the past two decades there has been a substantial increase in the prevalence of the metabolic syndrome in the Asia-Pacific region and developing nations in other continents⁶². Interestingly, only around 3% of Asians are classified as obese using a BMI cut-off of 30; it is recognised that obesity-related metabolic disorders occur at lower body weight than in Caucasians⁶³. Some studies have estimated the incidence of NAFLD to be around 15% in China⁶⁴ but extensive natural history studies are not available from that region. Despite the clinical differences between Western NAFLD and Asian NAFLD there is no current evidence to suggest that the histopathological features differ although to our knowledge this has not been comprehensively studied.

Diagnosis of NAFLD/NASH in other disease settings

Several therapeutic agents are known to be associated with liver injury resembling morphological changes typically occurring in NAFLD/NASH (termed chemotherapy- or drug-associated steatohepatitis, CASH or DASH, respectively)⁶⁵⁻⁶⁷ and have the potential to progress to cirrhosis and portal hypertension⁶⁸. The morphological features of drug- or chemotherapy-associated fatty liver disease broadly resemble that of metabolic syndrome-associated NAFLD but their location within the hepatic acinus may differ from adult metabolic syndrome-associated NAFLD that has a zone 3 accentuation pattern of injury. For example, ballooned hepatocytes may occur in a periportal distribution in amiodarone-induced hepatotoxicity. Moreover other drug-related changes including phospholipidosis or veno-occlusive disease⁶⁹ may also be present along with features resembling NAFLD. While a large number of drugs can cause steatosis (reviewed in⁷⁰), DASH/CASH is a relatively rare phenomenon. However, DASH/CASH due to amiodarone and irinotecan is well described⁷¹. Moreover, several steatogenic drugs such as tamoxifen, oestrogenic drugs and nifedipine may precipitate or exacerbate steatohepatitis in the presence of other risk factors. CASH in the setting of irinotecan treatment of liver metastases from colorectal cancer is associated

with an adverse prognosis^{66,72}, particularly in patients with pre-existing liver damage⁷². Other causes of steatosis and steatohepatitis including those secondary to gastrointestinal bypass surgery, exogenous toxins and inherited metabolic disorders were reviewed by⁷³.

Criteria for diagnosing NAFLD/NASH with concurrent disease

Given the high prevalence of NAFLD related to insulin resistance and/or the metabolic syndrome in Western populations, histological evidence of concurrent NAFLD and other liver disease, such as chronic hepatitis B (CHB) and C (CHC)⁷⁴⁻⁷⁶, human immunodeficiency virus (HIV) infection⁷⁷, autoimmune hepatitis⁷⁸⁻⁸⁰, biliary disease^{81, 82} or inherited metabolic liver disease⁸³ is a relatively frequent finding^{52,53,84,85}. In some but not all liver diseases concurrent NAFLD appears to aggravate liver injury and fibrosis progression^{75-77,85,86}. However, some diseases by themselves may be causally related to fatty liver development independently from insulin-resistance. Some of the more frequent ones include ALD, CHC, and drug induced liver injury (DILI). In such cases it is difficult to ascertain whether the concurrent condition leads to *de novo* steatosis or steatohepatitis or whether it exacerbates underlying NAFLD^{70, 87}.

Fatty change of hepatocytes in CHC can be found in 40-86% of cases, depending on the viral genotype⁸⁸. Steatosis in CHC cases may be due to virus-related insulin resistance or - in genotype 3 infections - to direct viral effects on hepatocellular lipid metabolism⁸⁹. Acinar inflammation is frequently present in CHC. However, there is no convincing evidence that CHC per se can lead to the form of ballooning seen in steatohepatitis, accompanied by MDB formation and pericellular fibrosis. Therefore, the diagnosis of NASH in CHC, as well as in most other liver diseases, is reliably achieved by these findings, provided that ALD can be excluded on clinical grounds. In CHC, NASH preferentially develops in cases with severe steatosis, and genotype 3 infection independently from the metabolic syndrome⁹⁰. Histological evidence of NAFLD in CHC⁹⁰ has been associated with a higher fibrosis stage, impaired treatment response to interferons and ribavirin and an increased risk of HCC in most studies^{76,91}. It is not yet clear whether fatty change and/or NASH impact on the efficacy

of newer protease-inhibitor based antiviral therapies⁴⁹. NAFLD in CHB is associated with metabolic host factors and is not related to increased risk of fibrosis or acceleration of disease progression^{92,93}.

Concurrence of ALD and NAFLD is probably not rare and is thought to promote liver injury and fibrogenesis. Although the spectrum of morphological lesions of ALD and NAFLD shows broad overlap⁹⁴ there are several histological changes that occur in ALD-associated liver damage that have not been described in NAFLD and may thus serve as indicators that the FLD may be alcohol-related. These include obliterative lesions of the terminal hepatic venules, canalicular cholestasis, alcoholic foamy degeneration, marked portal and/or acinar infiltration by neutrophils, and marked ductular reaction and cholangiolitis³⁵.

Histological definitions and classification

Although steatosis, steatosis with inflammation, NASH and cirrhotic NAFLD are considered components of a continuous spectrum, the initial concept of a two-hit phenomenon⁹⁵ whereby lipid accumulation is the first event in the pathogenesis of the disease while inflammatory mediators, endotoxin, mitochondrial dysfunction and ER stress represent a second stage which is a prerequisite for NASH development has recently been challenged. Many authorities now subscribe to a multiple, parallel-hit hypothesis⁹⁶. Recent evidence suggests that free fatty acids (FFA) and their metabolites may be major factors in the pathogenesis of NASH⁹⁷ and indeed there is evidence that triglyceride accumulation, the principal event in steatosis, may be a protective phenomenon as a sink for the more toxic FFA⁹⁶. This has led some to believe that steatosis and steatohepatitis may not be parts of a linear spectrum of NAFLD but rather discrete entities⁹⁸. Against this, however, are observations that NAFLD can regress either spontaneously or following therapeutic intervention and that in some cases there is not only a reduction in grade but even resolution of NASH³⁵.

Irrespective of whether the lesions are part of a continuum or distinct entities follow-up studies (discussed below) underline the need for an accurate diagnosis of NASH. Central

to this is recognition of the presence of ballooning. In the absence of immunohistochemistry this relies on sometimes-subtle changes by microscopy with clarification of the cytoplasm of hepatocytes and accentuation of the cell borders. The inter-observer reproducibility of identifying ballooning among general pathologists is at best moderate but in our opinion this has been contributed to by imperfect definitions. While it has been said that balloons may come in all shapes and sizes, reproducibility is likely to be considerably improved if enlargement of the cell is considered a pre-requisite for the identification of ballooning. In our respective referral practices we have frequently encountered over-diagnosis of NASH in cases in which there has been steatosis and chronic inflammation but where cytoplasmic glycogenosis has been mistaken for ballooning. Some pathologists use the term borderline NASH to describe cases that fall into this category; we would discourage the use of this misleading term.

Another area that requires further consideration is the definition of steatosis itself. In all recent scoring systems >5% is regarded as the cut-off for making a diagnosis. This however was based on early biochemical studies that indicated that normal liver contained 5% lipid by weight reviewed by⁹⁹. Adoption of this into histopathological definitions is somewhat arbitrary. A further pitfall is that not all papers have clearly defined whether it is 5% of area containing fat or 5% of hepatocytes. A similar argument about arbitrary cut-off levels could be made for the definition of mild, moderate and severe steatosis.

The NAFLD field has to some extent been plagued by the use of abundant terms and descriptors. Some authors have argued that non-alcoholic fatty liver disease is a misnomer and that it should be described using a positive criterion¹⁰⁰. We agree with this and our preference in assessing biopsies from patients with fatty liver disease is to follow the approach taken in viral hepatitis which (i) to identify the principal lesion i.e. steatosis (+/- inflammation) or steatohepatitis (ii) if steatohepatitis, then include an assessment of grade and stage (see below) and (iii) description of the known aetiology when available. The latter we would suggest should be one of the following: alcoholic, metabolic, mixed alcoholic/metabolic, drug/toxin-related or jejuno-ileal bypass surgery-related.

Paediatric NAFLD

NAFLD is the most common cause of steatosis in liver biopsies of children and adolescents followed by the effects of inherited metabolic disorders, cancer, and hepatitis C¹⁰¹. The increased prevalence of paediatric NAFLD (2.6%-7.1% in population-based studies and 0.7%-17.3% in autopsy studies) reflects the increase in prevalence of overweight and obesity in children over the last two decades, and it is linked with the development of the metabolic syndrome and its consequences¹⁰². Genome-wide association studies (GWAS) have shown a correlation of the I148M *PNPLA3* variant with NAFLD development in obese children of different ethnic backgrounds, as well as with histological severity of NASH and fibrosis (reviewed in¹⁰² and¹⁰³). It is worrisome that radiological imaging may be normal in up to 1/3 of children with histologically proven NAFLD even in advanced disease stages¹⁰¹, and that significant histological changes may be detected in children with NAFLD and normal or mildly elevated aminotransferase levels¹⁰⁴. This underlines the importance of liver biopsy in diagnosing paediatric NAFLD. NAFLD may advance to cirrhosis in 3%-5% of affected children^{73,101,104}. Unfortunately, non-invasive methods for diagnosing progressed NAFLD with fibrosis in children (hepatic fibrosis scores, transient elastography etc.) are not yet fully developed for implementation in routine practice¹⁰⁵. Binge alcohol drinking and its possible influence on underlying liver histology of obese teenagers is a cause of concern in adolescent NAFLD¹⁰⁶.

Since the prototypic study of Schwimmer et al¹⁰⁷ which highlighted histological differences between paediatric and adult NAFLD, several studies from different countries have confirmed that liver biopsies from children with NAFLD show more prominent portal-based chronic inflammation and fibrosis; more severe steatosis (panacinar or with a zone 1 predilection), less frequent hepatocyte ballooning and MDBs, and less commonly zone 3 sinusoidal fibrosis¹⁰⁸⁻¹¹⁰. Schwimmer et al first used the term “paediatric-type” or “type 2” NAFLD for this constellation of predominantly zone-1 based lesions in paediatric liver (51% of the 100 cases studied) in contrast to the less frequent (17%) zone-3 based histologic

pattern “adult type “ or “*type 1*” NAFLD. Type 2 NAFLD was thought to be more common in older boys of non-white ethnicity¹⁰⁷. However, it was shown in subsequent studies that most cases (up to 82%) have overlapping features¹⁰⁹. The National Institutes of Health (NIH)-sponsored multicentre NASH Clinical Research Network (NASH CRN) proposed the use of terms “borderline zone 1” or “borderline zone 3” NAFLD when liver histology does not fulfil set criteria for NASH but shows intermittent features corresponding to accentuation of lesions in zone 1 (periportal) or zone 3 (around the terminal hepatic venule)¹¹¹. Steatosis, portal inflammation, and fibrosis have been found to be less severe in NAFLD patients during or after puberty, compared to prepubertal cases, and postpubertal individuals had a lower prevalence of “borderline zone 1” NAFLD but more MDB¹¹². Activation of hepatic progenitor cells in portal/periportal areas exhibiting high Hedgehog pathway activity and expressing adipocytokines may explain the portal-based histological patterns of injury and fibrosis in paediatric NAFLD¹¹³. Unfortunately, to date there are no widely accepted criteria to reach an undisputable diagnosis of “steatohepatitis” in paediatric NAFLD when non-adult histological patterns are seen.

Recently, a histological scoring system for grading activity in paediatric NAFLD has been developed and validated taking into account portal-based inflammation in addition to steatosis, acinar inflammation and ballooning¹¹⁴. The new score showed an excellent correlation with a global histological diagnosis NASH but its value in routine practice remains to be determined.

Grading and staging of NAFLD

Evaluation of morphological features of NAFLD by semi-quantitative scoring is frequently performed to provide global and standardized assessment of grade of disease activity and stage of fibrosis for use in clinical trials and to support of clinical decision making^{22,56,60,115,116}.

One of the currently most widely used measures of grade, the NAFLD activity score (NAS)²², was developed by the NASH CRN¹¹⁷. This was based on a revision of the first grading system for NAFLD described by Brunt et al⁶⁰. The NAS is derived from the sum of

semi-quantitative numerical scores applied to steatosis (0-3), hepatocellular ballooning (0-2) and acinar (lobular) inflammation (0-3) which can range from 0-8 (Table 1). At thresholds of <3 and ≥ 5 the NAS showed good correlation with the histological diagnoses *not NASH* and *NASH*, respectively (22). However, the sensitivity and specificity of the NAS for a histological diagnosis of NASH at a threshold level of ≥ 5 is 57% and 95%, respectively¹¹⁸. The NAS cannot therefore be used (and indeed was never intended) to replace the histopathological classification of NAFLD types (i.e. steatosis versus NASH). Instead, the NAS is designed for use in clinical trials to reflect changes in individual histological key features of NASH. Interestingly, in contrast to fibrosis stage, NAS did not correlate with prognosis (liver-related death) in a recent study¹¹⁵. The NAS system has been externally validated¹¹⁸ and is currently among the most widely used. The CRN also issued a scoring system for the evaluation of fibrosis stage²². As in chronic viral hepatitis scoring systems, fibrosis is assessed independently from grade¹¹⁹.

Recently, a simple histological algorithm based on a scoring system for NAFLD was developed by the European Fatty Liver Inhibition of Progression (FLIP) consortium in an attempt to standardize and limit inter-observer-related variation in the histological diagnosis of NASH (Table 1). The FLIP algorithm is based on semi-quantitative scoring of the key features of NASH, steatosis (S), activity (A), and fibrosis (F). Activity is reflected by the sum of scores for hepatocellular ballooning and acinar (lobular) inflammation. Fibrosis is assessed using a 5-tier scale broadly similar to the NASH CRN fibrosis score. The SAF scoring system was originally developed for grading and staging of NAFLD in patients with morbid obesity undergoing bariatric surgery¹¹⁶ but was subsequently applied to patients with metabolic syndrome-associated NAFLD⁵⁶. The FLIP algorithm informed by the scores for steatosis, hepatocellular ballooning and inflammation allows for stratification into the two diagnostic categories: NASH versus steatosis. The diagnosis of NASH is only applied if all three key features, steatosis, hepatocellular ballooning and acinar inflammation are present. The SAF score was used to define two categories of NAFLD severity: mild disease when $A < 2$ and/or $F < 2$ and significant disease when $A > 2$ and/or $F > 2$ ⁵⁶. Disease severity is

therefore defined by hepatocellular ballooning, acinar inflammation and fibrosis, parameters of known prognostic significance in NAFLD. Steatosis, albeit a necessary component of NAFLD diagnosis but of only minor impact on prognosis remains a separate feature not included in this definition. The prognostic relevance of the dichotomous classification of mild and significant NAFLD remains to be evaluated in further studies.

Although not strictly a scoring system, one of the early morphological classification systems of NAFLD demonstrated the prognostic relevance of hepatocellular ballooning and fibrosis¹²⁰. In the study of Matteoni et al, NAFLD was classified into four distinct types comprising fatty liver without inflammation (type 1) or with inflammation (type 2), and fatty liver with hepatocyte ballooning (type 3) or with ballooning and either MDB or fibrosis (type 4). On long-term follow up in patients with types 3 and 4 NAFLD the prevalence of cirrhosis was 7-fold and liver-related death 5.5-fold higher than in patients with types 1 and 2 NAFLD. The prognostic utility of this simple morphological classification system of NAFLD and the prognostic impact of fibrosis stage was re-evaluated and confirmed in a recent study¹¹⁵.

However, several shortcomings inherent to any scoring system have to be considered. First, not all morphological features of injury can be accounted for. Several histological changes may provide important information with respect to prognosis (see below). Any scoring-based classification developed with simplicity and applicability in mind will not cover the whole range of complexity of the disease. Numeric score-based classification cannot therefore replace the descriptive interpretation of biopsy findings^{22,56}. Secondly, interpretation of morphological features of NAFLD is prone to observer-related variation^{22,55,56}. As noted above, this has been shown for some of the individual parameters of NAFLD scoring systems and is even more pronounced for the diagnosis of the different NAFLD types. In this respect the FLIP algorithm has been shown to significantly reduce the inter-observer bias of the diagnosis of NASH among expert hepatopathologists as well as for general pathologists trained in liver pathology⁵⁶.

Despite efforts to ensure standardized and reproducible classification of NAFLD some observer-related bias will most likely remain. DIA allows for quantitative assessment of

morphological features in histological sections. Although investigated in more detail for CHC^{121,122}, DIA could become a useful technique to overcome this obstacle in NAFLD. For example, DIA has been shown to unveil erroneous estimation of the extent of steatosis by conventional histology in NAFLD^{23,123}. While several reports relate to the role of DIA for quantification of steatosis only few studies have addressed the impact of DIA-assisted quantification of fibrosis or inflammation on semi-quantitative scoring or correlation with non-invasive methods like radiological fibrosis estimates and/or biochemical parameters/scores in NAFLD.

Prognostic lesions in NAFLD/NASH

Histological lesions of prognostic relevance

Results from several studies have indicated that in patients with steatosis with or without inflammatory acinar changes, NAFLD will follow a benign course with low probability to progress to NASH and/or fibrosis¹²⁴⁻¹²⁶. However, as mentioned above, recently it has been shown that both steatosis and steatosis with inflammation can infrequently progress to steatohepatitis with clinically significant fibrosis^{33,34}.

In contrast, histological NASH distinguished from steatosis with inflammation by the presence of hepatocellular ballooning has been shown to carry substantial potential for progression. This is evident from the seminal work of Matteoni et al described above¹²⁰. The prognostic relevance of this classification system was recently confirmed¹¹⁵. Furthermore, elevated mortality rates of NASH patients as compared to patients with steatosis were also reported by other investigators^{127,128}. Portal inflammation in NAFLD is strongly associated with disease severity (fibrosis stage)^{129,130}.

NASH is very frequently associated with fibrosis¹³¹. The fibrosis-promoting effect of NASH was confirmed in studies based on paired biopsies in which an increase of fibrosis stage over time was found in approximately 30-50% of NASH patients¹³²⁻¹³⁴ who may thus be at risk to develop cirrhosis. Indeed, as noted earlier, NASH is thought to represent one of the main causes of cryptogenic cirrhosis which is in most cases is considered burned-out

NASH^{135,136}. There is evidence to suggest that cryptogenic cirrhosis and NASH-related cirrhosis are both risk factors for the development of HCC^{137,138}. Recently, fibrosis was identified as the strongest predictor of overall and/or liver-related mortality in NAFLD^{115,139,140}.

Novel prognostic markers - clues from immunohistochemical and experimental studies

Oxidative and ER-stress and apoptosis are among the most important mechanisms driving injury and inflammation leading to NASH which is implicated in increased inflammation and fibrogenesis as well as hepatocarcinogenesis via damage-associated molecular pattern molecules (DAMPs) and morphogens like Hedgehog and Wnt. Several factors involved in these pathways have been shown to exhibit altered expression in the liver of patients with NASH as well as in mouse models of NASH (reviewed in¹⁴¹).

For example, components of the inflammasome are overexpressed in livers of NASH patients as compared to controls¹⁴². As noted above, induction of Sonic hedgehog (Shh) expression in ballooned cells is accompanied by enhanced fibrogenesis via Shh signalling by neighbouring stromal cells⁴⁴. Markers for hepatocellular ballooning may therefore be useful to identify these cells with higher sensitivity in individuals at risk for progressive disease. Furthermore, they may be helpful to detect and monitor treatment effects in clinical trials^{44,143}.

Recently, the aldose reductase AKR1B10 was found to be highly differentially expressed in steatohepatitis compared to steatosis and normal liver¹⁴⁴. AKR1B10 is involved in the detoxification of toxic aldehydes which may be prevalent in liver tissue in NASH or ASH. AKR1B10 was first described in human HCC¹⁴⁵. It is expressed in several human cancers¹⁴⁶ and may be involved in cell differentiation and proliferation¹⁴⁷. AKR1B10 expression may thus be a premalignant marker in the progression of steatohepatitis to HCC.

Increased numbers of CD68-positive macrophages in hepatic acini and portal tracts are found in NASH and advanced fibrosis^{130,148,149}. An association with NASH and fibrosis was also noted for ductular reaction (DR) which is thought to represent expansion of hepatic progenitor cell populations in response to injury. DR is a reactive lesion and consists of bile

ductules set in an inflamed stroma. DR may contribute to fibrogenesis and fibrosis progression in NAFLD. The extent of DR demonstrated by anti-keratin 7 antibodies has been shown to correlate with NASH and fibrosis stage¹⁵⁰. Immunohistochemical or molecular markers of inflammatory activity, hepatocellular injury and fibrogenesis may be important indicators of higher risk of disease progression but their prognostic utility has not yet been evaluated in clinical studies.

Liver biopsy in NAFLD – is there a role in 2015?

A recent clinical definition of NAFLD requires (i) evidence of hepatic steatosis by histology or imaging and (ii) exclusion of other causes of fat accumulation in hepatocytes, such as significant alcohol consumption, hereditary disorders or steatogenic drugs⁴⁹. The NAFLD spectrum is defined by morphological changes in the liver parenchyma discussed in detail above and carry increasingly adverse long-term prognosis ranging from no effects on overall survival for simple steatosis to increased mortality for NASH and cirrhosis mostly due to liver and/or cardiovascular disease^{120,151,152}. Incidence and natural history of NAFLD are still not well defined because ethical and logistic reasons preclude the use of liver biopsy as a screening test. From non-invasive radiological studies (ultrasonography, magnetic resonance imaging) it is estimated that NAFLD is present in approximately 30% of Western¹⁵³⁻¹⁵⁵ and 15% of Asian-Pacific populations^{64,156} thus representing the one of the most common forms of liver disease¹⁴¹. However, in populations with metabolic risk factors such as diabetes mellitus or obesity the prevalence is higher (70-90%)¹⁵⁷⁻¹⁶².

Simple steatosis alone affects the great majority of adult patients with NAFLD ¹²⁴. A minority (10-30%) have NASH^{120,161,163}, but this is the main progressive type of NAFLD associated with the development of fibrosis in 20-40% and cirrhosis in 20-30% of cases^{120,126,127,133,164} as well as significantly higher long-term mortality^{115,139}.

In adult patients with metabolic risk factors, in particular insulin resistance, clinical management is guided by the diagnosis/exclusion of NAFLD and discrimination of the NAFLD types. The diagnosis of NAFLD is based on the presence of hepatic steatosis which may be detected by imaging methods (e.g., ultrasound or MRI or novel techniques described above in the section of Steatosis)¹⁶⁵ or histology after other causes of chronic liver disease and steatosis have been excluded. Since there are currently no clinical or routine biochemical factors available to detect NASH with sufficient diagnostic accuracy, histological evaluation is still considered the gold standard to monitor NAFLD patients and to identify NASH despite the limitations referred to earlier regarding sampling and inter-observer variation. However, given the high prevalence of NAFLD the use of liver biopsy as a diagnostic tool is considered problematic. Apart from logistic and economic considerations, liver biopsy is an invasive procedure and associated with a small but not negligible risk of morbidity and mortality. There has therefore been considerable effort applied to the development non-invasive methods for the discrimination of steatosis and NASH which has resulted in the description of an ever growing list of novel NASH biomarkers as well as clinical models¹⁶⁶. The diagnostic accuracies of these markers for NASH diagnosis as indicated by the areas under the receiver–operating curve (AUROC) are in the range of 0.70 to 0.90. Lack of external validation, standardized definitions of optimal cut-offs and availability are currently among the most important drawbacks precluding their use as stand-alone tests in clinical practice¹⁶⁶. Among the radiological methods, magnetic resonance elastography (MRE) may have good accuracy for the diagnosis of NASH¹⁶⁷.

Recent technical progress has also given rise to non-invasive tools to assess fibrosis stage in NAFLD patients with good accuracy. Scores and indices such as the NAFLD fibrosis score^{168,169} or the FIB-4 index and radiological methods, in particular transient elastography (TE) have been evaluated in different cohorts of NAFLD patients. These methods have been shown to reliably exclude lower fibrosis stages and have become widely accessible in clinical practice^{166,170}.

Although new non-invasive techniques may eventually lead to a change in the role of liver biopsy, currently this is the definitive investigation for NAFLD providing at the same time information on steatosis, inflammation, hepatocellular injury, fibrosis, and concurrent liver disease. Most expert guidelines recommend liver biopsy for NAFLD patients at high risk for NASH and/or advanced fibrosis^{49,165,171,172}. Histological predictors of fibrosis progression have been described in longitudinal studies with sequential biopsies^{127,173}. Furthermore, it is not infrequent that NAFLD coexists with other liver diseases. In these settings clinical management is affected by potentially aggravated liver injury and accelerated disease progression^{50,85,174,175}. Detection of NAFLD or NASH as a coexisting condition is therefore reliably achieved by histology^{84,176,177} and is recommended by the European guidelines on NAFLD/NASH⁴⁸. In addition, most guidelines issued by international or national hepatological societies also consider the use of liver biopsy in suspected NAFLD patients in whom other causes of steatosis or chronic liver disease cannot be excluded on clinical grounds¹⁷⁸.

There is broad agreement that liver biopsy is an important tool in clinical trials and basic research on NAFLD defining the histological type of disease as well as assessing liver injury and effects of intervention³⁷. However, despite the utility of histological evaluation for clinical and research applications the limitations mentioned earlier have to be taken into account. These include sampling and observer-related biases which can partly be overcome by taking biopsies of adequate length (at least 16 mm) and diameter (preferable use of a 16 gauge or greater diameter needle) in similar fashion from the same liver lobe¹¹¹, using standardized scoring systems for the interpretation of histopathological findings (described above) and/or DIA for quantitative assessment of histological features^{23,122,179}. Besides histological evaluation, liver tissue analysis by immunohistochemistry and molecular methods has provided and is likely to continue to contribute importantly to the elucidation of mechanisms involved in pathogenesis and natural history of NAFLD (reviewed in⁵³).

However, studies evaluating the potential of these factors for clinical decision-making are rare.

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Figure legend

Figure 1. Expression of hepatocellular keratin 8 and 18 (K8/18) and sonic hedgehog (Shh) in steatohepatitis (A-D, serial sections, x100). (A) Ballooned hepatocytes with pale cytoplasm, few with small Mallory-Denk bodies (MDB, arrow-heads; haematoxylin & eosin, H&E) are surrounded by (B) collagen fibres (arrow-heads; chromotrope aniline blue, CAB). (C) The ballooned hepatocytes lack K8/18 immunostaining of the cytoplasm whereas the small MDB are K8/18-positive. (D) Ballooned hepatocytes are also decorated by antibodies specific for Shh.

Table 1: NASH Clinical Research Network (NASH CRN)²² and SAF¹¹⁶ histological scoring systems for NAFLD.

NASH CRN	SAF
Steatosis grade 0-3 0: <5%* 1: 5-33% 2: 34-66% 3: >66%	Steatosis grade (S) 0-3 S ₀ : <5%** S ₁ : 5-33% S ₂ : 34-66% S ₃ : >66%
Hepatocyte Ballooning 0-2 0: None 1: Mild, few 2: Moderate-marked, many	Hepatocyte Ballooning 0-2 0: None 1: Clusters of hepatocytes with rounded shape and pale and/or reticulated cytoplasm 2: Same as score 1 with enlarged hepatocytes (>2x normal size)
Lobular (acinar) Inflammation 0-3 0: None 1: <2 foci/20x field 2: 2-4/20x field 3: >4/20x field	Lobular (acinar) Inflammation 0-2 0: None 1: ≤ 2 foci per 20x field 2: > 2 foci per 20x field
NAFLD ACTIVITY SCORE (NAS): 0-8	ACTIVITY GRADE (A): 0-4
Sum of scores for steatosis, ballooning and lobular inflammation	Sum of scores for ballooning and lobular (acinar) inflammation A ₁ (A=1): mild activity A ₂ (A=2): moderate activity A ₃ & A ₄ (A>2): severe activity
FIBROSIS STAGE 0: No significant fibrosis 1: 1a mild (delicate) zone 3 perisinusoidal fibrosis (PSF) (requires collagen stain to identify) 1b moderate (dense) zone 3 PSF 1c portal fibrosis only 2: Zone 3 perisinusoidal fibrosis with periportal fibrosis 3: Bridging fibrosis 4: Cirrhosis	FIBROSIS STAGE (F) F ₀ : No significant fibrosis F ₁ : 1a mild zone 3 PSF 1b moderate zone 3 PSF 1c portal fibrosis only F ₂ : Zone 3 PSF with periportal fibrosis F ₃ : Bridging fibrosis F ₄ : Cirrhosis
	SAF SCORE S ₀₋₃ A ₀₋₄ F ₀₋₄

*percentage of parenchymal involvement by steatosis

**percentage of hepatocytes containing large and/or medium-sized intracytoplasmic lipid droplets

Figure 1