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Editorial note:

Non-alcoholic fatty liver disease (NAFLD) is the commonest metabolic liver disease worldwide and affects a quarter of general population in Hong Kong. Histological evaluation remains the gold standard for diagnosing NAFLD. This Topical Update reviews pathological features of NAFLD and highlights some practical points for our daily diagnostic work. We welcome any feedback or suggestions. Please direct them to Dr. Anthony Chan (e-mail: awh_chan@cuhk.edu.hk) of Education Committee, the Hong Kong College of Pathologists. Opinions expressed are those of the authors or named individuals, and are not necessarily those of the Hong Kong College of Pathologists.

Pathology of Non-Alcoholic Fatty Liver Disease

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a serious global health problem and associated with over-nutrition and its related metabolic risk factors including central obesity, glucose intolerance, dyslipidaemia and hypertension. It is the most common metabolic liver disease worldwide and its prevalence in most Asian countries is similar to that in the States, Europe and Australia. About 10-45% of Asian population have NAFLD.[1] With "westernized" sedentary lifestyle, the prevalence of NAFLD in general urban population in the mainland China is about 15%.[2] NAFLD is even more prevalent in Hong Kong. Our recent study demonstrated that NAFLD is found in 27.3% of Hong Kong Chinese adults by using proton-magnetic resonance spectroscopy.[3] We further realized that 13.5% of Hong Kong Chinese adults newly develop NAFLD in 3-5 years.[4] Both prevalence and incidence of NAFLD in Hong Kong are alarmingly high. Accurate diagnosis of NAFLD is crucial to allow prompt management of patients to reduce morbidity and mortality. NAFLD is composed of a full spectrum of conditions from steatosis to steatohepatitis (NASH) and cirrhosis. Various non-invasive tests, based on clinical, laboratory and radiological tests, have been developed to assess the degree of steatosis and fibrosis in NAFLD.[5, 6] However, liver biopsy remains the gold standard for characterizing liver histology 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.[7] This article reviews pathological features of NAFLD and highlights some practical points for our daily diagnostic work.

Pathological patterns of NAFLD

Non-Alcoholic Steatohepatitis Clinical Research Network (NASH-CRN) has provided numerous important data on the natural history, clinical features, management and pathology of NAFLD. Different pathological patterns of NAFLD described by NASH-CRN have been widely adopted in clinical practice and research studies.

Steatosis with or without inflammation

Hepatic steatosis (fatty change) is the accumulation of fat droplets, primarily triglyceride, in the cytoplasm of hepatocytes. The cutoff between physiologic and pathologic steatosis is 5% of affected hepatocytes, which is based on studies by lipid content measurement and imaging.[8] There are two morphological forms of steatosis: macrovesicular and microvesicular. Macrovesicular steatosis is typically featured by a hepatocyte containing a single large fat droplet displacing the nucleus to the periphery (Figure 1). However, hepatocytes containing multiple small to medium-sized fat droplets (Figure 2) in fatty liver disease are not uncommonly found in adjacent to those hepatocytes with a single large fat droplet. It has been demonstrated that a single large fat droplet is resulted from fusion of these small to medium-sized fat droplets. We should not hesitate to apply the term macrovesicular steatosis to those hepatocytes with small to medium-sized fat droplets. Some experts may prefer to use "mediovesicular" steatosis to describe them. In contrast, "genuine" microvesicular steatosis is characterized by the accumulation of much smaller uniform minute fat droplets dispersed throughout the hepatocytes, and sometimes requires special stain (e.g. oil red O) for better visualization. Diffuse microvesicular steatosis is typically found in conditions with serious mitochondrial dysfunction and fatty acid oxidation defect (e.g. Reye syndrome, acute fatty liver of pregnancy, acute alcoholic foamy degeneration). However, focal microvesicular steatosis is recognized up to 10% of liver biopsies in patients with NAFLD and associated with higher grades of steatosis, ballooning degeneration, inflammation and advanced fibrosis.[9]

In a liver biopsy of patients with NAFLD, the degree and distribution of steatosis should be evaluated at low magnification (at most 10x and usually 4x). Assessment at higher magnification may overestimate the severity of steatosis. The degree of steatosis is semi-quantitatively categorized as mild (5 to 33%), moderate (>33 to 66%) and marked (>66%).[10] The severity of steatosis is associated with lobular inflammation and perivenular fibrosis but there is no significant correlation with ballooning degeneration, Mallory-Denk bodies or portal/advanced fibrosis.[11] Predominant zonal distribution of steatosis should be also recorded unless steatosis is very mild or the biopsy is fragmented or inadequate. It is categorized into four patterns: zone 3 (perivenular), zone 1 (periportal), panacinar and azonal. Steatosis in NAFLD is usually present in zone 3 and panacinar distribution. Predominant zone 1 distribution is rare in adult (1%) but more commonly found in children and teenagers (12%).[10] Steatosis in an azonal distribution is more likely to be associated with ballooning degeneration, Mallory-Denk bodies and advanced fibrosis.[11]

When steatosis is accompanied by lobular and/or portal inflammation, a diagnosis of steatosis with inflammation is made. Inflammatory infiltrates are mainly lymphocytes, mononuclear cells and occasional eosinophils. Neutrophils are rarely seen in NAFLD in contrast to alcoholic liver disease (ALD). Lobular inflammation can be present in the form of small aggregates of macrophages (microgranuloma) or lymphocytes, similar to spotty necrosis in chronic viral hepatitis. Mild lobular inflammation (<2 foci/20x) is often found in about 80% of NAFLD with simple steatosis.[9] Portal inflammation is usually absent or mild (76% and 77% in adults and children, respectively).[12] There is no correlation between the severity of portal inflammation and lobular inflammation. "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. Its presence is associated with steatohepatitis and advanced fibrosis,[12] but should also raise the suspicion for other chronic hepatitis, particularly viral hepatitis C. Predominant portal inflammation exceeding lobular inflammation is more common in paediatric patients.[13]

Steatosis with or without inflammation is also known as simple steatosis and has been considered benign and non-progressive. However, our group demonstrated that 58% and 28% of patients with simple steatosis had increased disease activity and fibrosis progression in a 3-year interval, respectively.[14] Moreover, the accumulation of fat droplets is one the mechanisms leading to ballooning degeneration, the hallmark of steatohepatitis, through oxidative fat injury, endoplasmic reticulum dysfunction and abnormalities of the cytoskeleton.[15] Simple steatosis is not always quiescent and may mislead people on underestimation of the risk of disease progression.

Steatohepatitis

Steatohepatitis does not simply mean steatosis with inflammation but is a distinctive pathological pattern characterized by steatosis more than 5%, inflammation and ballooning degeneration. Ballooning degeneration is the key lesion to differentiate steatohepatitis from steatosis with inflammation. It is the hallmark of hepatocellular injury in steatohepatitis and is characterized by cellular swelling, rarefaction of the hepatocytic cytoplasm and clumped strands of intermediate filaments (Figure 3). It is associated with substantial accumulation of fat droplets as well as dilatation of the endoplasmic reticulum and cytoskeletal injury.[15] Ballooned hepatocytes are initially most frequently in the perivenular region in early stage of disease. This zonal distribution is lost when disease progresses or in very active disease. Ballooned hepatocytes often but not necessarily contain Mallory-Denk body. Mallory-Denk body, which is also known as Mallory body and Mallory hyaline, is a deeply eosinophilic, ropey intracytoplasmic inclusion (Figure 3), and an aggregate of misfolded intermediate filaments with other different classes of proteins, including p62 and ubiquitin. The identification of ballooned hepatocytes may not be always straightforward and the immunohistochemical stain (cytokeratin 8/18 [CK8/18]) is helpful in such situations. Ballooned hepatocytes are characterized by loss of cytoplasmic expression of CK8/18, whereas residual immunoreactivity is confined to their Mallory-Denk bodies if present (Figure 4).[16]

Fibrosis is an indicator of chronicity and disease progression. Although it is not necessary to establish a diagnosis of steatohepatitis, it is commonly found in adult (84%) and paediatric patients (87%) with NASH.[10] Its presence helps us to more confidently make a diagnosis of steatohepatitis in equivocal cases. Perivenular and pericellular/perisinusoidal fibrosis (Figure 5) is the distinctive pattern of fibrosis in fatty liver disease and not typically encountered in chronic viral hepatitis, autoimmune hepatitis and chronic cholestatic disease. It represents deposition of fibrous tissue in the space of Disse and is related to activation of stellate cells. It is typical in early stage of fibrosis in adult NAFLD/NASH, similar to that in ALD. However, the fibres tend to be thinner and less marked in NAFLD/NASH. As the disease progresses, periportal fibrosis will develop with fibrous strands entrapping periportal hepatocytes. Later, bridging fibrosis may occur between central regions (central-central fibrous bridging), between portal tracts (portal-portal bridging) or between central and portal regions (central-portal bridging). Cirrhosis is eventually established after progressive fibrosis, parenchymal extinction and hepatocellular regeneration. Two practical issues concerning pathological assessment of fibrosis are highlighted here. Firstly, a good quality connective tissue stain is crucial to highlight the earliest delicate fibrosis. Masson trichrome, Gordon-Sweets reticulin and Sirius red stains are common connective tissue stains widely used in hepatopathology. 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 pericellular fibrosis.[17] Secondly, aberrant arteries and microvessels in the perivenular region are commonly found in about 40% of patients with NASH, especially in those with advanced fibrosis (62%). Ductular reaction is present in 55% of arterialized scarred perivenular region.[18] The presence of aberrant artery and ductular reaction may cause misidentification of a perivenular region as a portal tract. Such misidentification could lead to erroneous interpretation of a portal-based process, potentially resulting in a missed NAFLD/NASH diagnosis and inaccurate assessment of fibrosis. To avoid this misidentification, proper appreciation of normal liver histology is necessary. 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. They are embedded within the fibrous stroma of the portal tract and separated from periportal hepatocytes by a limiting plate. However, in arterialized scarred perivenular region of NAFLD/NASH patients, aberrant artery and ductule may lie too far apart without an accompanied portal vein, or lie adjacent to or among hepatocytes without separation from the limiting plate in a portal tract.

Borderline steatohepatitis

Steatohepatitis may be further classified as definite or borderline. Definite steatohepatitis is applied for cases fulfilling all three diagnostic features of steatohepatitis (steatosis more than 5%, inflammation and ballooning degeneration), typically with a predominantly perivenular distribution. Borderline steatohepatitis is designated for those cases falling in the grey zone between steatosis with/without inflammation and definite steatohepatitis. Two forms of borderline steatohepatitis have been described by NAFLD-CRN. Zone 3 borderline steatohepatitis is applied for those do not have full-blown unequivocal histological features of definite steatohepatitis. It may include those cases with characteristic perivenular/pericellular fibrosis in absence of ballooning degeneration, and those cases with equivocal ballooning degeneration. However, this practice is controversial and not yet accepted universally. Some pathologists prefer to describe those cases with perivenular/pericellular fibrosis in absence of ballooning degeneration as steatosis with fibrosis or steatofibrosis in such cases. Zone 1 borderline steatohepatitis is characterized by portal-based injury (periportal steatosis, predominantly portal inflammation and portal fibrosis).[10] Ballooning degeneration is usually absent. This distinctive form of borderline steatohepatitis is a unique histological pattern that appears to predominantly affect paediatric patients with NASH (75%) and also known as type 2 (compared to usual "type 1" NASH in adult) or paediatric NASH in the literature. It more frequently affects boys, younger children, and Asian and Hispanic ethnicity.[13].

Cryptogenic cirrhosis

A diagnosis of cryptogenic cirrhosis is designated for those cases with minimal recognizable diagnostic features after exclusion of viral hepatitis, metabolic, autoimmune and cholestatic liver diseases. Cryptogenic cirrhosis accounts for 8-9% of liver transplantation in the States and NAFLD has been recognized as a common cause of cryptogenic cirrhosis.[19]

In patients with cryptogenic cirrhosis, the prevalence of diabetes mellitus and obesity is comparable to that of patients with NAFLD and far exceeds that of patients with cirrhosis associated with chronic viral hepatitis and autoimmune liver disease.[20] Typical histological features of steatosis and/or necroinflammatory activity in patients with NAFLD/NASH may resolve as disease progresses to advanced fibrosis. Recognition of residual ballooning degeneration, Mallory-Denk bodies and pericellular fibrosis, together with clinical evidence of metabolic risk factors, helps us to reach a diagnosis of "burnt-out" NAFLD cirrhosis.

Other pathological lesions in NAFLD

Some pathological changes may be found in NAFLD but have not been used to classify the pattern of the disease. Lipogranuloma is characterized by a loose aggregate of lymphocytes and macrophages surrounding a central fat globule (Figure 6). It can be found in NAFLD as well as ALD and ingestion of mineral oil in food and medication. It should be distinguished from fibrin ring granuloma, which has a characteristic fibrin ring highlighted by phosphotungstic acid-

haematoxylin stain o immunostaining for fibrin. Glycogenated nuclei are featured by nuclear clear vacuolation due to the accumulation of glycogen (Figure 7). They are found much more commonly in NAFLD than ALD. They may be considered evidence of impaired glucose tolerance or insulin resistance. However, they may occur physiologically in children and young adults (11% and 4% in the 20s and early 30s, respectively),[21] and pathologically in glycogen storage disease, Wilson disease, and other copper overload disorders. Giant mitochondria, also called megamitochondria, are eosinophilic oval or needle-shaped intracytoplasmic inclusions (Figure 8). Although they typically are found in alcoholic and non-alcoholic fatty liver diseases, they may be associated with a wide variety of physiologic and pathologic conditions, such as aging, acute fatty liver of pregnancy, glycogen storage disease and urea cycle defects. Glycogenic hepatocyte distension is characterized by marked enlargement of hepatocytes with cytoplasmic clearing by excessive accumulation of cytoplasmic glycogen. It occurs in glycogenic hepatopathy in poorly controlled diabetes, glycogen storage disease and urea cycle defects.

Metabolic syndrome is a significant risk factor for hepatocellular carcinoma (HCC; odds ratio 2.13) and intrahepatic cholangiocarcinoma (odds ratio 1.56).[22] Salomao et al. recently described a distinctive histological variant of HCC, steatohepatitic HCC. It is characterized by HCC with features resembling steatohepatitis (steatosis in more than 5% of tumour cells, ballooning degeneration, Mallory-Denk bodies, intratumoral inflammatory infiltrate and pericellular fibrosis) (Figure 9). It is associated with underlying NAFLD and metabolic risk factors but does not carry any prognostic significance.[23, 24]

Grading, Staging and Scoring Systems

To assess the severity of NAFLD in a liver biopsy, both activity (grade) and chronicity (stage) should be evaluated. Semi-quantification or scoring of the grade and stage are welcomed by some clinicians and pathologists to guide clinical management, standardize pathology reporting and facilitate research studies. In 1999, Brunt et al. proposed the first semi-quantitative grading and staging system based on liver biopsies from 51 patients with NAFLD. The disease activity grade was based by a combination of parameters including steatosis, lobular and portal inflammation, and ballooning degeneration. The fibrosis stage was assigned on fibrosis patterns of adult NAFLD from perivenular/pericellular to periportal, bridging and cirrhosis.[25]

In 2005, a revised Brunt's system by NASH-CRN was published (Table 1).[10] The disease activity grade, well-known as "NAFLD Activity Score (NAS), is the unweighted sum of scores for steatosis, ballooning degeneration, and lobular inflammation. In the fibrosis staging, early disease (stage 1) was subclassified into 1a (mild pericellular fibrosis), 1b (moderate pericellular fibrosis) and 1c (portal/periportal fibrosis only). The Asian-Pacific Working Party for NAFLD encouraged using this system for routine reports and research studies.[26] 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%); on the other hand, most cases with scores of 5 or more were diagnosed as definite steatohepatitis (86%).[27] Cases with NAS of 3 and 4 were distributed almost evenly between all three patterns: steatosis (27%), borderline steatohepatitis (32%) and definite steatohepatitis (41%). It has been repeatedly emphasized that NASH-CRN system should not be used as the diagnostic criteria for steatohepatitis (i.e., diagnosis of steatohepatitis only if NAS is 5 or more), although clinical trials have often selected patients with steatohepatitis based on an NAS value of 5 or more.[10, 27]

In 2012, Bedossa et al. proposed an algorithm and a scoring system based on a cohort of 679 obese patients underwent bariatric surgery (Table 2).[28] The FLIP (fatty liver inhibition of progression) algorithm is proposed for segregating lesions into normal liver, NAFLD or NASH by semiquantitative evaluation of steatosis, ballooning degeneration, and lobular inflammation. The SAF (steatosis, activity, fibrosis) score is the combination of scores of

steatosis, activity (ballooning degeneration and lobular inflammation) and fibrosis. It integrates both grade and stage together. Compared to the NASH-CRN system, steatosis is excluded from the activity score because there is no significant difference in transaminase levels between patients with normal liver and simple steatosis. A recent validation study involving 6 expert liver pathologists and 10 general pathologists showed that the FLIP algorithm significantly improve interobserver variations among pathologists at different levels of hepatopathology expertise.[29]

Differentiation between NAFLD and alcoholic liver disease

One may be asked by clinicians for the underlying aetiology of steatosis/steatohepatitis in a liver biopsy from a patient with concurrent drinking history and metabolic risk factors. Can pathological examination confidently differentiate between NAFLD and ALD? In most occasions, there is a considerable overlap between NAFLD and ALD at both the morphologic and clinical levels. Although ALD tends to have more Mallory-Denk bodies, satellitosis (hepatocyte with Mallory-Denk body surrounded by neutrophils) and less glycogenated nuclei, these changes are not unique to ALD. Few pathological lesions specific to ALD include acute foamy degeneration, sclerosing hyaline necrosis, acute/chronic cholestasis and veno-occlusive disease but they are present in a small portion of patients with ALD. The key distinguishing feature is in fact the amount of alcohol consumption obtained from clinical history. Low level of alcohol intake is beneficial to patients with NAFLD by reducing risk of developing steatohepatitis and fibrosis.[1] Intake levels of two standard drinks (20 g ethanol daily/140 g weekly) for men and one standard drink daily (70 g weekly) for women are endorsed as the acceptable threshold to define non-alcoholic.[26]

Conclusion

Histological evaluation remains the gold standard for diagnosing NAFLD. 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 patient management 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. Last but not latest, steatosis and even steatohepatitis are not only exceptional to NAFLD and ALD but also found in viral hepatitis C, drug-induced liver injury (e.g. methotrexate, tamoxifen, corticosteroid), Wilson disease and various metabolic liver diseases. Careful pathological examination, as well as good communication with clinicians, and proper correlation with clinical and laboratory parameters are essential for correct diagnosis of NAFLD and all other medical liver diseases.

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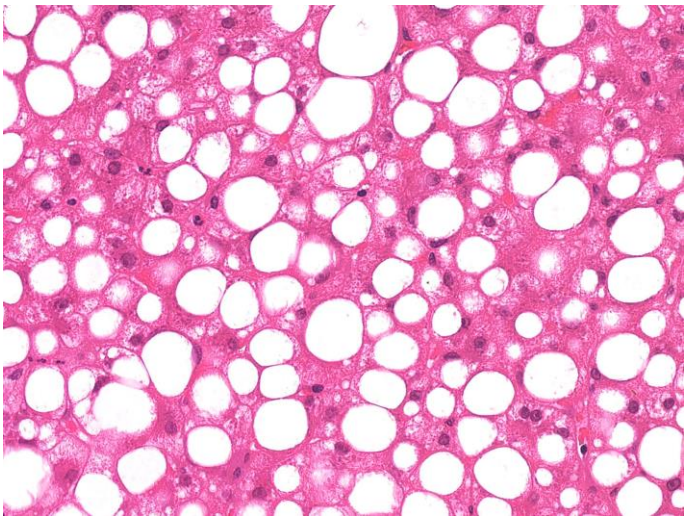
Figure 1: Macrovesicular steatosis.

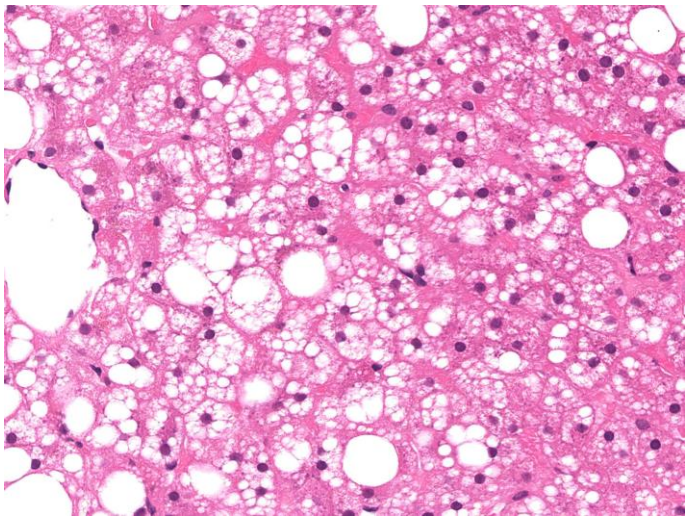
Figure 2: Macrovesicular steatosis with small to medium-sized fat droplets.

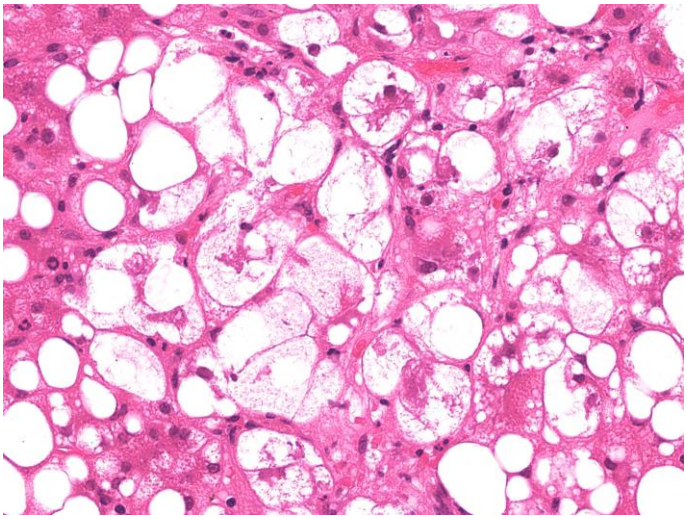
Figure 3: Ballooned hepatocytes with Mallory-Denk bodies

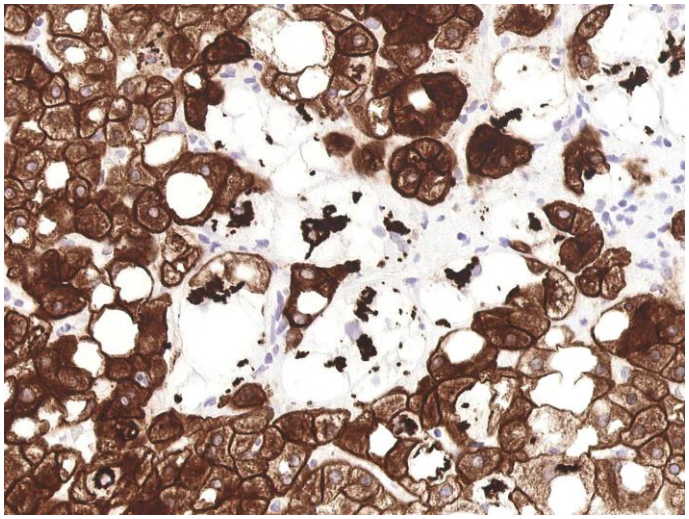
Figure 4: Ballooned hepatocytes are characterized by loss of cytoplasmic expression of CK8/18, whereas residual immunoreactivity is confined to their Mallory-Denk bodies. (Immunohistochemistry of CK8/18)

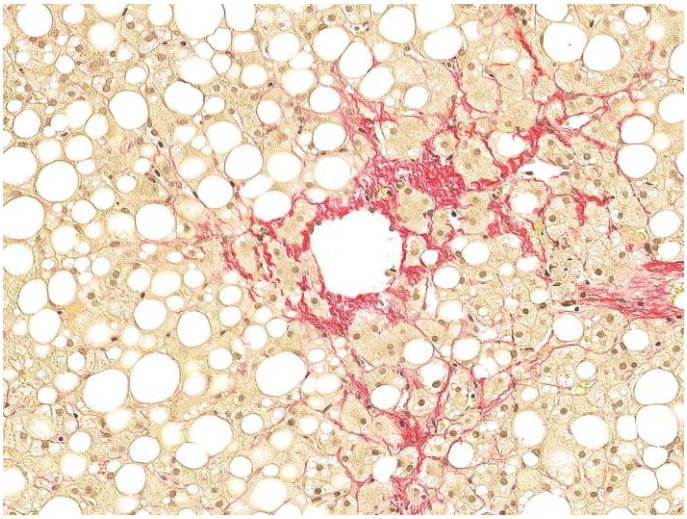

Figure 5: Perivenular and pericellular fibrosis. (Sirius red stain)

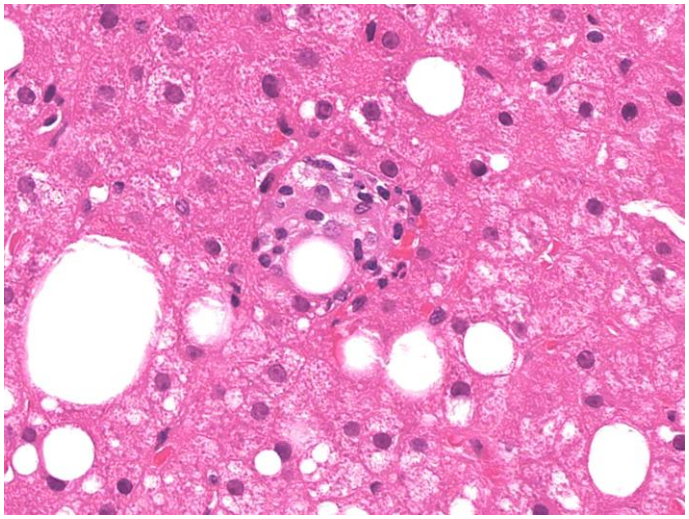

Figure 6: Lipogranuloma.

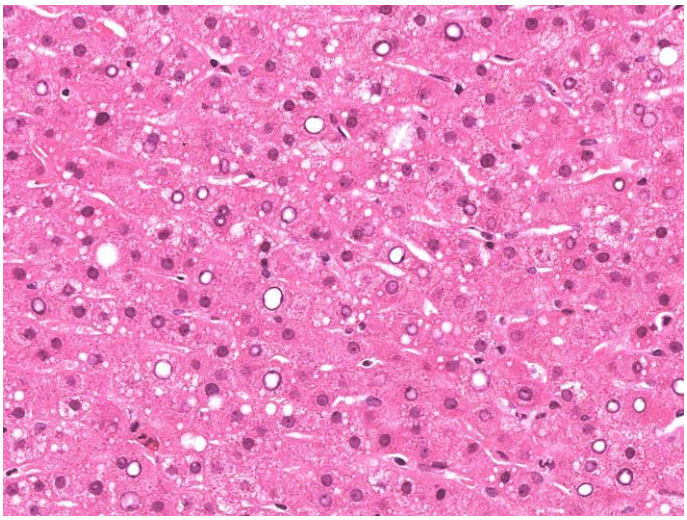

Figure 7: Glycogenated nuclei.

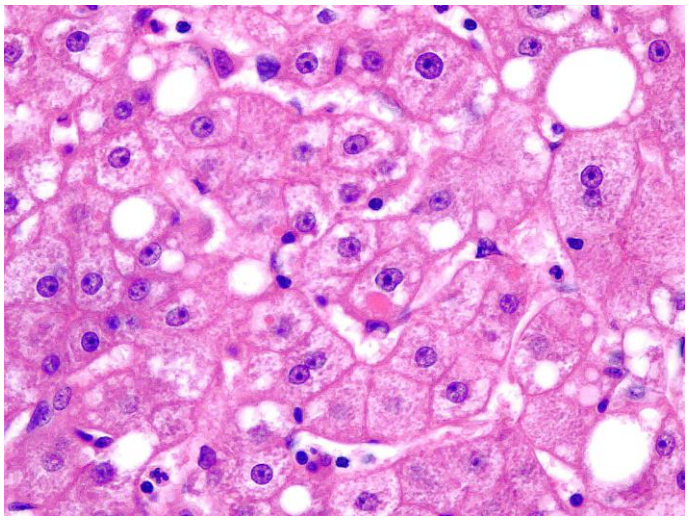

Figure 8. A giant mitochondrion.

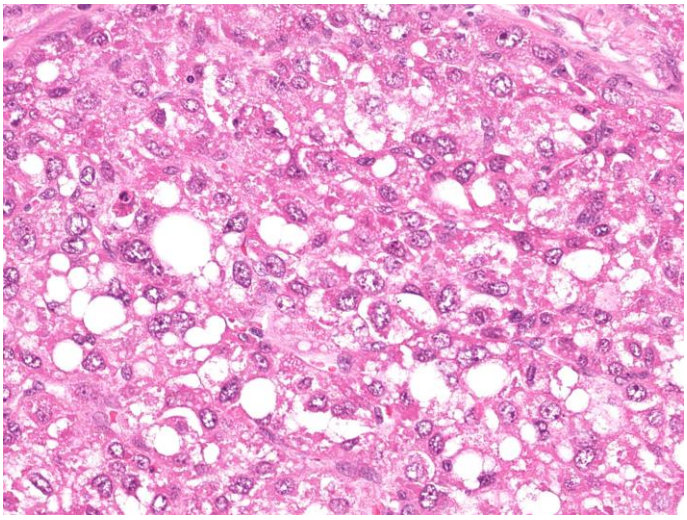

Figure 9: Hepatocellular carcinoma with steatohepatitic features.

Table 1: NAFLD Activity Score (NAS) and fibrosis stage by NASH-CRN.[10]

NAFLD Activity Score			
Score	Steatosis	Lobular inflammation	Ballooning degeneration
0	<5%	None	None
1	5-33%	<2 foci/20x field	Few
2	>33-66%	2-4 foci/20x field	Many
3	>60%	>4 foci/20x field	

Fibrosis Score	
Stage	Histological findings
1a	Mild pericellular fibrosis (only seen on connective tissue stain)
1b	Moderate pericellular fibrosis (readily seen on H&E)
1c	Portal/periportal fibrosis without pericellular fibrosis
2	Pericellular and portal/periportal fibrosis
3	Bridging fibrosis
4	Cirrhosis

Table 2: SAF (steatosis, activity, fibrosis) score and FLIP algorithm.[29]

SAF (steatosis, activity, fibrosis) Score			
Steatosis	Steatosis		
S0	<5%		
S1	5-33%		
S2	>33-66%		
S3	>60%		
Activity	Score	Lobular inflammation (LI)	Ballooning degeneration (BD)
A0-4 (LI+BD)	0	None	None
	1	≤2 foci/20x field	Hepatocytes with a rounded shape and pale cytoplasm usually reticulated. Size is quite similar to that of normal hepatocytes
	2	>2 foci/20x field	Hepatocytes with a rounded shape and pale cytoplasm usually reticulated. Some cells are twice of the size of normal hepatocytes
Fibrosis	Histological findings		
F1a	Mild pericellular fibrosis (only seen on connective tissue stain)		
F1b	Moderate pericellular fibrosis (readily seen on H&E)		
F1c	Portal/periportal fibrosis without pericellular fibrosis		
F2	Pericellular and portal/periportal fibrosis		
F3	Bridging fibrosis		
F4	Cirrhosis		

FLIP Algorithm			
Steatosis	Ballooning degeneration	Lobular inflammation	Diagnosis
0	0, 1 or 2	0, 1 or 2	Not NAFLD
1, 2 or 3	0	0, 1 or 2	NAFLD
1, 2 or 3	1 or 2	0	NAFLD
1, 2 or 3	1 or 2	1 or 2	NASH