*Title*

# Hepatocytic ballooning in non-alcoholic steatohepatitis: dilemmas and future directions.

**Short Title:** Ballooning in NASH

**Authors:**

Yang-Yang Li[1], Tian-Lei Zheng[2,3], Shu-Yuan Xiao[4], Peng Wang[5], Wen-Jun Yang[6], Li-Lin Jiang[7], Li-Li Chen[8], Jun-Cheng Sha[9], Yi-Jin[1], Sui-Dan Chen[1], Christopher D. Byrne[10], Giovanni Targher[11], Jian-Min Li[1], Ming-Hua Zheng[8,12,13*]

**Affiliations:**

[1]Department of Pathology, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China;

[2]Artificial Intelligence Unit, Department of Medical Equipment Management, Affiliated Hospital of Xuzhou Medical University, Xuzhou, China;

[3]School of Information and Control Engineering, China University of Mining and Technology, Xuzhou, China;

[4]Department of Pathology, Shanghai Jiahui International Hospital, China;

[5]Department of Pathology, Beijing Ditan Hospital, Capital Medical University, China;

[6]Department of Pathology, the Affiliated Hospital of Hangzhou Normal University, Hangzhou, China;

[7]Department of Pathology, Wuxi Fifth People's Hospital, Wuxi, China;

[8]MAFLD Research Center, Department of Hepatology, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China;

[9]Department of Interventional Radiology, Affiliated Hospital of Xuzhou Medical University, Xuzhou, China;

[10]Southampton National Institute for Health Research Biomedical Research Centre, University Hospital Southampton, and University of Southampton, Southampton General Hospital, Southampton, UK;

[11]Section of Endocrinology, Diabetes and Metabolism, Department of Medicine, University of Verona, Verona, Italy;

[12]Institute of Hepatology, Wenzhou Medical University, Wenzhou, China;

[13]Key Laboratory of Diagnosis and Treatment for the Development of Chronic Liver Disease in Zhejiang Province, Wenzhou, China.

***Corresponding author:**

Ming-Hua Zheng, MD, PhD

MAFLD Research Center, Department of Hepatology, the First Affiliated Hospital of Wenzhou Medical University; No. 2 Fuxue Lane, Wenzhou 325000, China.

E-mail: zhengmh@wmu.edu.cn; fax: (86) 577-55578522; tel: (86) 577-55579611.

**Word count: 3841**

**Number of figures and tables:** 3 figures and 2 tables

**Abbreviation list:**

NASH, non-alcoholic steatohepatitis; H&E, hemotoxylin and eosin; MDBs, Mallory-Denk bodies; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH-CRN, NASH Clinical Research Network; ER, endoplasmic reticulum; UPR, unfolded protein response; PERK, Protein kinase RNA-like ER kinase; eIF2α, eukaryotic initiation factor 2 alpha; NF-κB, nuclear factor kappa B; IF, intermediate filament; keratin 8/18, K8/18; ATG7, autophagy-related 7; VAS, visual analog scale; HSP, heat shock protein; SHh, sonic Hedgehog; HSCs, hepatic stellate cells; TAC, total antioxidant capacity; PIVENS, Pioglitazone versus Vitamin E versus Placebo for the Treatment of Nondiabetic Patients; MicroS, microvesicular steatosis; SAF, steatosis, activity, fibrosis; AI, artificial intelligence; ML, machine learning; SHG, second harmonic generation; TPEF, two-photon excitation fluorescence; CNN, convolutional neural network; VGG, visual geometry group; MLP, multilayer perceptron.

**Competing interests:** All authors have no conflicts of interest.

**Funding source:**

This work was funded by grants from the National Natural Science Foundation of China (82070588), High Level Creative Talents from Department of Public Health in Zhejiang Province (S2032102600032) and Project of New Century 551 Talent

Nurturing in Wenzhou. GT is supported in part by grants from the School of Medicine, University of Verona, Verona, Italy. CDB is supported in part by the Southampton NIHR Biomedical Research Centre (NIHR203319), UK.

**Author Contributions:**

Ming-Hua Zheng: Study supervision and funding acquisition;

Yang-Yang Li: Study concept and design;

Tian-Lei Zheng and Jun-Cheng Sha: Formal analysis;

Shu-Yuan Xiao, Peng Wang, Wen-Jun Yang, Li-Lin Jiang: Review and editing;

Li-Li Chen: Specifically visualization presentation;

Yi-Jin, Sui-Dan Chen, Jian-Min Li: Acquisition of data;

Christopher D. Byrne and Giovanni Targher: Writing-review and editing.

All authors contributed to the manuscript for important intellectual content and approved the submission.

**Acknowledgements:** None.

**Abstract**

Hepatocytic ballooning is a key histological feature in the diagnosis of non-alcoholic steatohepatitis (NASH) and is an essential component of the two most widely used histological scoring systems for diagnosing and staging non-alcoholic fatty liver disease (NAFLD) [namely, the NAFLD activity score (NAS), and the steatosis,

activity, and fibrosis (SAF) scoring system]. Due to the increasing incidence of NASH globally, the diagnostic challenges of hepatocytic ballooning are unprecedented. Despite the clear pathological concept of hepatocytic ballooning, there are still challenges in assessing hepatocytic ballooning in “real life” situations. Hepatocytic ballooning can be confused with cellular edema and microvesicular steatosis. Significant inter-observer variability does exist in assessing the presence and severity of hepatocytic ballooning. In this review article, we describe the underlying mechanisms associated with hepatocytic ballooning. Specifically, we discuss the increased endoplasmic reticulum stress and the unfolded protein response, as well as the rearrangement of the intermediate filament cytoskeleton, the appearance of Mallory-Denk bodies and activation of the sonic Hedgehog pathway. We also discuss the use of artificial intelligence in the detection and interpretation of hepatocytic ballooning, which may provide new possibilities for future diagnosis and treatment.

**Key Points:**

1. Significant inter-observer variability exists in assessing the presence and severity of hepatocytic ballooning, which may be reduced with relevant training and preparation.
2. We describe the underlying mechanisms associated with hepatocytic ballooning and the associations between hepatocytic ballooning and fibrosis, with other clinical phenotypes.
3. Artificial intelligence (AI) in the detection and interpretation of hepatocytic ballooning may provide new possibilities for the future diagnosis and improved

treatment of NASH.

**Key words:** Hepatocytic ballooning; Keratin 18; Sonic Hedgehog; Fibrosis; Artificial intelligence

## 1. Introduction

"Hepatocytic ballooning" is a widely used term in liver histopathology that indicates hepatocyte degeneration associated with enlargement, swelling, rounding and characteristic reticulated cytoplasm. Hepatocytic ballooning is commonly observed not only in non-alcoholic steatohepatitis (NASH), but also in alcoholic steatohepatitis, viral hepatitis, cholestatic liver diseases, and certain drug-induced liver injuries. Hepatocytic ballooning is a unique form of hepatocyte injury. These cells are typically large, round, and usually have a diameter about 1.5-2 times greater than normal hepatocytes, and have a pale staining reticulated cytoplasm, rendering a "spider web" appearance by hemotoxylin and eosin (H&E) staining, often found near steatotic hepatocytes or areas with perisinusoidal fibrosis.

Hepatocytic ballooning has been associated with greater severity of liver disease and higher risk of developing liver-related complications, cardiovascular disease and chronic kidney disease.[1, 2] Matteoni et al.[3] reported that liver-related deaths occurred more frequently in patients in whom liver biopsies showed hepatocytic ballooning, Mallory-Denk bodies (MDBs) or fibrosis.

Enlarged ballooned hepatocytes with obvious MDBs may be readily identified on liver histology specimens. However, in clinical practice, expert liver pathologists recognize and interpret multiple visual cues when assessing the presence of hepatocytic ballooning. Ballooning degeneration is not specifically defined in the

histological non-alcoholic fatty liver disease (NAFLD) activity score (NAS) proposed by the NASH Clinical Research Network (NASH-CRN), nor there is guidance for scoring severity of hepatocytic ballooning beyond the generic descriptors “few” or “many”. In addition, it is not always easy to distinguish between hepatic edema and ballooning, because cellular swelling is a continuous process. For example, the new NASH-CRN histological scoring system uses an expanded five-tier ballooning score in its database and one component of this newly expanded scoring system, i.e., the presence of non-classical ballooning, is used for the hepatic edema and ballooning, which is difficult to distinguish.

## 2. Hepatocytic ballooning in NASH: possible mechanisms

### 2.1 Mitochondrial ultrastructural abnormalities

The main feature of hepatocytic ballooning is a high degree of cellular swelling. Water retention in cells leads to an increase in volume, which is achieved by the hepatocyte $Na^+/K^+$ pump. The main causes of water retention are hypoxia, lack of substrates for ATP synthesis; oxidation and destruction of enzymes (ATPase) and infection. Hepatocyte mitochondria are damaged and swollen and form giant mitochondria. A decrease in mitochondrial activity results in a reduction in intracellular ATP and dysfunction of the $Na^+/K^+$ pump on membranes, which causes an increase in intracellular $Na^+$ and water-sodium retention. The cytoplasm has a highly spongy matrix, the microvilli are distorted and lost, and the nuclei are expanded. Finally, the classical ballooned hepatocytes develop. Mitochondrial

ultrastructural abnormalities have been noted with borderline NASH and definite NASH.[4] Abnormalities in ultrastructural morphology and function of mitochondria linked with increasing NAFLD severity are likely to explain the higher mitochondrial $H_2O_2$ emission as well as the greater lobular inflammation and hepatocytic ballooning in NASH **(see schematic Figure 1 which illustrates the evolution of hepatocytic ballooning).**[5]

## 2.2 Endoplasmic reticulum stress activates the unfolded protein response

In NASH, the damaged hepatocytes undergo a continuous transition from mild edema to ballooning and then to lysis and necrosis. Cellular edema can be quite marked, with few long-term consequences if the injurious stimuli are removed.[6] These stimuli include lipotoxicity, abnormal lipid and glucose metabolism, hepatocyte mitochondrial dysfunction, as well as oxidative and endoplasmic reticulum (ER) stress and increased production of proinflammatory cytokines, whose unwarranted activation may exacerbate hepatocytic ballooning, as illustrated in **Figure 1.**[7]

The ER is an essential subcellular compartment responsible for the synthesis and folding of proteins that traffic through the secretory pathway in the cell. Protein folding is sensitive to alterations in ER homeostasis, including $Ca^{2+}$ levels and energy, nutrient availability, as well as the protein-folding load in the ER. Perturbations in these pathways may interfere with protein folding in the ER, thus leading to proteotoxic ER stress, which in turn may activate the unfolded protein response

(UPR). The UPR is initially an adaptive signaling pathway, which elicits global cellular changes (such as attenuation of translation) and activates specific pathways of protein folding for restoring ER homeostasis. Protein kinase RNA-like ER kinase (PERK) is one of protein kinases that phosphorylates eukaryotic initiation factor2 alpha (eIF2α), and the ability of PERK to induce eIF2α phosphorylation can regulate not only translation but also activation of the nuclear factor kappa B (NF-κB).[8] Thus, activation of this UPR branch links disruption of ER homeostasis to low-grade inflammation (mostly via NF-κB activation) and redox balance activation **(see Figure 1).**

### 2.3 Damage to intermediate filaments of the cytoskeleton

Hepatocytic ballooning is associated with accumulation of hepatic fat droplets, ER expansion and damage to intermediate filaments (IF) of the cytoskeleton, as evidenced by loss of cytoplasm and increased MDB formation **(Figure 2)**. Ballooned hepatocytes exhibit increased ER stress and represent an extreme morphological manifestation of abnormal protein turnover. As cellular swelling progresses, clear spaces or vacuoles may form in the cytoplasm of hepatocytes; these usually represent dilated portions of the ER and/or Golgi apparatus. If severe enough, ER cisternae may be ruptured, and “cytoplasmic lakes” not confined by membranes may form. If solubilized protein also accumulates in these lakes, the clear vacuole may bind eosin and be visualized histologically as a hyaline droplet. This histologic change is termed “vacuolar degeneration” or “vacuolar change”. Sometimes vacuoles are not present in

the affected hepatocytes; instead, the cytoplasm is “diluted”, and organelles are widely dispersed within the rarefied (electron-lucent) cytoplasm. These changes can be attributed to damage to the cytoskeleton.

The structure of MDBs was first described in the early nineteenth century by Dr. Mallory as hyaline inclusion bodies in alcoholic steatohepatitis. MDBs are protein aggregates, consisting of keratin 8/18 (K8/18), ubiquitin and p62. MDBs can be easily recognized on H&E staining and can be marked with K18 staining **(Figure 2 C-D)**; MDBs are present in approximately 30% of NASH liver biopsies and can vary in size from tiny little cytoplasmic granules to large cytoplasmic inclusions. With an increased oxidative stress, abnormal cytokeratin may accumulate along with HSPs, ubiquitin, tissue transglutaminase, proteasome subunits, tubulin, and p62. Young, tiny and then well-formed MDBs become evident in ballooned hepatocytes. Impairment of autophagy, possibly involving defective clearance of damaged mitochondria and protein aggregates, are likely to lead to protein loss-of-function. In individuals with NAFLD and advanced fibrosis or hepatocellular carcinoma, Baselli et al.[9] found that rare mutations in a gene called autophagy-related 7 (ATG7) led to accumulation of p62 and ballooning degeneration, and increased the risk of developing severe liver disease. ATG7 genetic variants caused protein dysfunction and impairment of autophagy (thus facilitating hepatocyte ballooning and inflammation). In particular, the most frequent variant p.V471A was associated with an increased risk of hepatocytic ballooning, irrespective of steatosis grade. From these studies, it is

reasonable to assume that there is a close interconnection between autophagy and hepatocytic ballooning, which needs to be better clarified in future studies. Besides, the presence of MDBs may help to identify ballooned hepatocytes, thus supporting the notion that MDBs are important markers for diagnosing NASH. Pai et al.[10] reported that the correlations with disease activity visual analog scale (VAS) were strongest for hepatocytic ballooning and MDBs. Based on these studies, the value of MDBs may not be discounted for the diagnosis of hepatocytic ballooning and disease activity assessment in the future.

**2.4 Hepatocytic ballooning, fibrosis and clinical prognosis**

NAFLD prognosis is strongly associated with the histological severity of liver fibrosis.[11] Previous studies have shown that hepatocytic ballooning is also associated with greater fibrosis severity and cytoskeletal damage.[12, 13] Hepatocytes undergoing ER stress produce sonic Hedgehog (SHh) ligands, which act as paracrine profibrogenic factors for Hedgehog-responsive stromal cells. Injured and dying hepatocytes release SHh ligands, which promote hepatic inflammation and increased fibrogenesis by activated hepatic stellate cells (HSCs).[14] These effects may explain why the stage of liver fibrosis correlates with hepatocytic ballooning in NASH.[15] Interestingly, hepatocytic ballooning is usually noted first in zone 3, near the central vein **(Figure 2 A-B)**. There may be lobular inflammation and perisinusoidal fibrosis in the immediate vicinity.[16, 17] Trichrome staining showed that ballooned hepatocytes accumulate in areas of matrix deposition, and the amount of SHh-positive hepatocytes

correlates with the degree of hepatocytic ballooning and fibrosis stage.[18] As summarized in **Table 1**, several studies have explored the association between hepatocytic ballooning and fibrosis stage, supporting a link between these two histopathological features.[18-24] According to the aforementioned studies, SHh not only identifies hepatocytic ballooning, but may also predict liver fibrosis undetected by hepatopathologist. However, there are few applications of SHh to the liver biopsy nowadays.

### 2.5 Association between hepatocytic ballooning and clinical phenotypes

Hepatocytic ballooning has been reported to be associated with some clinical phenotypes. Compared with those without ballooning, patients with hepatocytic ballooning were more likely to have overweight/obesity, dyslipidemia, abnormal glucose tolerance, insulin resistance, and higher serum biomarkers of necroinflammation.[25] Some studies have also reported a strong association between hepatocytic ballooning, stage of fibrosis, and adverse liver-related outcomes.[3, 21, 26, 27] In particular, patients with hepatocytic ballooning, with or without coexisting fibrosis and MDBs, had an increased risk of adverse liver-related outcomes. In addition, although a number of histopathological features (e.g., ballooning, portal inflammation, and MDBs) seemed to be associated with liver-related mortality in univariate analyses, however, the histologic stage of fibrosis remained the strongest predictor of liver-related mortality in multivariate analyses.[27] Hepatocytic ballooning was associated with advanced fibrosis in some studies, but it was not a significant

predictor of mortality in NAFLD.[21]

Some studies have reported that NASH patients with higher levels of dietary total antioxidant capacity (TAC) had fewer ballooned hepatocytes than those with lower dietary TAC levels, thereby suggesting a beneficial role of foods naturally high in antioxidant capacity in reducing free radical production and oxidative stress.[28] Data from the Pioglitazone versus Vitamin E versus Placebo for the Treatment of Nondiabetic Patients (PIVENS) trial showed that Vitamin E supplementation reduced hepatocytic ballooning and serum aminotransferase levels in patients with NASH, possibly due to Vitamin E-induced reduction in stress-related mechanisms triggering hepatocyte SHh production. These results suggest that given the strong association between ballooning degeneration and liver-related clinical outcomes, it could be appropriate also to add this histologic feature to other secondary endpoints in future NASH clinical trials.[29]

In the PIVENS trial it was also reported that a proteomics-based signature of individual features of NAFLD could efficiently detect hepatocytic ballooning, thus supporting further validation of these proteomic models to enable a “liquid biopsy”-based assessment of NAFLD.[30] It is expected that proteomic models and tissue biopsy will combine to achieve more accurate clinical diagnosis and drug efficacy measurement in future clinical trials for NASH. It is also reasonable to assume that through a better understanding of hepatocytic ballooning, a more solid evidence-base

will be established for supporting the use of a “liquid biopsy”-based assessment of NAFLD.

### 2.6 Hepatocytic ballooning: is it reversible or irreversible?

Cellular edema is a reversible injury that may develop into irreversible extreme cellular edema, lysis and necrosis. Why is there a distinction between mild-moderate edematous degeneration and ballooning degeneration in this pathological process? We do not know exactly whether hepatocytic ballooning represents an adaptive (physiological, reversible) process, or whether degenerative changes (pathological, most likely irreversible) of hepatocytes can occur because of the changed environment. Swelling of hepatocytes occurs because of different stressors, particularly oxidative stress. It has been reported that mild volume changes (up to 10% increase in the hepatocyte volume) occur physiologically and are adaptive. However, high-grade swelling (causing up to nearly 30% increase in the volume of hepatocytes) is a pathological process that induces hepatocyte apoptosis and necrosis.

## 3. Dilemmas in hepatocytic ballooning diagnosis

The identification of hepatocytic ballooning is required to exclude the interference of other factors in the pathological diagnosis, such as microvesicular steatosis (MicroS) of hepatocytes, which morphologically manifests as multiple tiny fat droplets around the nucleus, and which may also occur in some liver diseases such as acute fatty liver in pregnancy, Reye’s syndrome or after the intake of certain drugs/toxins.

Liver biopsy preparation can also affect the detection of hepatocytic ballooning. Problems with the sectioning technique include the presence of foreign matter (fungal hyphae), knife marks that affect the image recognition of cells, excessive staining, and too little liver tissue leading to a negative diagnosis. Digital imaging issues, including unclear scans, are unavoidable in real life and may influence how hepatocytic ballooning is defined, and result in differences across different hospitals and institutions.

In previously published studies, hepatocytic ballooning was the most challenging histologic feature in terms of inter-observer variability.[31, 32] The κ statistics for hepatocytic ballooning ranged from 0.43-0.66 in the histological evaluation systems. This difference exists not only between general pathologists and hepatopathologists,[33] but also amongst expert hepatopathologists. A recent study showed that both the intra-rater and inter-rater reliability of existing histologic NAFLD indices and fibrosis staging systems may be improved with relevant training and preparation of expert hepatopathologists.[34] Although the inter-observer variability cannot be completely eliminated, the results of this study show promise in improvement of histological diagnosis of hepatocytic ballooning.

## 4. Current Status and Research Opportunities

### 4.1 Value of hepatocytic ballooning in histological scoring systems

Hepatocytic ballooning is a key component of the histological scoring systems that are widely used to diagnose and stage NAFLD, including the NASH-CRN NAS, and the steatosis, activity, and steatosis, activity, fibrosis (SAF) score. These two histological scoring systems grade hepatocytic ballooning on a 3-point scale (0-2), but there are subtle differences. In NAS scoring system, hepatocytic ballooning is graded from zero to two as follows: grade 0: no ballooned hepatocytes; grade 1: few ballooned hepatocytes; and grade 2: many ballooned hepatocytes. Ballooned hepatocytes are the swollen hepatocytes which are 1.5 times the size of adjacent normal hepatocytes with voluminous clear to rarified cytoplasm and small bits of eosinophilic material. A NAS score of less 3 and greater than 5 shows a good correlation with the histologic diagnosis of “not NASH” and “definite NASH”, respectively. However, due to missing hepatocytic ballooning, which is the key feature of NASH, the diagnosis would be steatosis with lobular inflammation rather than NASH. A failing of the NAS score is not having an essential pre-requisite for hepatocytic ballooning. In the SAF scoring system, detection of hepatocytic ballooning is a requirement for NASH diagnosis, and is graded from 0 to 2 as follows: grade 0: normal hepatocytes; grade 1: presence of clusters of hepatocytes with a rounded shape and pale cytoplasm usually reticulated; and grade 2: same as grade 1 with some enlarged hepatocytes, at least 2-fold that of normal cells. Thus the SAF scoring system focuses on cellular changes rather than quantity.

According to a flowchart proposed by Japanese researchers, the identification of

ballooned hepatocytes can be improved by the presence of transparent cytoplasm in hepatocytes, irregular granular structures within the cytoplasm, expanded and rounded cells, and whether fatty degeneration is the significant pathogenic change.[35] Given the importance of improving the hepatocyte ballooning assessment in NASH as suggested by a systematic Research and Development/University of California Los Angeles study, Pai et al. assessed the reliability of seven hepatocytic ballooning items, including the NAS (0-2), the SAF scoring system (0-2), the Goodman classification (0-3), the expanded NAS ballooning item (0-4), a ballooning item based on percent involvement of ×20 fields (0-3), an alternate expanded ballooning item based on clusters of ballooned cells (0-4), and a ballooning VAS.[10] In this study, the expanded NAS ballooning criterion was found to be most robust for identifying hepatocyte ballooning severity; it was graded as follows: grade 0: none, grade 1: non-classical ballooned hepatocytes (either few or many), grade 2: few classical ballooned hepatocytes, grade 3: many classical ballooned hepatocytes, but not severe; and grade 4: severe ballooned hepatocytes (many classical ballooned cells visible from ×40 fields). Further formal validation of this expanded ballooning criterion is required in larger cohorts of NAFLD patients.

**4.2 Hepatocytic ballooning and MicroS**

In NASH, hepatic steatosis is usually due to macrovesicular steatosis that refers to hepatocytes with a single large intracytoplasmic fat droplet or smaller well-defined droplets, expanding the cell and displacing the nucleus to cell periphery.[36] MicroS,

which is typically characterized by distended hepatocytes with foamy appearing cytoplasm and small lipid vesicles, may also be detectable in NASH.[37] Tandra et al. reported that presence of MicroS was associated with greater severity of steatosis, ballooning injury, MDBs and megamitochondria, as well as higher levels of NAS score, greater stages of fibrosis, and presence of borderline or definite NASH.[38] The coexistence of MicroS and hepatocytic ballooning was found to be a strong predictor for the development of hepatocyte damage caused by oxidative stress.[39] To date, there is very little information about the prevalence and significance of MicroS in NAFLD. Celebi et al. reported that the prevalence of MicroS was about 30% in NAFLD.[39] Further studies are needed to better understand the role of MicroS in the natural history of NAFLD.

**4.3 Immunohistochemistry for detecting ballooned hepatocytes**

IF of the cytoskeleton are crucial for stabilization and topographical organization of a cell and its organelles. In hepatocytes, IFs consist of K18 and K8 noncovalently assembled in an equimolar ratio as heteropolymers. Studies have shown that ballooned hepatocytes show absent or reduced cytoplasmic K18 staining, whereas normal-sized hepatocytes are K18 positive. A combination of oil red O staining with K18 staining has proved to be a reliable method for detecting ballooned hepatocytes. Guy et al.[40] showed that K8/18 plus ubiquitin staining can improve detection of hepatocyte injury in NAFLD. However, hepatocyte injury not only includes ballooned hepatocytes but also some normal-sized hepatocytes. These authors showed that

K8/18 plus ubiquitin staining technique may be useful for revealing hepatocytes which are not yet ballooned, but manifest loss of typical hepatocyte keratins or contain ubiquitin aggregates. Kusano et al.[41] found that combined SHh and K8/18 immunohistochemistry are useful for detecting ballooned hepatocytes (regardless of background liver disease) and improving pathological diagnosis accuracy. Anti-K8/18 negative hepatocytes are not the same as balloon-shaped hepatocytes. From our observations, it was possible to detect hepatocytic ballooning, a few of non-classical ballooned hepatocytes or even normal-sized hepatocytes using K18 staining **(Figure 3).** Thanks to the availability of more markers to detect ballooned hepatocytes, it will become easier to identify and deeply understand this histopathological change. Further studies of IF and K8/18 may improve the detection of hepatocytic ballooning or cellular injury in NASH, since cellular volume might not be the gold standard for ballooned hepatocytes in future studies.

### 4.4 Hepatocytic ballooning and machine learning

The use of artificial intelligence (AI) in health and pathology is growing exponentially. Whereas the histological scoring system of "NAS" is accepted worldwide, it still has its drawbacks.[31] First, it relies on expert hepatopathologists whose availability is limited. In addition, the task of histopathological review is time-consuming and tiring, thereby affecting the diagnostic accuracy. Second, even amongst expert hepatopathologists, there is poor agreement regarding the number of ballooned hepatocytes seen on the same digitized histology images.

Currently, there are few studies that focussed on quantifying hepatocytic ballooning by AI algorithms,[42, 43] and there is no complete agreement on hepatocytic ballooning as a criterion **(Table 2)**. One of the first machine learning (ML) classification analysis of hepatocytic ballooning was undertaken by Vanderbeck et al. in 2015[44] The ML model proposed by these authors had a good accuracy for identifying hepatocytic ballooning with a ROC curve of 0.983. Teramoto et al. implemented and tested a topological data analysis methodology combined with ML techniques and applied this to the classification of tissue images into NAFLD subtypes using the Matteoni classification in liver biopsies.[42] The authors obtained accuracy rates of more than 90% for the classification between NASH and non-NASH patient groups. These two studies showed that application of ML methods for identifying hepatocytic ballooning is feasible, but its reproducibility should be further tested in larger cohort studies. Recently, Forlano et al.[45] used ML to develop fully automated software for quantification of steatosis, inflammation, ballooning, and fibrosis in biopsy specimens from 246 patients with NAFLD. The results from the software analysis correlated with those from histopathologists, with high levels of inter-observer and intra-observer agreement (ranging from 0.95 to 0.99). The results of this study also pointed out that there was no “gold standard” for quantitative analysis of hepatocytic ballooning at this stage, so its clinical application value has yet to be confirmed. In another study, Liu et al.[46] evaluated a second harmonic generation/two-photon excitation fluorescence (SHG/TPEF) imaging-based tool to provide an automated

quantitative assessment of histological features pertinent to NASH. The authors developed and validated qFIBS, a computational algorithm that quantifies key histological features of NASH. Performance of qFIBS was best when assessing degree of steatosis and fibrosis, but performed less well when distinguishing severe inflammation and higher ballooning grades. To further demonstrate the value of ML for the detection of ballooning lesions, Brunt et al.[43] invited nine expert hepatopathologists to label only hepatocytic ballooning in liver tissue, and later invited these expert hepatopathologists again by rotating and mirroring images from the same database, and found that there was a substantial divergence in hepatocytic ballooning identified amongst these expert hepatopathologists. Conversely, Heinemann et al.[31] performed an experimental study in rats and mice, using a pathologist to label liver biopsy images of each animal with ballooning lesions, and used them for semi-quantitative analysis. These authors constructed four types of convolutional neural networks (CNN) based on different deep learning network architectures in order to identify ballooning lesions, inflammation, steatosis, and fibrosis. A 94% identification rate was achieved for ballooning lesions. Using a fully automated quantitative deep learning network based on CNN to analyze liver pathological images, Arjmand et al.[47] reported that the constructed CNN model achieved better classification accuracy than visual geometry group (VGG)-16 (94%) and multilayer perceptron (MLP) (90.3%), while AlexNet emerges as the most efficient classifier (97%). In 2021, Taylor et al.[48] used liver biopsy samples from three clinical trials to construct and validate the feasibility of deep learning for automated

quantitative analysis of key histological features of NASH. These authors confirmed the good diagnostic performance of automatic quantitative analysis of deep learning for NASH assessment. Finally, Qu et al.[49] also developed a CNN network for predicting NAFLD activity score and fibrosis staging. They found the best diagnostic performance for hepatic steatosis (AUC=90.5%) and a relatively good performance for diagnosing hepatocytic ballooning (AUC=81%).

The use of AI algorithms may be useful for improving the assessment of ballooned hepatocytes, although AI algoirthms are only as good as the quality of the inputted data. Thus the development of better AI tools will require also improved understanding of hepatocytic ballooning and how these features are perceived, interpreted, and related to clinical prognosis.

## 5. Conclusions, and future perspectives

Histological analysis of liver biopsy samples is essential in the management of NASH patients, especially in terms of diagnosis, definition of severity, and prediction of prognosis. Histologically assessed hepatocyte ballooning is a key feature discriminating NASH from simple steatosis. Since the challenges of studying hepatocytic ballooning are real, we expect breakthroughs in hepatocytic ballooning will improve development of better more precise treatments for NASH in the future. Improved AI-based approaches may also provide a more reliable tool to quantification of ballooned hepatocytes.

## References


1 Zhou X D, J Cai, G Targher, C D Byrne, et al. Metabolic dysfunction-associated fatty liver disease and implications for cardiovascular risk and disease prevention. *Cardiovasc Diabetol*. 2022;21(1):270.

2 Sun D-Q, G Targher, C D Byrne, D C Wheeler, et al. An international Delphi consensus statement on metabolic dysfunction-associated fatty liver disease and risk of chronic kidney disease. *HepatoBiliary Surg Nutr*. 2023.

3 Matteoni C A, Z M Younossi, T Gramlich, N Boparai, et al. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology*. 1999;116(6):1413-1419.

4 Elferink R O The start of a new era in bile physiology. *J Hepatol*. 2004;41(6):902-904.

5 Moore M P, R P Cunningham, G M Meers, S A Johnson, et al. Compromised hepatic mitochondrial fatty acid oxidation and reduced markers of mitochondrial turnover in human NAFLD. *Hepatology*. 2022;76(5):1452-1465.

6 Haschek-Hock W M, C G Rousseaux, M A Wallig ,B Bolon NEW: Haschek and Rousseaux's Handbook of Toxicologic Pathology, Volume 1: Principles and Practice of Toxicologic Pathology, 4th Edition - 2021. *Int J Toxicol*. 2022;41(3):253-254.

7 Afonso M B, R E Castro ,C M P Rodrigues Processes exacerbating apoptosis in non-alcoholic steatohepatitis. *Clin Sci (Lond)*. 2019;133(22):2245-2264.

8 Wang Y C, X Li, Y Shen, J Lyu, et al. PERK (Protein Kinase RNA-Like ER Kinase) Branch of the Unfolded Protein Response Confers Neuroprotection in Ischemic Stroke by Suppressing Protein Synthesis. *Stroke*. 2020;51(5):1570-1577.

9 Baselli G A, O Jamialahmadi, S Pelusi, E Ciociola, et al. Rare ATG7 genetic variants predispose patients to severe fatty liver disease. *J Hepatol*. 2022;77(3):596-606.

10 Pai R K, V Jairath, M Hogan, G Zou, et al. Reliability of histologic assessment for NAFLD and development of an expanded NAFLD activity score. *Hepatology*. 2022;76(4):1150-1163.

11 Angulo P, D E Kleiner, S Dam-Larsen, L A Adams, et al. Liver Fibrosis, but No Other Histologic Features, Is Associated With Long-term Outcomes of Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology*. 2015;149(2):389-397.e310.

12 Amidi F, B A French, D Chung, C H Halsted, et al. M-30 and 4HNE are sequestered in different aggresomes in the same hepatocytes. *Exp Mol Pathol*. 2007;83(3):296-300.

13 Zatloukal K, S W French, C Stumptner, P Strnad, et al. From Mallory to Mallory-Denk bodies: what, how and why? 2007;313(10):2033-2049.

14 Verdelho Machado M ,A M Diehl Role of Hedgehog Signaling Pathway in NASH. *Int J Mol Sci*. 2016;17(6).

15 Gao B, K Sakaguchi, K Matsuura, T Ogawa, et al. In Vitro Production of Human Ballooned Hepatocytes in a Cell Sheet-based Three-dimensional Model. *Tissue Eng Part A*. 2020;26(1-2):93-101.

16 Lai J, H L Wang, X Zhang, H Wang, et al. Pathologic Diagnosis of Nonalcoholic Fatty Liver Disease. *Arch Pathol Lab Med*. 2022;146(8):940-946.
17 Brunt E M, C G Janney, A M Di Bisceglie, B A Neuschwander-Tetri, et al. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol*. 1999;94(9):2467-2474.
18 Rangwala F, C D Guy, J Lu, A Suzuki, et al. Increased production of sonic hedgehog by ballooned hepatocytes. *J Pathol*. 2011;224(3):401-410.
19 Fujii H, Y Ikura, J Arimoto, K Sugioka, et al. Expression of perilipin and adipophilin in nonalcoholic fatty liver disease; relevance to oxidative injury and hepatocyte ballooning. *J Atheroscler Thromb*. 2009;16(6):893-901.
20 Guy C D, A Suzuki, M F Abdelmalek, J L Burchette, et al. Treatment response in the PIVENS trial is associated with decreased Hedgehog pathway activity. *Hepatology*. 2015;61(1):98-107.
21 Younossi Z M, M Stepanova, N Rafiq, L Henry, et al. Nonalcoholic steatofibrosis independently predicts mortality in nonalcoholic fatty liver disease. *Hepatol Commun*. 2017;1(5):421-428.
22 Kakisaka K, Y Suzuki, Y Fujiwara, T Abe, et al. Evaluation of ballooned hepatocytes as a risk factor for future progression of fibrosis in patients with non-alcoholic fatty liver disease. *J Gastroenterol*. 2018;53(12):1285-1291.
23 Kleiner D E, E M Brunt, L A Wilson, C Behling, et al. Association of Histologic Disease Activity With Progression of Nonalcoholic Fatty Liver Disease. *JAMA Netw Open*. 2019;2(10):e1912565.
24 Brunt E M, D E Kleiner, L A Wilson, A J Sanyal, et al. Improvements in Histologic Features and Diagnosis Associated With Improvement in Fibrosis in Nonalcoholic Steatohepatitis: Results From the Nonalcoholic Steatohepatitis Clinical Research Network Treatment Trials. *Hepatology*. 2019;70(2):522-531.
25 Brunt E M, B A Neuschwander-Tetri, D Oliver, K R Wehmeier, et al. Nonalcoholic steatohepatitis: histologic features and clinical correlations with 30 blinded biopsy specimens. *Hum Pathol*. 2004;35(9):1070-1082.
26 Richardson M M, J R Jonsson, E E Powell, E M Brunt, et al. Progressive fibrosis in nonalcoholic steatohepatitis: association with altered regeneration and a ductular reaction. *Gastroenterology*. 2007;133(1):80-90.
27 Younossi Z M, M Stepanova, N Rafiq, H Makhlouf, et al. Pathologic criteria for nonalcoholic steatohepatitis: interprotocol agreement and ability to predict liver-related mortality. *Hepatology*. 2011;53(6):1874-1882.
28 de Oliveira D G, F de Faria Ghetti, A P B Moreira, H H M Hermsdorff, et al. Association between dietary total antioxidant capacity and hepatocellular ballooning in nonalcoholic steatohepatitis: a cross-sectional study. *Eur J Nutr*. 2019;58(6):2263-2270.
29 Sanyal A J, E M Brunt, D E Kleiner, K V Kowdley, et al. Endpoints and clinical trial design for nonalcoholic steatohepatitis. *Hepatology*. 2011;54(1):344-353.
30 Sanyal A J, S A Williams, J E Lavine, B Tetri, et al. Defining the serum proteomic signature of hepatic steatosis, inflammation, ballooning and fibrosis in nonalcoholic fatty liver disease. *J Hepatol*. 2022.
31 Heinemann F, G Birk ,B Stierstorfer Deep learning enables pathologist-like scoring of

NASH models. *Sci Rep*. 2019;9(1):18454.
32 Kuwashiro T, H Takahashi, H Hyogo, Y Ogawa, et al. Discordant pathological diagnosis of non-alcoholic fatty liver disease: A prospective multicenter study. *JGH Open*. 2020;4(3):497-502.
33 Juluri R, R Vuppalanchi, J Olson, A Unalp, et al. Generalizability of the nonalcoholic steatohepatitis Clinical Research Network histologic scoring system for nonalcoholic fatty liver disease. *J Clin Gastroenterol*. 2011;45(1):55-58.
34 Davison B A, S A Harrison, G Cotter, N Alkhouri, et al. Suboptimal reliability of liver biopsy evaluation has implications for randomized clinical trials. *J Hepatol*. 2020;73(6):1322-1332.
35 Sakamoto M, H Tsujikawa, K Effendi, H Ojima, et al. Pathological findings of nonalcoholic steatohepatitis and nonalcoholic fatty liver disease. *Pathol Int*. 2017;67(1):1-7.
36 Brunt E M Nonalcoholic fatty liver disease: what the pathologist can tell the clinician. *Dig Dis*. 2012;30 Suppl 1:61-68.
37 Kleiner D E ,H R Makhlouf Histology of Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis in Adults and Children. *Clin Liver Dis*. 2016;20(2):293-312.
38 Tandra S, M M Yeh, E M Brunt, R Vuppalanchi, et al. Presence and significance of microvesicular steatosis in nonalcoholic fatty liver disease. *J Hepatol*. 2011;55(3):654-659.
39 Celebi G, A F Cicek, H Gurel, H Genc, et al. Microvesicular steatosis: a missed item in the management of nonalcoholic fatty liver disease? *Acta Gastroenterol Belg*. 2020;83(4):565-570.
40 Guy C D, A Suzuki, J L Burchette, E M Brunt, et al. Costaining for keratins 8/18 plus ubiquitin improves detection of hepatocyte injury in nonalcoholic fatty liver disease. *Hum Pathol*. 2012;43(6):790-800.
41 Kusano H, R Kondo, S Ogasawara, M Omuraya, et al. Utility of sonic hedgehog and keratin 8/18 immunohistochemistry for detecting ballooned hepatocytes. *Histopathology*. 2022;80(6):974-981.
42 Teramoto T, T Shinohara ,A Takiyama Computer-aided classification of hepatocellular ballooning in liver biopsies from patients with NASH using persistent homology. *Comput Methods Programs Biomed*. 2020;195:105614.
43 Brunt E M, A D Clouston, Z Goodman, C Guy, et al. Complexity of ballooned hepatocyte feature recognition: Defining a training atlas for artificial intelligence-based imaging in NAFLD. *J Hepatol*. 2022;76(5):1030-1041.
44 Vanderbeck S, J Bockhorst, D Kleiner, R Komorowski, et al. Automatic quantification of lobular inflammation and hepatocyte ballooning in nonalcoholic fatty liver disease liver biopsies. *Hum Pathol*. 2015;46(5):767-775.
45 Forlano R, B H Mullish, N Giannakeas, J B Maurice, et al. High-Throughput, Machine Learning-Based Quantification of Steatosis, Inflammation, Ballooning, and Fibrosis in Biopsies From Patients With Nonalcoholic Fatty Liver Disease. *Clin Gastroenterol Hepatol*. 2020;18(9):2081-2090.e2089.
46 Liu F, G B Goh, D Tiniakos, A Wee, et al. qFIBS: An Automated Technique for Quantitative Evaluation of Fibrosis, Inflammation, Ballooning, and Steatosis in Patients With Nonalcoholic Steatohepatitis. *Hepatology*. 2020;71(6):1953-1966.

47 Arjmand A, C T Angelis, V Christou, A T Tzallas, et al. Training of Deep Convolutional Neural Networks to Identify Critical Liver Alterations in Histopathology Image Samples. *Appl.Sci*. 2020;10(1):42.
48 Taylor-Weiner A, H Pokkalla, L Han, C Jia, et al. A Machine Learning Approach Enables Quantitative Measurement of Liver Histology and Disease Monitoring in NASH. *Hepatology*. 2021;74(1):133-147.
49 Qu H, C D Minacapelli, C Tait, K Gupta, et al. Training of computational algorithms to predict NAFLD activity score and fibrosis stage from liver histopathology slides. *Comput Methods Programs Biomed*. 2021;207:106153.

## Table Legends

**Table 1. Association between hepatocytic ballooning and fibrosis stage in patients with NASH.**

**Table 2. Machine learning algorithms applied to hepatocytic ballooning in NASH.**

## Figure Legends

**Figure 1.** Evolution of hepatocytic ballooning. Due to the metabolism of excess free fatty acids, free cholesterol, ceramides and inflammation, hepatocytes go from normal cell to swelling, experiencing increase in volume, accumulation of large and small fat droplets in the cytoplasm, and increase in ER stress. ER stress leads to ER expansion and ballooning, activates the UPR (HSP precursor), increases HSPs, and eventually forms MDBs, while elevated SHh can activate HSCs, leading to fibrosis, and PERK activates eIF2α, thereby exacerbating cellular lysis and necrosis.

Abbreviations: PERK: protein kinase RNA-Like ER kinase

**Figure 2.** (A) Hepatocytic ballooning is mostly located in zone 3, two ballooned

hepatocytes are large (black arrows), have a diameter more than 30μm (1.5-2 times the normal hepatocyte diameter), round, and have pale staining reticulated cytoplasm rendering a “spider web”, appearance by H&E staining (original magnifications ×100). (B) Ballooning coexisting with lipid droplets can often appear in the same hepatocyte (original magnifications ×200). (C) Lack of K18 staining in four ballooned hepatocytes (red arrows) compared to anti-K18 positive neighboring cells (original magnifications ×200). (D) Anti-K18 staining of ballooned hepatocytes (red arrows) and MDBs (yellow arrow, original magnifications ×400).

**Figure 3.** (A) A cluster of ballooned hepatocytes, including classical and non-classical ballooned cells, is present (original magnifications ×100). (B) Several classical ballooned cells are large (yellow arrows), have a diameter more than 30μm (1.5-2 times the normal hepatocyte diameter), round, and have pale staining reticulated cytoplasm rendering a “spider web”, appearance, whatever, non-classic ballooned cells are similar to normal-sized hepatocytes (red arrows) (original magnifications ×200). (C) It may be efficient to detect hepatocytic ballooning using K18 staining; K18 staining technique is also useful for revealing other hepatocytes that are not yet ballooned (original magnifications ×100). (D) Ballooned hepatocytes show absent cytoplasmic K18 staining (yellow arrows), whereas non-classical ballooned cells or several normal-sized hepatocytes are anti-K18 negative (red arrows, original magnifications ×200).