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Review Article

Liquid Liver Biopsy for Disease Diagnosis and Prognosis

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Abstract

Liver diseases are a major burden worldwide, the scope of which is expected to further grow in the upcoming years. Clinically relevant liver dysfunction-related blood markers such as alanine aminotransferase and aspartate aminotransferase have limited accuracy. Nowadays, liver biopsy remains the gold standard for several liver-related pathologies, posing a risk of complication due to its invasive nature. Liquid biopsy is a minimally invasive approach, which has shown substantial potential in the diagnosis, prognosis, and monitoring of liver diseases by detecting disease-associated particles such as proteins and RNA molecules in biological fluids. Histones are the core components of the nucleosomes, regulating essential cellular processes, including gene expression and DNA repair. Following cell death or activation of immune cells, histones are released in the extracellular space and can be detected in circulation. Histones are stable in circulation, have a long half-life, and retain their post-translational modifications. Here, we provide an overview of the current research on histone-mediated liquid biopsy methods for liver diseases, with a focus on the most common detection methods.

Introduction

Chronic liver disease (CLD)

CLD is a clinical entity involving a process of progressive destruction and regeneration of the liver parenchyma. CLD refers to a hepatic disease that lasts over six months, and the related deteriorations include the decreased synthesis of clotting factors and other proteins, the detoxification of harmful products of metabolism, and excretion of bile.[1] Accordingly, CLD consists of a wide range of liver pathologies which include inflammation, liver cirrhosis, and hepatocellular carcinoma (HCC).[1] CLD can be caused by viral infection, including hepatitis B and C, by alcohol misuse as in alcoholic fatty liver disease (AFLD), or by obesity/excessive nutrient intake as in nonalcoholic fatty liver disease (NAFLD). NAFLD is present when > 5% of hepatocytes are steatotic in patients who do not consume excessive alcohol consumption (< 20 g/day for women and < 30 g/day for men) and ranges in severity from simple steatosis (fat without significant hepatic inflammation or hepatocellular injury) to steatohepatitis (fat with hepatocellular injury and hepatic inflammation), through to advanced fibrosis and cirrhosis, which is a risk factor for HCC.[2] AFLD broadly consists of three stages, each increasing in severity. Drinking a large amount of alcohol, even for just a few days, can lead to simple steatosis. Most individuals consuming > 40 g/day of alcohol per day develop simple steatosis; however, only a subset of individuals will develop more advanced disease over a longer period. Between 10–35% of individuals with alcohol-related steatosis who continue drinking heavily will develop it.[3] The third stage of alcohol-related liver disease is cirrhosis, where healthy liver tissue has been replaced permanently by scar tissue, involving up to one in every five long-term heavy drinkers who will develop alcohol-related liver cirrhosis.[3] AFLD also increases the risk of developing HCC. Chronic alcohol use of more than 80 g/day for more than 10 years increases the risk of HCC by approximately five-fold.[4,5] In many patients, AFLD, NAFLD, and hepatitis infection can coexist and synergize to worsen CLD.[5,6]

The societal burden and the epidemiological figures of CLD are tremendous. An estimated 1.5 billion persons have CLD worldwide, with increasing prevalence and mortality.[7] Cirrhosis is the eleventh leading cause of death, accounting for ~2% of deaths in 2016.[8] According to the results from the Global Burden of Diseases, Injuries, and Risk Factors Study 2017 on the burden of cirrhosis and its trends since 1990, by cause, sex, and age, for 195 countries and territories,
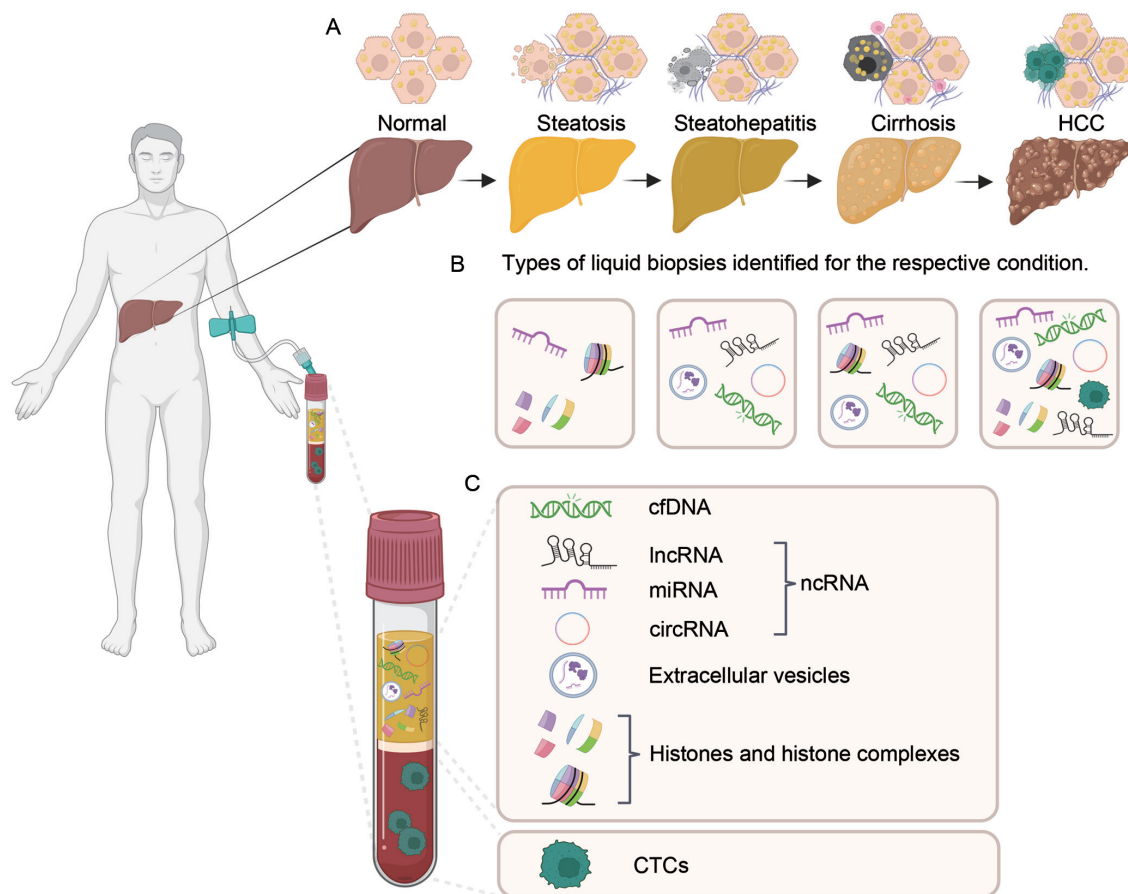
Fig. 1. Liquid biopsies in CLD. (A) Mechanisms of NAFLD. NAFLD progression is marked by: the accumulation of fatty liver (steatosis, illustrated by fat droplets accumulation in the cells and increased collagen production in the extracellular space), inflammation (steatohepatitis, activated immune cells are shown in light gray); cirrhosis (necrosis, necrotic cells are shown in dark gray); HCC with cancer cells shown in green). (B) Liquid biopsy types reported for the respective stages indicated above. (C) Liquid biopsy analytes, acquired by peripheral blood collection, fractionation, and subsequent extraction. CTC, circulating tumor cells; cfDNA, cell-free DNA; circRNA, cicular RNA; lncRNA, long noncoding RNA; miRNA, microRNA. Obtained with BioRender software.

CLD caused ~1.32 million deaths in 2017, with approximately two-thirds inmen.[9] In the last three decades, the epidemiology of CLD has changed,[10] reflecting the implementation of large-scale hepatitis B vaccination and hepatitis C treatment programs, and CLD is nowadays more often the result of the increasing prevalence of the obesity- and/or metabolic syndrome-associated NAFLD, and increasing alcohol misuse, triggering AFLD.

As CLD is a leading cause of years of working-life loss, second only to ischemic heart disease in Europe, increasing attention has been given to policy actions for marketing, pricing, and taxation of alcohol and unhealthy foods.[11]

AFLD/NAFLD: MAFLD?

AFLD and NAFLD are CLDs that have similar pathological spectra, ranging from simple hepatic steatosis to steatohepatitis, liver cirrhosis, and HCC (Fig. 1A). In their initial to advanced stages, they are both characterized by the presence of hepatic steatosis, hepatocellular ballooning, and lobular inflammation with or without fibrosis. They are both frequently accompanied by extrahepatic complications, including cardiovascular disease, metabolic syndrome, and type 2 diabetes (T2DM). Among chronic drinkers and obese patients, about 35% and 65–85% develop CLD, respectively.[12,13] Since the 1980s, there has been a tendency to

separate NAFLD and AFLD as clinical entities. In particular, it was defined that NAFLD develops in the absence of known factors that cause fat accumulation such as alcohol consumption defined as < 30 g/day in men and < 20 g/day in women, viral liver disease, and hereditary disorders.[14] Instead, AFLD is caused by heavy chronic alcohol consumption, defined as consumption of more than three standard drinks per day in men, and more than two drinks per day in women, or binge drinking defined as more than five standard drinks in men and more than four in women in a 2 h period.[14] Upon liver biopsy, only two subtle histological changes may help to distinguish AFLD from NAFLD, as they are present in AFLD but not in NAFLD: (1) pronounced cholestasis (reduced or stopped bile flow), which is usually indicative of acute decompensation (hepatocyte keratin 7 immunostaining, in this case, becomes more intense), and (2) sclerosing hyaline necrosis, in which the central vein is almost completely obliterated.[15] However, these features are not universal, and there is otherwise a substantial overlap in the histological findings between AFLD and NAFLD patients; in the absence of knowledge about the clinical history indicating the nature of the patient's disease, the histology cannot be decisive for the diagnostic process. At the molecular level, AFLD and NAFLD implicate the involvement of common but also distinct cell signaling mechanisms in the liver parenchyma. A study from

Sookoian *et al*.[16] employing integrated omics and system biology approaches cross-comparing enrichment analyses showed that NAFLD is associated with pathways that include insulin signaling, caspases, and mitochondrial-related apoptosis, stress induction of heat shock proteins, cellular proliferation, hypoxia induction, and protein associated with epigenetic regulation. Conversely, AFLD is associated with a more reduced network of disease pathways, mostly focused on modulation of the immune response, toll-like receptor signaling, and cytokines.[16] The same study postulated more pervasive systemic complications of NAFLD vs. AFLD, in particular at the cardiovascular level.[16] A more recent comprehensive literature review indicated that NAFLD is regulated by the Nrf2/FXR/LXRα/RXR/SREBP-1c, PI3K/AKT/SREBP-1c, AMP-activated protein kinase (AMPK)/Sarcoendoplasmic reticulum Ca2+-ATPase 2b (SERCA2b), LILRB4/SHP1/TRAF6/NF-κB/MAPK, TXNIP/NLRP3, and TAZ/Ihh signaling pathways, while AFLD is regulated by the SIRT1/AMPK/Lipin-1, PI3K/AKT/Nrf2/PPARγ, p62/Nrf2/KEAP1, STING-IRF3-Bax, C3/CYP2E1/Gly-tRF/SIRT1 and LRP6/Wnt/β-catenin/CYP2E1 signaling pathways, respectively.[17] Despite the molecular differences revealed by the mechanistic studies, it is clear that NAFLD and AFLD more often coexist and have overlapping physiopathology. This underscored the need for a change in nomenclature to account for the dual etiology of CLD, which is present in a likely significant proportion of patients with concurrent alcohol consumption and metabolic disturbances. Accordingly, in recent years, NAFLD was re-defined by experts from the European Liver Patients' Association as metabolic (dysfunction)-associated fatty liver disease, or MAFLD, a more appropriate nomenclature encompassing clinical features independent of alcohol consumption: hepatic steatosis, in addition to one of the following three criteria, namely overweight/obesity, presence of T2DM, or evidence of metabolic dysregulation.[18,19] Results from the large NHANES III study demonstrated that MAFLD has a closer association to all-cause and cause-specific mortality, compared with NAFLD, because it excluded participants with lower mortality risk and included participants with higher risk.[20] In turn, MAFLD may coexist with other types of CLD, such as viral hepatitis. The relationship and the synergy between these two disease entities are outside the scope of this review and it has been summarized elsewhere.[21]

As mentioned, in a minority of NAFLD and AFLD (or MAFLD) patients, there is a progression to steatohepatitis, fibrosis, and ultimately HCC and liver failure (Fig. 1A). Steatohepatitis can begin to be symptomatic and patients may complain of fatigue, malaise, and dull right-upper-quadrant abdominal discomfort. The molecular mechanisms that combine to define the transition to steatohepatitis and progressive disease are complex.[22] It is increasingly appreciated that this transition is multifactorial and in addition to genetic factors, alcohol or fat-induced hepatocyte damage, reactive oxygen species, and gut-derived microbial components result in steatosis and inflammatory cell (macrophage and neutrophil leukocyte) recruitment and activation in the liver, as reviewed elsewhere.[23–26]

There are still not many options in terms of Food and Drug Administration (FDA)-approved pharmacological or nutritional therapies for treating patients with MAFLD. For MAFLD patients, intake of pioglitazone, vitamin E, and abstinence from alcohol consumption are beneficial.[27] Also, weight loss and healthy nutrition are common therapeutic strategies.[28] In some instances, bariatric surgery may be recommended to achieve and maintain the necessary extent of weight loss required for therapeutic effect.[29] Obeticholic acid , identified in 1999 and originally approved for the treatment of

primary biliary cholangitis , is the only FDA-approved treatment for steatohepatitis.[27] Obeticholic acid is a semisynthetic bile acid analog that has the chemical structure of 6α-ethyl-chenodeoxycholic acid. It is a FXR agonist which regulates the expression of transcription factors that reduce bile acid synthesis and hepatic steatosis, and its anti-inflammatory and anti-fibrotic properties have been demonstrated in several clinical trials.[27] Many drugs are in various stages of research, but only a few have currently entered phases II and III, such as FGF-21, PPAR agonists, GLP-1 receptor agonists, THR-β agonists.[30,31]

Cirrhosis

Liver cirrhosis is highly prevalent worldwide. Its prevalence is increasing in middle-high developed regions and Eastern Europe, while it is decreasing in low-developed regions and Western Sub-Saharan Africa, as ranked by the sociodemographic index.[32] It is an end-stage liver disease, where impaired liver function is caused by the formation of fibrosis consequent to damage.[33] The latter can be in turn a consequence of different causes, such as obesity, MAFLD, high alcohol consumption, viral hepatitis B (HBV) and/or viral hepatitis C (HCV) infection, autoimmune diseases, cholestatic diseases, and iron or copper overload. Liver cirrhosis typically develops slowly over months or years, and over a long period of inflammation resulting in the substitution of the healthy liver parenchyma with fibrotic tissue and regenerative nodules, in turn, inducing portal hypertension.[34] Liver cirrhosis evolves from an asymptomatic phase (compensated) to a symptomatic phase (decompensated). As cirrhosis worsens, symptoms may include itchiness, swelling in the lower legs, fluid build-up in the abdomen that can become spontaneously infected, jaundice, development of spider-like blood vessels in the skin, and dilated veins in the esophagus, stomach, or intestines. More serious complications, resulting in hospitalization, impaired quality of life, and high mortality, include hepatic encephalopathy and HCC. Notably, HCC is the leading cause of death in cirrhotic patients, with a yearly incidence of up to 6%.[35] The severity of liver cirrhosis was commonly classified with the Child-Pugh score, which was used for a long time to determine patients who are candidates for liver transplantation, before being replaced by the European Foundation for the Study of Chronic Liver Failure Organ Failure score and by the Model for End-Stage Liver Disease in previous decades.[36,37]

HCC

Liver cancer is one of the most aggressive types of cancer, characterized by a lower than 20% 5-year overall survival rate and increasing disease burden and death incidence. HCC is by far the most common liver malignancy, representing approximately 90% of all cases. Among the most significant risk factors for the development of HCC are cirrhosis, HBV and HCV, chronic alcoholism, and NAFLD.[38] There are several scoring systems developed to measure the risk of HCC development. However, these systems lack universal acceptance due to the variability of HCC etiology between geographic regions.[39,40] HCC can be generally subdivided into two main classes: proliferative and nonproliferative, which exert distinct clinical features.[41] Tumor burden/HCC staging is generally defined by the number and the size of nodules, the presence of vascular invasion, and extrahepatic spread.[42,43] Clinical management includes surgical therapies, tumor ablation, transarterial therapies, and systemic therapies.[44] HCC is usually diagnosed at an advanced stage when the disease has already spread further than the liver. The multikinase inhibitor Sorafenib was the first FDA-approved systemic drug

to treat HCC and is currently a standard first-line therapy.[45,46] Nevertheless, the increasing occurrence of drug resistance to Sorafenib in HCC patients[47] and the generally very low numbers of targetable somatic mutations in HCC[48] indicate the need for novel therapeutic strategies development and early detection biomarker identification. The poor prognosis of HCC has been linked to diagnostic delays,[49] and failure to identify high-risk individuals owing to inadequate early detection screening methods.[50,51]

CLD diagnostic approaches

Patients with simple steatosis are considered at low risk of disease progression. Therefore, NAFLD/MAFLD detection at an early-stage is of utmost importance for appropriate preventive strategies. Noninvasive diagnostic tools are becoming increasingly significant for NAFLD staging and severity assessment: the European Association for the Study of the Liver (EASL);[52] the American Association for the Study of Liver Disease (AASLD) and the Asian Pacific Association for the Study of the Liver (APASL) produced recent guidelines in this regard.[53–55] In general, the diagnosis of NAFLD currently requires (1) evidence of hepatic steatosis by imaging or histology, (2) no significant alcohol consumption, (3) no competing causes of hepatic steatosis, and (4) no coexisting causes of CLD. Consistently, with the adoption of MAFLD new nomenclature, clinical practice guidelines have been debated by the APASL on MAFLD and have been recently produced by EASL, AASLD, and APASL.[18,56,57] Although overall many similarities exist across guidelines, there are several key areas of guidelines from Europe, Asia, and the USA, including the definition of alcohol consumption, screening, fibrosis assessment, lifestyle intervention, and pharmacological intervention of MAFLD.[53] Regardless, current imaging approaches present disadvantages. For instance, ultrasound is insensitive to mild steatosis.[31] Computed tomography has low sensitivity and specificity, combined with patient exposure to ionizing radiation.[31] Magnetic Resonance Imaging approaches have shown promising results in steatosis/nonalcoholicsteatohepatitis (NASH) detection.[58] However, the requirement for trained operators, the high cost, and the inspection time limit their screening application in the clinic. The progression of simple steatosis to NASH poses an increased risk of the development of fibrosis, cirrhosis, and HCC,[59,60] indicating the need for accurate methods for steatosis–NASH differentiation. To distinguish NASH from steatosis, current guidelines indicate the necessity of liver biopsy to confirm NASH diagnosis, severity grade, and the level of fibrosis.[52,54,55] However, liver biopsies are inconvenient due to the invasiveness of the procedure, and they have limited heterogeneity assessment of the tissue sample since they represent a tiny fraction of the liver parenchyma.[61] Current noninvasive diagnostic approaches for assessment of fibrotic stage and disease progression to cirrhosis aregenerally based on tissue stiffness quantification by tissue elastographyapproach, as recommended by the EASL; the AASLD and the APASL.[62,63] However, tissue elastography approaches are marked by several drawbacks.[64] Finally, HCC surveillance, in cirrhotic or noncirrhotic patients, currently remains an unmet need.[65] In general, histopathological diagnosis upon liver biopsy remains a mainstay according to EASL,[66] AASLD,[67] and APASL guidelines.[68] Noninvasive imaging strategies, such as Computed Tomography or Magnetic Resonance Imaging should be used first; ultrasound and FDG PET-scan, have limited sensitivity in particular for early-stage HCC.[51,66] Therefore, there is an increasing interest in the identification of circulating HCC biomarkers for early detection.

Liquid Biopsy for Liver-Related Diseases

Liquid biopsy represents a minimally invasive, convenient, and cost-effective method of molecular diagnosis that can provide comprehensive information on the molecular landscapes of liver diseases and can represent approaches to overcome tumor heterogeneity and monitor them in real-time (Fig. 1B, C). A detailed description of the histone/nucleosome-independent liquid biopsy types is beyond the scope of the current review and is therefore briefly discussed below.

Circulating DNA

Cell-free DNA (cfDNA) fragments are shed into the circulation from dead cells both in healthy and diseased individuals (Table 1).[69–97] However, a much larger amount of cfDNA is normally detected in cancer patients.[98–100] Studies have found that cfDNA that originates from tumors, referred to as circulating tumor DNA (ctDNA) is notably shorter, compared with nonmutated cfDNA,[69,101,102] and could be used to enrich tumor-derived fragments for further analysis on genomic and epigenomic level. Furthermore, targeted sequencing of ctDNA can detect tumor-associated mutations with high sensitivity, in cases without prior knowledge of their presence in the tumor tissue, indicating strong application for cancer diagnostics and patient stratification for targeted therapy.[70,71,103] Positive ctDNA detection and gene analysis in HCC patients prior to surgery was also associated with an increased risk of early recurrence and extrahepatic metastasis.[72] Furthermore, continuous assessment of ctDNA could inform on therapy response and disease progression.[72,73] Study of cfDNA in liquid biopsies is highly focused on cancer diagnosis, therapy response, and prognosis. However, cfDNA has also shown promising results in NAFLD patient stratification and disease severity. Specifically, levels of 90bp and 222bp cfDNA fragments in the plasma of NAFLD patients, diagnosed with fatty liver, inflammation, and liver stiffness were significantly elevated, compared with healthy individuals, and correlated with disease severity.[74] Similarly, levels of methylated cfDNA are significantly higher in nonfibrotic NAFLD patients, compared with NAFLD patients with confirmed fibrosis.[75,76] Fibrosis was indicated as the strongest predictor of NAFLD progression and NAFLD-related mortality.[104,105] Therefore, circulating cfDNA methylation may be a biomarker for therapy response and stratification of NAFLD patients at high risk of disease progression. However, despite the undeniable value of cfDNA in approaching liver-related disease, cfDNA has a short half-life[106] and requires genetic differences to distinguish tumor-derived material.

Circulating noncoding RNAs:long noncoding RNA(lncRNA), micro RNA(miRNA), and circular RNA(circRNA)

NcRNAs are endogenous RNA transcripts that do not code for a protein product. NcRNAs are highly abundant and relatively stable signaling molecules, crucial for gene expression regulation.[107] The different types of ncRNAs use distinct regulatory mechanisms: lncRNAs are linear transcripts of > 200 nucleotides, which can function in cis or trans as signals, guides, decoys, scaffolds, or enhancers.[108] miRNAs are the most abundant ncRNAs, exerting their inhibitory effect on mRNA stability and translation initiation by binding to DNA, RNA, and proteins.[109,110] circRNAs is a covalently closed RNA that is produced by exon skipping or back-splicing of a precursor mRNA. circRNAs modulate various mechanisms such as transcription and translation by acting as transcriptional regulators, protein/RNA sponges, and templates.[111] Research on circRNAs is limited. However, due to their closed

Table 1. cfDNA in liver diseases

Liver-related pathology	Target	Method of detection	Level/result	Suggested function	Reference
Pediatric NAFLD	cfDNA methylation	Methylamp global DNA methylation quantification	↑ in NASH patients, compared with HC and NAFLD children without NASH, positive correlation with histological traits	Risk assessment, diagnosis	77
NAFLD	Total cfDNA, gene-coding cfDNA, Alu repeat sequences, mitochondrial DNA copies, 5-methyl-2′-deoxycytidine in serum	qPCR, RNAseP detection, ELISA	↓ cfDNA and RNAse P coding DNA levels, ↑ levels of 5-methyl-2′-deoxycytidine in cirrhotic vs. noncirrhotic patients		78
Fibrosis in NAFLD and AFLD	Methylation of the PPARγ gene promoter in plasma	Pyrosequencing	Hypermethylation reflects a signature related to severe fibrosis	Differentiation of fibrosis stage	75
Fibrosis in NAFLD	cfDNA methylation	DNA methylation array	↑ in patients with nonsignificant fibrosis, compared with significant fibrosis	Differentiation of fibrosis stage	76
NAFLD	cfDNA levels	qPCR	90bp cfDNA ↑ in NAFLD patients, compared with HC, correlation with disease severity	Detection, staging	74
Chronic Hepatitis C, PEG-IFN-alpha and ribavirin treatment	Serum cfDNA: Methylation of SOCS-1 promoter region	Quantitative methylation-specific polymerase chain reaction	↑ SOCS-1 methylation post-treatment associated with better sustained virologic response	Monitoring of treatment response	79
Liver cirrhosis	Plasma circulating cfDNA, quantification and sequencing	Somatic mutation analysis by NGS	20 unique variants, including single nucleotide variation s or insertions and deletions	Early detection	80
Primary HCC in NAFLD patients	TERT mutation in serum cfDNA	Wild-type blocking polymerase chain reaction, Sanger sequencing	Positivity	Early detection	81
Nonviral liver cancer with fatty liver disease	TERT C228T	Wild-type blocking polymerase chain reaction, Sanger sequencing	Positivity rate ↑ compared with HBV and HCV	Early detection	82
HCC	Plasma cfDNA – size profile	Chromosome arm-level z-score analysis, qPCR	Shorter DNA fragments associated with tumor-associated copy number alterations,↑ mtDNA in HCC patients, compared with HC, HBV, and cirrhotic patients	Detection, profiling, monitoring	69
HCC	Plasma cfDNA	Deep sequencing	Detection of somatic mutations in HCC-associated genes	Detection, profiling, monitoring	70
HCC	Plasma cfDNA	Targeted sequencing with ultra-high coverage and molecular barcoding	Detection of oncogenic mutations in HCC	Detection, profiling, monitoring, prognosis	71
Recurrent liver cancer post hepatectomy or liver transplantation	Plasma cfDNA	Whole-genome sequencing, Sanger sequencing, PCR, qPCR	↑ ctDNA with disease progression, ctDNA as microscopic vascular invasion predictor	Detection, profiling, monitoring, prognosis	72

(continued)

Table 1. *(continued)*

Liver-related pathology	Target	Method of detection	Level/result	Suggested function	Reference
HCC	Ultra-deep sequencing, Droplet digital PCR of TERT promoter in cfDNA	Sequencing, Droplet digital PCR	Mutation profiles predicting prognosis and therapy response	Profiling, monitoring, therapy response, prognosis	73
HCC	Plasma circulating Cell-free DNA integrity	qPCR	↓ cfDNA integrity than those with benign diseases and HC	Diagnosis and surveillance	83
HCC	Plasma circulating cfDNA, quantification and sequencing	Somatic mutation analysis by NGS	↑ cfDNA levels post-therapy, associated with disease progression, 28 variants identified, including single nucleotide variationsor insertions and deletions	Prognosis, therapy response prediction	84
HCC	Methylation profiling of cfDNA, based on 2,321 tissue-based differentially methylated blocks	DNA bisulfite sequencing	Multilayer HCC screening distinguishing early-stage HCC from HC, asymptomatic Hepatitis B surface antigen + or cirrhotic patients	Early detection	85
HCC	Serum cfDNA: HCCS1 promoter hypermethylation	Methylation-specific polymerase chain reaction	↑ HCCS1 methylation in HCC, compared with HC and CHB patients, positive correlation with tumor node metastasis stage	Diagnosis, prognosis	86
HCC	Somatic copy number alterations in cfDNA	Low-pass sequencing	1q+ and 8q+ : significantly associated with early cancer or high-grade dysplasia	Diagnosis, early detection, surveillance	87
HCC	CfDNA	NGS technology to acquire genome-wide 5-hmc,Nucleosome footprint, 5' end motif and fragmentation profiles	Distinct patterns/ landscapes compared with HC, Combining all four methods differentiated differentiate HCC from LC	Diagnosis, surveillance	88
HCC	HCC-specific methylation marker panel, developed based on HCC tissue	Bisulfite sequencing, molecular-inversion (padlock) probes,prognostic prediction model	401 selected markers discriminating HCC patients from HC and individuals with HBV/HCV infection, or fatty liver	Determine tumor burden, therapy response, and HCC stage, prognosis	89
HCC in cirrhotic patients	Plasma cfDNA	HCC blood test (epigenomics AG), DNA methylation panel established by NGS	mSEPT9 positivity, methylation biomarker panel	Early detection, HCC surveillance	90
HCC in cirrhotic patients	cfDNA methylation profile of p16, SFRP1, LINE1	Bisulfite modification, multiplex methylated PCR	Methylation of p16, SFRP1, LINE1, and an overall increase in the number of aberrantly-methylated genes was associated with HCC development	Surveillance	91
HCC in HBV-diagnosed patients	cfDNA methylation	Low-pass whole-genome bisulfite sequencing	cfDNA in intergenic and repeat regions, hypomethylation nearby HBV integration sites differentiates HCC patients from hepatic and cirrhotic patients	Early detection, detecting minimal tumoral residual disease after surgical resection	92

(continued)

Table 1. *(continued)*

Liver-related pathology	Target	Method of detection	Level/result	Suggested function	Reference
HCC, average/high risk for HCC (viral hepatitis, cirrhosis)	cfDNA fragmentation	Whole-genome sequencing	cfDNA fragmentation changes, exhibiting liver cancer-related genomic and chromatin alterations	HCC detection	93
HCC	cfDNA methylation	DNA methylation datasets processing, machine learning model	Methylation signatures differentiated HCC patients from HC and patients with cirrhosis or with other types of cancers such as colorectal and breast	Detection	94
HCC and CHB	cfDNA fragmentation	NGS	Somatic mutations, tumor-associated preferred DNA ends	Diagnosis	95
HBV	cfDNA quantification	Duplex PCR	↑ in HBV patients, in combination with other markers differentiate inflammation severity	Diagnosis, assessment of liver injury	96
Liver transplantation	cfDNA quantification	qPCR	↑ cfDNA levels post-transplantation associated with portal hepatitis and systemic inflammation	Prognosis	97

AFLD, alcoholic fatty liver disease; ELISA, enzyme-linked immunosorbent assay; HBV, viral hepatitis B; HC, healthy controls; HCC, hepatocellular carcinoma; HCV, viral hepatitis C; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NGS, next-generation sequencing; cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; mtDNA, mitochondrial DNA.

conformation, circRNAs are highly stable, suggesting promising blood biomarker properties.[111] NcRNAs are often deregulated and aberrantly expressed in liver diseases and in many cases are suggested to contribute to disease pathogenesis and disease progression.[112,113]

ncRNAs present in plasma or serum have shown promising results in distinguishing NAFLD and chronic hepatitis C (CHC)patients from healthy individuals (Table 2).[114–134] Particularly, liver fibrosis was characterized by a distinct miRNA, lncRNA, and circRNA profile in circulation, correlating with staging.[114–116,135] Furthermore, specific ncRNAs were shown to differentiate between patients with simple steatosis and NASH.[115,117] Similarly, GAS5 lncRNA levels in plasma are decreased in patients with cirrhosis, compared with patients with advanced fibrosis.[116] Changes in the levels of circulating miRNA have also been highly studied as potential HCC biomarkers.[136] As an example, circulating miRNA-16 levels were decreased in the circulation of HCC patients and correlated with tumor size, and other clinical parameters.[104,105] Importantly, circulating miRNA-16 was elevated in NAFLD and HCV patients,[114] rendering miRNA-16 as a potential biomarker for disease progression. Similarly, plasma miRNA-21 expression was significantly elevated in HCC patients, discriminating HCC patients from healthy individuals and HCC patients from chronic hepatitis. Furthermore, levels of circulating miRNA-21 were decreased following tumor resection and correlated with post-operative tumor recurrence.[118] While individual miRNAs have shown promising results, creating panels of several circulating miRNAs/lncRNAs is likely more robust, as indicated for HCC even in early-stage patients,[119] cirrhosis, and acute liver failure (ACLF) (Table 2).[120]

Circulating HCC cells

Circulating tumor cells (CTCs) are tumor cells of primary or metastatic origin that are detected in the blood or lymphatic circulation following intravasation. CTCs have undergone epithelial-mesenchymal transition and are considered highly metastatic.[137,138] To survive in the circulation CTCs can form clusters, increasing their metastatic potential, stemness features, and plasticity.[139] CTCs are a scarce population of cells, carrying crucial features that can inform on the tumor characteristics and impact HCC diagnosis and treatment regimen.

The value of CTCs in the field of liquid biopsies is undisputed. CTC research has led to remarkable progress in the noninvasive diagnostic, prognostic, and therapy response monitoring of cancer, including HCC.[140–142] Sequencing of CTCs isolated from metastatic HCC patients revealed liver cancer-characteristic mutations, including low-frequency variants.[143] Furthermore, CTC count predicted poor prognosis and post-operative disease recurrence.[143,144] PD-L1+ CTCs have been shown to distinguish between early and advanced stage HCC and are suggested as a promising biomarker for immunotherapy patient stratification and therapy response monitoring.[145,146] Compared with ctDNA and circulating ncRNAs, CTCs can be analyzed at the genomic, epigenomic, transcriptomic, and proteomic levels as single cells or in bulk, providing a detailed scope of tumor-associated signatures.[147] However, because of their limited presence in circulation, many clinically relevant challenges occur regarding their effective, pure, unbiased, and affordable capturing, which are critical for downstream analysis. CTCs can be detected based on several characteristics such as size, charge, density, and expression of cell-surface marker,[148] even *in vivo*.[149,150] The first and only FDA-approved CTC capturing method relies on the selection of EpCAM+ CTCs as EpCAM has been universally recognized as a CTC detection marker.[151,152] Nevertheless, it has been increasingly appreciated that solely EpCAM+ ex-

Table 2. Circulating noncoding RNAs in liver diseases

Liver-related pathology	Target	Method of detection	Level/result	Suggested function	Reference
NAFLD	Serum miRNA-34a and miRNA-122	qPCR	Higher levels of miRNA-34a and miRNA-122 in NAFLD patients, compared with HC	Surveillance, early detection	121
NAFLD	Postulated 18 different serum miRNAs	Serum RNAseq analysis	miRNA-192, -27b, -22, -197, and -30c were associated with NAFLD severity, but not with drug-induced liver injury	Detection, diagnosis	122
Steatosis/NASH	Circulating miRNAs in serum	miRNA expression array and qPCR	A panel of ↑ miRNAs in steatotic or NASH patients, compared with HC, association with increased cardiovascular disease risk and atherogenesis, differentiation of NASH from steatosis, miRNA-122 differentiated liver fibrosis	Surveillance, diagnosis, prognosis	115
CHB	Circulating miRNAs in serum	miRNA expression array and qPCR	Differential expression of miRNAs in CHB vs. HC and CHB vs. NASH vs. HC	Detection of liver injury	123
CHB	Serum lincRNA-p21	qPCR	↓ lincRNA-p21 in CHB patients than in HC, negative correlation with fibrosis stage in CHB patients	Diagnosis, staging	24
CHC	Serum miRNA let-7a-5p	qPCR	↓ in CHC patients with cirrhosis, positive correlation with severity of fibrosis	Diagnosis, staging	125
CHC, NAFLD	Serum miRNAs	qPCR	↑ miRNA-122, miRNA-34a, and miRNA-16 in NAFLD and CHC patients than in HC, miRNA-122 and miRNA-34a correlated with disease severity	Detection, staging	114
NASH	Plasma lncRNA LeXis	qPCR	↑ LeXis in NASH patients than in steatotic individuals	Diagnosis	126
NASH	Serum miRNAs	Small RNA sequencing and quantitative reverse transcription PCR	miRNA-21-5p, miRNA-151a-3p, miRNA-192-5p, and miRNA-4449 differentiated NASH from steatotic patients	Diagnosis	117
NAFLD, fibrosis	Plasma lncRNA GAS5	qPCR	↑ GAS5 in advanced fibrosis, ↓ in cirrhotic NAFLD patients	Diagnosis, monitoring of disease progression	116
AC	Plasma lncRNAs AK054921 and AK128652	Global transcriptomic profiling by lncRNA microarray, qPCR	LncRNA signature, specific for excessive drinkers, not found in HC and AC, ↑ AK128652 and AK054921, correlating with alcoholic cirrhosis severity and patient survival	Diagnosis, prognosis	127
Cirrhosis, ACLF	Serum miRNAs	Open array	MiRNAs profiles differentiating cirrhosis disease progression, kidney or liver failure, poor outcome	Monitoring, prognosis	120
HCC	Serum miRNAs	miRNA expression array and qPCR	miRNA classifier differentiating HCC patients from non-HCC and at-risk patients	Surveillance, early detection	128
HCC	Plasma lncRNA	LncRNA microarray	HCC patients showed ↑ RP11–160H22.5, XLOC_014172, and LOC149086, compared with HC, association with metastasis, ↓ post-surgery	Surveillance, metastasis prediction, monitoring of disease progression	129
HCC	Plasma ZFAS1	qPCR	↑ ZFAS1 in HCC patients, compared with HC, cirrhotic, or hepatitis B patients	Detection	130

(continued)

Table 2. *(continued)*

Liver-related pathology	Target	Method of detection	Level/result	Suggested function	Reference
HCC	Plasma miRNA-21	qPCR	↑ in HCC than in chronic hepatitis patients and HC. ↓ miRNA-21 post-surgery, correlating with a lower risk of recurrence	Detection, monitoring, prognosis	118
HBV-Related HCC	Plasma miRNAs	Microarray and qPCR	MicroRNA panel differentiated HCC from HC, CHB, and cirrhosis	Surveillance, early detection	119
HCV and HCV-associated HCC	Serum NEAT1 and TUG1 in HCV	qPCR	↓ NEAT1 and TUG1 in HCV and HCC, compared with HC, ↓ TUG1 in HCC patients, compared with HCV and HC	Diagnosis, monitoring of disease progression	131
HCV-positive cirrhosis and HCC	Serum miRNAs	qPCR	A panel of deregulated miRNAs in HCV-positive cirrhotic and HCV-positive HCC patients	Surveillance, early detection	132
HCC, Sorafenib treatment	Serum miRNA-221 levels	qPCR	Positive therapy response associated with ↓ miRNA-221 pretreatment levels and ↑ miRNA-221 post-treatment	Therapy response prediction and monitoring	133
Biliary tract cancer	Plasma miRNA-21	qPCR	↑ miRNA-21 in Biliary tract cancer patients, compared with HC benign biliary disease patients	Detection, diagnosis	134

AC, alcoholic hepatitis; ACLF, acute liver failure; CHB, chronic hepatitis B; CHC, chronic hepatitis C; HBV, viral hepatitis B; HC, healthy controls; HCC, hepatocellular carcinoma; HCV, viral hepatitis C; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; lncRNA, long noncoding RNAs; miRNA, microRNA.

pression could be insufficient for CTC enrichment[153,154] as EpCAM[low/negative] CTCs are missed. Furthermore, EpCAM-based detection of CTC was found highly inefficient in HCC with approximately 25% CTC detection rate.[155] Instead, novel methods are focused on distinct hallmarks such as ploidy subtraction enrichment and immunostaining-fluorescence *in situ* hybridization , which has shown promising results in capturing CTCs expressing distinct biomarkers and establishing their prognostic value.[156] Nevertheless, there is no perfect system for CTC caption, and future work is likely marked by the combined application of several methods.

Extracellular vesicles (EVs)

EVs are cargo-carrying particles, released from cells in the extracellular space, inducing crucial cell-cell signaling cascades in normal physiology or pathological processes.[157] EVs vary in size, release mechanism, and the nature of the loaded cargo (protein, lipids, metabolites, and nucleic acids). The molecules found in EVs may carry essential information on the cell-of-origin or the underlying trigger of EV release. Increasing evidence implicates EVs as key players in the pathology of several liver diseases, including NAFLD, AFLD, viral hepatitis, and HCC.[158] EVs can signal to cells in close proximity or be transported to distal sites to act as long-range signals.[159] EVs have been purified from most mammalian cells and bodily fluids. Furthermore, cellular compounds transferred by EVs are protected from the hostile environment of the circulation and other degradation stimuli in the extracellular space, making them highly stable. Given that EVs are loaded with only a subset of molecules, which could be otherwise barely detected in the total volume of body fluids, EVs have emerged as a promising liquid biopsy approach (Table 3).[160–177] Several EV-based biomarkers, including proteins and miRNAs, have been reported in the serum or plasma of people with liver disease. For instance, decreased levels of miRNA-718 in serum EVs of HCC patients were associated with aggressiveness and recurrence following liver transplantation,[160] while high levels of EV-associated miRNA-21 correlated with cirrhosis and advanced tumor stage.[161] Similarly, elevated levels of several circulating EV-associated miRNAs were found in alcoholic hepatitis (AH), ASH, and AFLD patients.[178,179] EVs enriched with six sphingolipids were significantly elevated in AH patients, compared with healthy individuals, heavy drinkers, NASH patients, and alcoholic cirrhosis patients.[158] Mitochondrial DNA (mtDNA) encapsulated in EVs was also shown to promote inflammation, which is in line with the EV-enclosed mtDNA in NASH patients.[180] Furthermore, proteomic analysis of circulating EVs has identified differential proteomic profiles, differentiating precirrhotic NASH, cirrhotic NASH, and healthy individuals.[162]

However, in line with the capturing-related challenges with CTCs, the detection and enrichment methods of EVs come with various limitations and factors affecting the yield.[181,182] Despite the substantial progress in EV isolation, standardization of EV procedures and nomenclature is yet to progress, delaying their clinical applications.

Histones

Methods for isolation and analysis of liquid biopsies have rapidly evolved over the past few years, thus providing details on the development and progression of liver diseases, treatment strategies, and patient stratification. Their long half-life and stability in the bloodstream render histones and histone complexes valuable liquid biopsies. Anti-nucleosome antibodies are more than two-fold more sensitive, compared with antibodies against DNA.[183] Furthermore, in contrast to CTC and EVs, circulating histones do not require complex

Table 3. EVs in liver diseases

Liver-related pathology	Target	Method of detection	Level/result	Suggested function	Reference
NASH	Total circulating EVs and hepatocyte-derived EVs	Differential centrifugation and size-exclusion chromatography, flow cytometry, electron microscopy, western blotting, and dynamic light scattering	Positive correlation between NASH characteristics and total or hepatocyte-derived EVs, proteomic signatures differentiating precirrhotic/cirrhotic patients from HC	Surveillance, detection, diagnosis	162
NAFLD – pre and post-weight loss	Total EVs, hepatocyte-specific EVs, lipid and sphingolipids analysis	Differential ultracentrifugation and quantified by nanoparticle tracking analysis	↓ total EVs and hepatocyte-specific EVs post weight loss, a positive correlation between hepatocyte-specific EVs and NAFLD clinical parameters	Detection, diagnosis, monitoring	163
ALD vs NAFLD	Proteomic analysis of serum extracellular vesicles	Centrifugation, Liquid chromatography-mass spectrometry, protein identification, and label-free quantification using the MaxQuant platform	A panel of proteins differentiated between ALD and NAFLD	Diagnosis	164
NAFLD/NASH, CHC	Microparticles from immune cells in serum	Differential centrifugation, Fluorescence-activated cell scanning	EV profiles correlating with inflammation severity and fibrotic stage	Surveillance, early detection, diagnosis	165
CHB, DeCi	EVs and EV miRNA in serum	Centrifugation, miRNA-seq, and qPCR arrays	Severe liver injury associated with the highest concentration of EVs, compared with DeCi and HC patients, miRNAs as predictors of disease progression	Surveillance, early detection	166
AIH	EV-encapsulated miRNAs in serum	Microarray, digital PCR	↑ EV-miRNA-557 in AIH, compared with patients with NASH, Primary biliary cholangitis, and HC that are correlated with relapse	Diagnosis	167
AH	Sphingolipids encapsulated in EVs	Tandem mass spectroscopy	↑ EVs and EV sphingolipid cargo in AH patients, compared with HC, heavy drinkers, end-stage-liver disease, and DeCi	Detection, survival prediction, monitoring	168
CHC	Soluble CD81 in the exosomal serum fraction	Differential centrifugation, immunoblotting, and densitometry	Patients with CHC – ↑ CD81, associated with inflammation and fibrosis severity, cured CHC patients – CD81 levels, similar to HC	Detection, diagnosis, monitoring	169
CHC	EV proteome in serum	Affinity purification, shotgun and targeted proteomics	SAP and PPBP were ↓ with liver fibrosis severity	Diagnosis, staging	170
HCV	Exosome-encapsulated miRNA-19a	Exosome isolation (ExoQuick), qPCR	↑ in HCV patients with fibrosis, compared with HC and fibrotic patients with non-HCV-related liver pathology	Diagnosis	171
HCC	MiRs in exosomes from serum	Ultracentrifuge, microarray	↓ exosomal miRNA-718 in patients with tumor recurrence following liver transplantation	Monitoring, prediction of recurrence	160
HCC and CCA	AnnexinV+ EpCAM+ CD147+ taMPs in serum	Differential centrifugation, Fluorescence-activated cell scanning	↑ in HCC and CCA, differentiating nonliver cancers or other liver disorders. ↓ taMPs post tumor resection	Detection, diagnosis, monitoring	172

(continued)

Table 3. *(continued)*

Liver-related pathology	Target	Method of detection	Level/result	Suggested function	Reference
HCC, CCA, Primary sclerosing cholangitis	Proteome of serum EVs	NTA, mass spectrometry	Differentially expressed proteins in EVs, showing promising diagnostic capacity between the distinct groups	Diagnosis	173
CCA	EVs in human bile	Nanoparticle tracking analysis, qPCR miRNA arrays, qPCR	Development of biliary vesicle miRNA-based panel differentiating CCA from biliary obstruction and bile leak syndromes	Surveillance, detection	174
HCC	Extracellular vesicle-derived lncRNAs	qPCR based on differentially expressed lncRNAs in HCC tissue	↑ EV-derived LINC00853 in all-stage HCC, including AFP-negative HCC, compared with HCC, chronic hepatitis, and liver cirrhosis	Surveillance, early detection	175
HCC	Serum exosomal microRNA	qPCR	↑ exosomal miRNA signature in HCC patients, compared with CHB and LC,↓ miRNA signature when compared with CHB	Surveillance, early detection	176
HCC	miRNA-21 in serum exosomes	qPCR	↑ in HCC than CHB or HC, correlating with cirrhosis and tumor stage	Detection, diagnosis	161
HCC recurrence	Exosomal miRNAs in serum	Ultracentrifugation, microarray, qPCR	↓ miRNA-718 levels correlated with HCC tumor aggressiveness	Monitoring, prognosis	160
Liver metastasis in CRC	Plasma EV	Ultracentrifugation, tethered cationic lipoplex nanoparticlestechnology	CRC-derived sEVs with enriched microRNA-21-5p positively correlated with liver metastasis	Detection, disease progression	177

AH, alcoholic hepatitis; AIH, autoimmune hepatitis; ALD, alcoholic liver disease; CCA, cholangiocarcinoma; CHC, chronic hepatitis C; CRC, colorectal carcinoma; DeCi, decompensated cirrhosis; EVs, extracellular vesicles; HCC, hepatocellular carcinoma; HCV, viral hepatitis C; LC, liver cirrhosis; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NTA, nanoparticle tracking analysis; lncRNA, long noncoding RNA; miRNA, microRNA; taMPs, tumor-associated microparticles.

extraction methods, which affect the downstream analysis. The purpose of the following sections of this review is to provide an overview of circulating histones as liquid biopsies and their application for the detection, prognosis, and monitoring of hepatic disease progression and outcomes.

Extracellular/circulating histones

DNA in eukaryotic cells is compacted into chromatin by wrapping around histone proteins, generating protein-DNA complexes referred to as nucleosomes. Each nucleosome comprises a histone octamer: one tetramer H2A-H2B, two H3-H4 dimers, and approximately 147 base pairs of DNA.[184] Neighboring nucleosomes are connected through a short DNA stretch bound by histone H1, referred to as a linker histone creating the structure of chromatin.[184] Nucleosomes are highly dynamic structures,[185,186] regulating key cellular processes, including gene transcription, replication, and DNA repair through particularly ordered signaling cascades.[186] Both core and linker histones can be epigenetically modified through post-translational modifications (PTMs), altering protein-protein and protein-DNA interactions, resulting in nucleosome occupancy or position changes, activation/suppression of gene expression, and cell division.[185,187,188] Nucleosome function can also be altered on a structural level by the incorporation of histone variants, which have defined functions such as macroH2A1.2, H2AX, and H2AZ in DNA repair,[189,190] or tissue-specific expression as the histone variants H3T and H3.5 in testicular cells.[189] It is widely accepted that the correct organization and regulation of nucleosomes and chromatin are crucial for genome stability.[185,191]

Studies have shown that histones and histone complexes are also detected in the extracellular space and the bloodstream, following release by damaged cells and activated immune cells. Upon release, extracellular histones function as damage-associated molecular pattern molecules, exerting cytotoxic and pro-inflammatory activity.[192–194] Neutrophils have been shown to utilize a distinct immune defense mechanism, referred to as neutrophil extracellular traps (NETs), in which histones, DNA, and other factors such as granule proteins are released in the extracellular space.[195] NETs can cause a further histone release by inducing a specialized form of neutrophil cell death,[195] resulting in a self-sustained inflammatory cascade and cell death.[196–199] Furthermore, studies have shown that NETs are associated with pathogenesis and could act as effectors in disease maintenance or progression, as found in HCC.[200–202] Importantly, histone H3 citrullination is an essential epigenetic change in NETs formation and it is widely utilized as NETs marker.[203,204] Given that NETs have been suggested as potential biomarkers for disease prognosis and therapy response,[205,206] accurate detection of extracellular histones is essential. Furthermore, while previously NETs have been considered neutrophil-specific, current research has found that other innate immune cells, including macrophages, mast cells, and eosinophils[207] can fight pathogens by extracellular traps, further indicating the role of histones in the extracellular space.

An increasing number of studies suggest that circulating histones, post-translationally modified histones, and histone complexes are differentially detected in the plasma or serum of patients with liver-associated diseases, including HCC, indicating potential biomarker function.

Extracellular histones in liver diseases

Histones have been mostly studied as a component of NETs and have been studied in the context of liver disease for many years, most significantly concerning the epigenetic

changes involved in liver pathogenesis and epigenetic-based therapeutics. The role of histones in liver disease is a vast topic and has been reviewed elsewhere.[208–213] For this review, we will focus on the role of extracellular histones in liver pathology. Wang *et al*.[214] recently showed that NETs are associated with cancer development and progression by modulating gene expression in naïve CD4^{+} T-cells in a TLR4-mediated manner, favoring Treg differentiation, resulting in repressed immunosurveillance in NASH mouse model and increased incidence of HCC development. Inhibition of NETs suppressed Treg activity and tumor burden in NASH-HCC models, directly linking histones with liver disease progression. Similarly, in the surgical stress murine model induced by liver ischemia-reperfusion injury, downregulation of NETs formation caused a reduction in the development and progression of metastatic liver disease.[200] HCC is often caused by the progression of liver fibrosis to liver cirrhosis and subsequent cancer nodule formation. Recently, Wang *et al*.[215] showed that induction of fibrosis in mice caused a drastic increase in circulating histones. Furthermore, direct histone treatment of LX2 human hepatic stellate cells caused activation of collagen I production and α-SMA through TLR4-MYD88 signaling. The administration of either noncoagulant heparin (NAHP) or TLR4-blocking antibody resulted in decreased Aminotransferase levels and histology-based liver injury score, suggesting histone inhibition as a potential therapeutic approach. However, it should be mentioned that no difference in extracellular histones was observed between carbon tetrachloride and carbon tetrachloride+ NAHP treated groups.[215] Therefore, heparin could have exerted its liver protective function irrespective of coagulation and extracellular histone neutralization.

NETs have also been implicated as drivers of earlier stages of liver disease. Tanshinone IIA has been suggested to exert its anti-inflammatory and anti-steatotic effect in NASH-induced mice at least partially by regulating NETs.[216] NET production is also drastically elevated in the circulation of AH patients and mice, together with a specific subpopulation of low-density neutrophils that exert defective properties. The authors showed that alcohol induces the activation of cultured human neutrophils, causing nonlytic NETs release in high-density neutrophils, which subsequently become low-density neutrophils with diminished homing capacity and clearance.[217]

Chen *et al*.[218] further showed that extracellular H3 could induce ferroptosis in hepatic macrophages and ACLF model mice. Importantly, treatment with anti-H3 antibody suppressed cell damage and pro-inflammatory cytokine production *in vitro* and *in vivo*. Histone H4 has been also shown to directly induce hydrogen peroxide production in neutrophils in a calcium- and cell adhesion-dependent manner, resulting in degranulation and pro-inflammatory cytokine release. Mechanistically, the authors showed that histone H4 causes a prolonged increase in neutrophil intracellular calcium, membrane depolarization, and rapid permeabilization,[219] suggesting a potential molecular mechanism behind the drastic neutrophil activation in liver diseases.[220]

Together, previous studies have indicated the role of histones in liver disease, the potential molecular mechanisms, and the promising therapeutic benefit of anti-histone therapy. Nevertheless, many questions remain, the main one being: can we use circulating extracellular histones as a biomarker for liver disease detection and monitoring in patients?

Methods of detection

Circulating histones and histone complexes can be detected by several methods, including enzyme-linked immunosorbent assay (ELISA), proteomic analysis, and imaging approaches including single-molecule imaging and ImageStream.

ELISA: There are several ELISAs developed to detect histone subtypes, specific PTMs, or nucleosomes. In a study comparing patients with local, locally advanced, or metastatic prostate cancer (PC) following therapy by ELISA-based detection of specific plasma components, H3K27me3 levels were inversely correlated with metastatic PC and showed the ability to differentiate patients with localized and metastatic disease.[221] A subsequent study utilized an ELISA-based assay consisting of antibody-mediated nucleosome immobilization and incorporation of antibodies detection histone variants or histone modifications of interest, to show the diagnostics potential of circulating nucleosomes in distinguishing PC patients from healthy controls or individuals with benign disease.[222] The authors identified a panel of five nucleosome-associated marks in serum that achieved a higher disease-predictive score, compared with the common pancreatic tumor biomarker, carbohydrate antigen 19–9. The same assay was subsequently applied to the serum of people referred for colorectal cancer (CRC)-related endoscopic screening. Of the 12 epigenetic epitopes measured on circulating nucleosomes, the assay revealed two groups of four markers that differentiated early-stage CRC patients from healthy individuals, and healthy individuals from people with benign polyps, respectively.[223] These results indicate that ELISA is a powerful approach, detecting differences in circulating histones and histone complexes between groups and could be used to assess their biomarker properties. Nevertheless, ELISA is characterized by antigen detection bias such as histone PTMs with high concentration due to the generally low sensitivity of the assay. Furthermore, while multiplexed ELISAs have been developed, increasing the assay significance and power as a detection and quantification approach, they are currently limited to the multiplexed detection of H3, H4, and post-translationally modified H3/H4.[224,225]

Proteomics: While ELISA is a time-efficient assay, providing multiplex opportunities that do not require a highly specialized scientist, ELISA has considerable bias in regards to antibody sensitivity and specificity, detection of known histone modifications, antigen concentration, and accessibility of antigen target.[226] To tackle these limitations, one can choose to rely on proteomic analyses. Van den Ackerveken *et al*.[227] developed an epigenetic profiling approach on circulating nucleosomes, based on intact H3.1-positive nucleosome capturing by immunoprecipitation, liquid chromatography, and tandem mass spectrometry. An alternative nucleosome isolation approach to immunoprecipitation is a previously described acid-based extraction, which applies trichloroacetic acid -mediated total protein precipitation, followed by histone extraction by 0.2 M H_2SO_4.[228] Multiple reaction monitoring targeted mass spectrometry, involving heavy-isotope labeled Spike-In peptides,[229] is another approach proven powerful in identifying and quantifying histone proteins[230] and histone PTMs.[231] Alternative MS approaches are isobaric tags for relative and absolute quantification and tandem mass tag MS, both of which have been previously applied in plasma proteome assessment.[232] Overall, MS allows for an antibody-independent measurement of histone levels and a systemic nonbiased analysis of histone-associated PTMs.

Single-molecule imaging and ImageStream: Recently, Fedyuk *et al*.[233] developed a single-molecule imaging approach EPINUC that assesses the epigenetic signature of circulating nucleosomes, detecting individual histone PTMs and their combinatorial pattern on individual nucleosomes by total internal reflection microscopy. EPINUC differentiated patients with late-stage CRC from healthy individuals based

on the epigenetic profile of circulating nucleosomes. EPINUC is yet to be utilized on other cancer types such as HCC and early-stage patients. ImageStream device combines the sensitivity of flow cytometry with the detailed phenotypic abilities of cellular imaging, allowing for the detection of multiple biomarkers and the acquisition of up to 12 channels. Furthermore, ImageStream is able to detect particles in the range of 1 µm to 20 nm as shown by analysis of calibration beads.[234] ImageStream acquires a large number of images per sample, providing for fast collection of morphology-based and fluorescent signal-based data. Furthermore, ImageStream can be coupled with open-source artificial intelligence software, creating the possibility for a fully automated quantification. ImageStream has been proven valuable in the liquid biopsy field, especially in the detection and characterization of CTCs. Previously, the focus was on developing reliable methods for CTC extraction and quantification and determining their prognostic ability in terms of disease progression and therapy response. Current research aims to further increase and better estimate the clinical potential of CTCs by characterizing their features and behavior. ImageStream detects the expression of multiple markers on a single CTC, allowing for simultaneous positive and negative selection, as shown for esophageal, hepatocellular, thyroid, ovarian, and lung cancers.[235,236]

Similarly, an increasing number of studies indicate the value of ImageStream in detecting circulating histones and addressing their effect on the surrounding cells in various contexts. For instance, ImageStream was applied to assess NETs levels in both murine and human whole blood samples, based on the expression of NETs components, including positive staining for H3Cit,[237,238] bypassing time-consuming analysis and potential bias. Similarly, anti-histone antibodies detecting histones H1, H2A, H2B, H3, and H4 were utilized as a marker of Eosinophil extracellular traps , allowing tracing the origin of Eosinophil extracellular traps -associated particles of interest as nuclear, rather than mitochondrial.[239] ImageStream-mediated analysis showed that following trauma extracellular H4 exerts cytotoxic function on platelets, resulting in ballooning and H4-retaining microparticle secretion, which binds to leukocytes. These findings strongly suggest that ImageStream is a powerful tool not only to detect circulating histones but also to track extracellular histones-mediated cell-cell communication and the resulting intracellular changes. ImageStream-mediated detection of circulating histones has also been applied for liver-associated diseases that will be discussed in the following section.[77,240] Nevertheless, ImageStream-based analysis of circulating histones in blood samples of HCC patients or animal models is currently lacking.

Circulating histones as markers and predictors of NAFLD/NASH

To decrease the risk of disease progression and determine the most optimal treatment approach, it is essential to discriminate between NAFLD and NASH patients. Current data on promising noninvasive NAFLD/NASH diagnostic approaches including imaging and biomarkers is scarce. Nevertheless, circulating histones and histone complexes have been indicated as potential NAFLD biomarkers. Circulating nucleosomes have been found elevated in obese individuals and correlated with fatty liver and poor metabolic health.[241] Given that obesity and metabolic syndrome are among the main risk factors of NAFLD development, it could be hypothesized that nucleosomes render prognostic value. ImageStream has been recently applied to the serum and plasma of lean MAFLD[240] and NAFLD[77] patients, respectively.

While high levels of circulating nucleosomes are reported in various diseases and conditions, including obesity-associated MAFLD,[241] nucleosomes showed poor association with non-obese (lean or overweight) MAFLD or NASH. However, serum nucleosomes were found significantly elevated in grade 3 steatosis, compared with grade 1 lean MAFLD patients. Looking at histones onan individual level, circulating macroH2A1.1 and macroH2A1.2 histones, either as individual proteins or as a dimer with H2B, were significantly decreased in steatotic grade 3 lean/nonobese MAFLD patients, compared with grade 1. Conversely, H2A and H2A/H2B complex were significantly elevated in overweight MAFLD, but not in lean MAFLD patients. Together, circulating histones macroH2A1.2, H2B, and H4 were associated with MAFLD disease severity, further suggesting potential prognostic and patient stratification properties (Table 4).[77,240-250] Interestingly, pediatric NAFLD patients were characterized by contrasting histone expression signature in circulation. Specifically, serum levels of macroH2A1.2 in NAFLD children were significantly elevated compared with healthy controls. However, no difference in circulating macroH2A1.2 was observed between children with or without NASH. Furthermore, macroH2A1.2 showed an inverse correlation, having the strongest correlation with early-stage steatosis and the weakest with NAFLD Activity Score (NAS).[77] Together, these findings indicate the prognostic significance of macroH2A1.1 in early NAFLD stages but not in disease progression to NASH. It should be noted that high-risk individuals such as those with T2DM or metabolic syndrome were not included in the studies. It would be interesting to evaluate whether such a histone signature could differentiate between people at risk and those that have developed the initial stages of liver disease.

The progression of early-stage NAFLD to NASH is marked by the induction of inflammation, which among other pathways can be directly induced by NETs-associated histone release.[193,251,252] In line with that, increased levels of NETs were detected in the circulation of patients diagnosed with NASH,[201] cirrhosis, or HCC,[253] compared with normal livers.

Recently developed DNA sequencing-coupled bioinformatic approaches indicated that the retained epigenetic profile on plasma/serum nucleosome-associated DNA could predict the tissue-of-origin of the circulating fragments. Sadeh *et al*.[254] applied active marks-mediated chromatin immunoprecipitation of nucleosomal DNA on the plasma of patients with diverse liver-associated pathologies, including NAFLD/NASH patients. Interestingly, based on the region characterized by the active mark, the authors determined differential gene expression profiles that could be traced back to specific liver zones or condition-related transcriptional pathways.[254] The method allows for noninvasive genetic-independent identification of differentially regulated pathways through unbiased analysis, which could potentially distinguish crucial clinical features of the disease. Nevertheless, such an approach requires extensive technology and trained personnel, making it currently challenging for potential routine clinical practice.

Circulating histones in liver dysfunction, transplantation, and HCC

An increasing number of studies are reporting the strong diagnostics and monitoring value of circulating nucleosomes in several solid malignancies.[222,223,227,233] Nevertheless, no difference in nucleosome levels was found between chronic hepatitis B (CHB) and HCC,[246,255] (Table 4) suggesting that HCC development following CHB is not associated with further elevated nucleosome content. This finding also indicates that solely measuring nucleosome levels is unable to distinguish HCC cases in high-risk individuals. The lack of specificity of

Table 4. Circulating histones and histone complexes in liver diseases

Liver-related pathology	Target	Method of detection	Level in circulation	Suggested function	Reference
Obesity, MAFLD	Nucleosomes	ELISA	Increased	Diagnosis, fatty liver, poor metabolic health	241
Lean MAFLD grade 3 steatosis	Nucleosomes	ELISA	Increased	Disease/severity staging	240
Lean/nonobese MAFLD	macroH2A1.1, macroH2A1.2	ImageStream	Decreased	Diagnosis	240
Overweight MAFLD	H2B, H2A/H2B complex	ImageStream	Increased	Diagnosis	240
MAFLD	macroH2A1.2, H2B, and H4	ImageStream	Decreased	Disease/severity staging	240
Pediatric NAFLD	macroH2A1.2	ImageStream	Increased	Detection	77
Pediatric NAFLD	macroH2A1.2	ImageStream	Decreased in advanced steatotic children	Disease/severity staging	77
HCC post locoregional transarterial chemoembolization therapy	Nucleosomes	ELISA	Increased	Detection of a positive therapy response	242
HCC post Sorafenib treatment	H3K27me3, H3K36me3	ELISA	Decreased	Detection of apositive therapy response	243
HCC post-Sorafenib treatment	H3K27me3/H3K36me3 ratio	ELISA	Increased	Prediction of therapy resistance and disease progression	243
HCC post-RFA	Nucleosomes	ELISA	Transiently increased and subsequently decreased	Detection of a positive therapy response	244
HBV-related ACLF	H4	ELISA	Increased	Detection, prognosis	245
Cirrhosis, ACLF	Nucleosomes	ELISA	Increased cirrhosis < ACLF	Detection	246
ACLF, grade I–IV	Nucleosomes	ELISA	Increased grade I/II < grade III/IV	Detection, staging, prognosis	247
ACLF (HBV-related), conventional therapy and Qingchangligan	Nucleosomes	ELISA	Decreased nucleosomes and pro-inflammatory cytokines following conventional therapy + Qingchangligan, compared withconventional therapy alone	Therapeutic	248
PGD	Nucleosomes	ELISA	Transiently increased and subsequently decreased	Detection, prognosis	249
Hepatectomy	Histone H3	ELISA	Low levels of post-surgical (24h) H3 levels were associated with delayed liver function recovery	Prognostic	250

ACLF, acute liver failure; ELISA, enzyme-linked immunosorbent assay; HBV, viral hepatitis B; HCC, hepatocellular carcinoma; MAFLD, metabolic (dysfunction)-associated fatty liver disease; NAFLD, nonalcoholic fatty liver disease; PGD, primary graft dysfunction; RFA, radiofrequency ablation.

circulating nucleosomes is further supported by the fact that nucleosomes in plasma and serum are elevated in several other types of cancer and benign conditions.[256] However, elevated nucleosome content in the serum of HCC patients 24 h post locoregional transarterial chemoembolization therapy was found as an independent marker of therapy response, with elevated circulating nucleosomes 24 h post-therapy correlating with disease progression.[242] Changes in nucleosome levels were also detected following hepatic radiofrequency ablation (RFA),[244] which is also applied as a first-line HCC treatment.[257,258] Specifically, a drastic transient increase in circulating nucleosomes was observed 24 h post-treatment, compared with the pre-RFA state, which correlated with liver damage and upregulated pro-inflammatory markers MPO, interleukin (IL)-6, and IL10. Interestingly, the values of circulating nucleosomes decreased back to pretreatment levels after four weeks.[244] However, how these values correlate with the patient therapy response four weeks post-treatment was not addressed. Furthermore, the serum histone content in the HCC-induced mouse model was significantly increased, compared with HCC-free animals, and recapitulated the elevated extracellular histone levels in HCC tissues.[228] More important, circulating histones in HCC rat serum had epigenetic signatures characteristic of both human and rat HCC tumor tissues, including hypo-acetylation at H4K16 and hypomethylation H4K20.[228] Changes in post-translationally modified circulating histone levels from baseline to post-therapy were also shown to predict patient response and outcomes following Sorafenib treatment. A decrease of H3K27me3 and H3K36me3 post-treatment was associated with a detectable response to Sorafenib. Conversely, an increased H3K27me3/H3K36me3 ratio was correlated with therapy resistance and disease progression.[243] It should be noted that a clear definition of a time point for post-treatment sample collection is not specified. Furthermore, changes in the post-translational modification levels are not addressed in the patients that showed stable disease at first visit, but progressive disease at follow-up assessments. Therefore, we cannot address how changes in the modifications of interest correlate with the disease alteration. These findings suggest that epigenetic profiling of circulating histones can be used as a patient stratification approach and therapy response biomarker. However, whether changes in circulating histone level post-therapy reflect disease progression or remission, and whether pretreatment levels of circulating histones are indicative of short/sustained therapeutic response is currently unclear as longitudinal studies are lacking.

Circulating H4 histones were drastically increased in ACLF patients, compared with healthy individuals/CHB/HBV-related liver cirrhosis.[245] In line with these findings, nucleosomes were significantly elevated in the serum of ACLF patients[246,247] and primary graft dysfunction (PGD),[249] compared with healthy individuals and patients without PGD development, respectively. Blasi *et al*.[246] also reported significantly elevated plasma nucleosome levels in patients with acutely decompensated cirrhosis (DeCi) , compared with healthy individuals, and in ACLF patients, compared with acutely DeCi. While cirrhotic patients were also included in the study of Wen *et al*.,[247] patients were grouped as CLD, which includes inflammation, liver cirrhosis, and HCC. Importantly, circulating histones correlated with ACLF severity and predicted patient prognosis.[245,247] Upon incubation of ACLF[245,247]and PGD[249] from patient serum with human L02 hepatocytes and monocytic U937 cells, the authors found elevated L02 cell death and cytokine induction in U937 cells. Importantly, these effects were abrogated following heparin treatment, which binds histones,[259–261] and anti-histone an-

tibody administration.[249] In ACLF mouse models, NAHP was able to diminish inflammation and liver injury,[247] indicating (1) that histones exert their function irrespective of coagulation; and (2) the potential therapeutic value of anti-histone therapy in ACLF and liver transplantation-related dysfunction. In line with these findings, a subsequent study showed that a transiently increased nucleosome level followed by a partial decrease, instead of a decline back to baseline values, in patient plasma following liver transplantation was associated with early complications, including acute kidney injury, early allograft dysfunction, and decreased survival.[262]

Studies on individual circulating histones and histone PTMs are currently scarce compared with studies addressing nucleosomes in plasma or serum. Furthermore, while differences in plasma nucleosome levels have been detected between some patient groups, for example, cirrhosis vs. ACLF patients,[246] and low vs. high-grade ACLF[247]), nucleosome levels vary substantially between the cohorts of patients with ACLF,[246,247] and might be influenced by patient stratification, and the methodological approach, indicating the need for standardization.

Translational impact of liver liquid biopsy

Liquid biopsy is a continuously growing field, with new development in biomarker/s panel selection, methods, and ameliorated standardization approaches. It is essential to establish models that characterize the most suitable liquid biopsy type for a specific disease or condition, aiming at diagnosis, characterization, monitoring, or patient stratification.

Early detection of liver diseases is crucial for prognosis and patient quality of life. While currently applied enzyme biomarkers have long-established value for liver disease detection, they often present low sensitivity and specificity. For early-stage liver disease monitoring and detection, a biomarker should be suitable for routine screenings, fast, cost-effective, and easy to perform. Liquid biopsy biomarkers such as cfDNA-derived variants and epigenetic alterations, ncRNAs, and EVs have been shown to differentiate specific diseases not only from healthy individuals but also from patients with closely related diseases/conditions. Furthermore, compared with cfDNA and lncRNAs, EV isolation and analysis currently requires approaches that might not be applicable in every clinical laboratory. In comparison, while data suggest that circulating histones and histone complexes might be further studied as an early detection biomarker for several liver diseases, current research lack comparison with other liver pathologies.

Liver disease staging relies on tissue biopsy and imaging analysis. Liquid biopsy markers like circulating histones, nucleosomes, cfDNA, ncRNAs, and EVs are associated with liver disease severity and could provide diagnostic value at a relatively low cost and analysis time. EVs may contain important information regarding liver disease severity but require more elaborate processing and analysis. Furthermore, such noninvasive analysis could be clinically valuable when a set of detection biomarkers is developed to replace the need for invasive tissue biopsy.

The genetic signature is of utmost importance for appropriate diagnosis and treatment regimen definition, especially in liver malignancy diagnosis. To that end, CTCs and ctDNA have the greatest potential. Nevertheless, the concentration of CTCs and ctDNA in circulation is a crucial factor of such a liquid biopsy method. CTCs enter the circulation during intravasation, the initial stage of the metastatic cascade. Therefore, patients with a nonmetastatic early-stage of the respective malignant liver disease might not benefit from such analysis. However, ctDNA is released in circulation upon

cell death. Cell turnover (proliferation and apoptosis) is often high in cancer, favoring the detection of ctDNA in plasma/serum of patients with localized disease. That allows for early detection and characterization of the disease phenotype such as the identification of potentially targetable markers and therefore, timely intervention through local or systemic treatment. It should be noted that advanced sequencing technology should be in place to perform such analysis. Alternatively, external institutions might be involved, which would likely increase the processing time. Furthermore, frequent genetic and epigenetic alterations, targetable mutations, or clinical-trial-relevant targets could be easily addressed by targeted polymerase chain reaction (PCR), which drastically decreases the processing time.

Circulating histones and post-translationally modified histones have shown promising results for treatment monitoring (surgical, conservative, or palliative). It is unclear whether specific histones, histones variants, or histones modifications might detect responses to particular treatments. However, changes in circulating nucleosomes, H3K27me3/H3K36me3, and histone H3 levels were shown to occur rapidly following RFA, Sorafenib therapy, and hepatectomy. Similarly, while the levels of circulating nucleosomes in plasma or serum lack sensitivity in detecting specific organ-specific diseases, they might be a valuable method for therapy response monitoring and disease progression surveillance. Subsequent studies should focus on acquiring data from longitudinal studies with large cohorts that would shed light on some essential questions such as (1) how the circulating histone levels correlate with disease progression and progression-free survival; and (2) could circulating histones be used for patient therapy stratification?

Conclusions

There is a growing need for minimally invasive biomarkers for the diagnosis, staging, prognosis, monitoring, and personalized management of liver diseases. Increasing evidence indicates the potential diagnostic, prognostic, and monitoring value of circulating nucleosomes, histones, and histone complexes in liver diseases. In this respect, histones joined DNA-based biomarkers in the market arena of epigenetic-based companion diagnostic tests (CDx), used as a companion to a therapeutic drug to determine its applicability to a specific patient.[263] Nevertheless, results and suggested conclusions should be taken with caution, since data has been gathered from retrospective studies, some of which predominantly have a small sample size and/or lack high-risk groups. Furthermore, the currently nonstandardized circulating histone analysis and the interstudy variability pose challenges to the clinical application of circulating histones in liver disease assessment. Patients with early-stage liver diseases often do not show differential levels of circulating histones or histone complexes, limiting their value for disease diagnosis and prevention. While circulating histones have shown promising results for monitoring HCC therapy response and disease progression, current data on their potential in HCC detection is limited. Future studies should aim for large patient cohorts including various liver-related and liver-independent diseases. To increase the specificity of circulating histones, studies could focus on developing liver disease-predictive models combining several histone variants, complexes, and PTMs.

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Conflict of interest

MV has been an editorial board member of *Journal of Clinical and Translational Hepatology* since 2022. The other authors have no conflict of interests related to this publication.

Author contributions

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